FRUIT AND WINE COMPOSITION IN RELATION TO PROCESSING AND PRODUCT QUALITY

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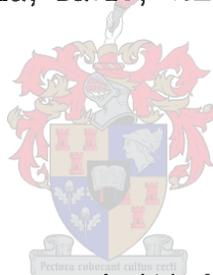
A Dissertation of published papers submitted for the Degree of Doctor of
Science (Agriculture) in the Department of Oenology of the University of
Stellenbosch

This submission consists of a selection of 33 papers published over the period 1961 - 1982 drawn from a more complete list (attached) authored or coauthored by the candidate. The papers are presented in chronological order. The selection was intended to present a cross-section of the work accomplished in relation to the title of the dissertation and retain modest size. Excluded are other topics, all work involved in previous degrees of the candidate, and books. Seven of the papers (63, 64, 71, 72, 83, 86, 111) represent thesis research of graduate students working directly under the candidate's supervision and have, therefore, been used by them toward their degrees. The candidate's contribution will be specified in more detail subsequently. Papers 29, 42, and 61 involved collaboration with Dr. C.S. Ough who has received the D.Sc. from the University of Stellenbosch and may have been included in his Dissertation.

To the candidate's best knowledge and belief, this Dissertation contains no material previously written or published by another person, except where due reference is made.

I wish to express my gratitude to the Faculty of the University of Stellenbosch, notably Professor C.J. van Wyk and to other members of the South African wine community including the K.W.V., the Cape Wine and Spirits Institute, the Viticultural and Oenological Research Institute (Nietvoorbij), and Stellenbosch Farmers' Winery who have directly and indirectly assisted in research and made my visits memorable.

I also wish to thank my wife for her understanding and support. The University of California and particularly the faculty of the Department of Viticulture and Enology, deserve my deep appreciation for many years of help and fruitful collaboration. Professors M.A. Amerine, J.F. Guymon (deceased), H.W. Berg, C.S. Ough, R.E. Kunkee, and A.D. Webb have been especially helpful. Dr. W.A. Gortner, Head of the Department of Chemistry when I was in it at the Pineapple Research Institute in Honolulu, is also a revered mentor.

Signed:

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Date: 1 June, 1983

Publications by
VERNON L. SINGLETON
1949 - 1959

1. Studies on a new mold metabolite. V. L. Singleton. M. S. Thesis, Purdue University, 44p., 1949.

2. Factors which affect the amino acid composition of alfalfa. V. L. Singleton. Ph. D. Thesis, Purdue University, 95p., 1951.

3. The effect of sulfur deficiency on the amino acids of alfalfa. E. T. Mertz, V. L. Singleton, and C. L. Garey. Arch. Biochem. Biophys. 38, 139-45 (1952).

4. The hydrolysis and amino acid assay of alfalfa, and the methionine range in 100 selections. V. L. Singleton, E. T. Mertz, and R. L. Davis. Agron. J. 44, 346-8 (1952).

5. Biochemistry of antibiotics. B. M. Duggar and V. L. Singleton. Ann. Rev. Biochem. 22, 459-96 (1953).

6. Variations in fruit quality measured by chemical criteria. V. L. Singleton and D. P. Gowing. PRI News 3, 73-82 (1955).

7. Pineapple fruit composition, flavor, and quality. V. L. Singleton. Research Rept. PRI No. 38, 1-46 (1955).

8. Possibilities of prediction of Cayenne pineapple fruit composition based upon few analyses. V. L. Singleton and D. P. Gowing. PRI News 3, 150-9 (1955).

9. Quality and yield data for varieties in the 1955 industry field tests - chemical composition. V. L. Singleton. PRI News 3, 275-9 (1955).

10. Practical quality separations of whole pineapple fruit. V. L. Singleton. PRI News 4, 8-12 (1956).

11. Nucleocidin, a new antibiotic with activity against trypanosomes.
    S. O. Thomas, V. L. Singleton, J. A. Lowery, R. W. Sharpe,
    L. M. Pruess, J. N. Porter, J. H. Mowat, and N. Bohonos.
    Antibiotics Annual 4, 716-21 (1956-7).

12. The nature of the separation produced by flotation of Cayenne pine-
    apple fruits. V. L. Singleton. PRI News 5, 1-10 (1957).

13. Selection and handling of pineapple fruits for shipment fresh. V. L.
    Singleton. Research Rept. PRI No. 49, 1-10 (1957).

14. Herstellung und Gewinnung eines neuen Antibiotikums. Samuel
    Owen Thomas, James Alfred Lowery, and Vernon LeRoy Singleton.
    German Patent 1,026,482; Mar. 20, 1958.

15. Decumbin, a new compound from a species of Penicillium. V. L.
    Singleton, N. Bohonos, and A. J. Ullstrup. Nature 181, 1072-3
    (1958).

16. Calcium deficiency, taste, and plant metabolism related to crystals
    in pineapple. V. L. Singleton. PRI News 6, 76-83 (1958).

17. A test for the degree of bruising of pineapple flesh. V. L. Singleton.
    PRI News 6, 111-14 (1958).

18. Pineapple fruit composition and biochemical quality research, April,
    1954 - September, 1958. V. L. Singleton. Research Rept. PRI
    No. 69, 1-68 (1959).

19. Antibiotic designated nucleocidin and preparation thereof. Assigned
    to American Cyanamid Co. British Patent 815,381; June 24, 1959.

20. Some possibilities of rapid aging. Vernon L. Singleton. Wines &
    Vines 40(7), 26 (1959).

21. Nucleocidin and the process for obtaining the same. Samuel Owen
    Thomas, James Alfred Lowery, and Vernon LeRoy Singleton.
    U. S. Patent 2,914,525; Nov. 24, 1959.

Publications by
VERNON L. SINGLETON
1960-1964

22. Automatic tools for the laboratory. Vernon L. Singleton. Wines & Vines 41(8), 29-31 (1960).

23. Liquid-liquid partition chromatography. V. L. Singleton. Am. J. Enol. Viticult. 11(3), 129-36 (1960).

24. Carotenoid pigments of pineapple fruit. I. Acid-catalyzed isomerization of the pigments. V. L. Singleton, Willis A. Gortner, and H. Y. Young. J. Food Sci. 26(1), 49-52 (1961).

25. Carotenoid pigments of pineapple fruit. II. Influence of fruit ripeness, handling, and processing on pigment isomerization. Willis A. Gortner and V. L. Singleton. J. Food Sci. 26(1), 53-5 (1961).

26. An extraction technique for recovery of flavors, pigments, and other constituents from wines and other aqueous solutions. V. L. Singleton. Am. J. Enol. Viticult. 12(1), 1-8 (1961).

27. Wood chips and wine treatment; the nature of aqueous alcohol extracts. V. L. Singleton and Diana E. Draper. Am. J. Enol. Viticult. 12(4), 152-8 (1961).

28. A crystallization technique. V. L. Singleton. Chemist-Analyst 51, 18 (1962).

29. Complexity of flavor and blending of wines. V. L. Singleton and C. S. Ough. J. Food Sci. 27(2), 189-96 (1962).

30. Aging of wines and other spiritous products, acceleration by physical treatments. V. L. Singleton. Hilgardia 32(7), 319-92 (1962).

31. Adsorbents and wines. I. Selection of activated charcoals for treatment of wine. V. L. Singleton and Diana Draper. Am. J. Enol. Viticult. 13, 114-25 (1962).

32. Ultrasonic treatment with gas purging as a quick aging treatment for wine. V. L. Singleton and Diana E. Draper. Am. J. Enol. Viticult. 14(1), 23-35 (1963).

33. Changes in quality and composition produced in wine by cobalt-60 gamma irradiation. V. L. Singleton. Food Technology 17(6), 112-15 (1963).

34. The possible role of ultrasound and ionizing radiation in wine aging. Vernon L. Singleton. Wines & Vines 44(8), 31-2, 34 (1963).

35. A test of fractional addition of wine spirits to red and white port wines. V. L. Singleton and J. F. Guymon. Am. J. Enol. Viticult. 14(3), 129-36 (1963).

36. Application of charcoal in wine clarification. Vernon Singleton. Wines & Vines 45(3), 29-31 (1964).

37. Chemical characterization of the mold product decumbin. V. L. Singleton and N. Bohonos. Agr. Biol. Chem. 28(2), 77-81 (1964).

38. The transfer of polyphenolic compounds from grape seeds into wines. V. L. Singleton and Diana E. Draper. Am. J. Enol. Viticult. 15(1), 34-40 (1964).

Color
39. Anthocyanin level in port-type wines as affected by the use of wine spirits containing aldehydes. V. L. Singleton, H. W. Berg, and J. F. Guymon. Am. J. Enol. Viticult. 15(2), 75-81 (1964).

40. A comparison of normal and stressed-time conditions on scoring of quality and quantity attributes. C. S. Ough, V. L. Singleton, M. A. Amerine, and G. A. Baker. J. Food Science 29(4), 506-19 (1964).

41. The natural flavonoids and polyphenolics and their properties and reactions. Vernon L. Singleton. In: Lecture Summaries of Selected Topics Presented during the First Fermented Beverage Technology Short Course. G. M. Cooke, Editor, University of California Extension, Davis, 9p., 1964.

42. Chemical and sensory effects of heating wines under different gases. V. L. Singleton, C. S. Ough, and M. A. Amerine. Am. J. Enol. Viticult. 15(3), 134-45 (1964).

43. Characterization of flavonoids in Fragaria. L. L. Creasy, E. C. Maxie, and V. L. Singleton. Proc. Am. Hort. Soc. 85, 325-31 (1964).

44. Chemical and physical development of the pineapple
fruit I. Weight per fruitlet and other physical
attributes. Vernon L. Singleton. J. Food Sci.
30(1), 98-104 (1965).

45. Chemical and physical development of the pineapple
fruit II. Carbohydrate and acid constituents.
V. L. Singleton and Willis A. Gortner. J. Food
Sci. 30(1), 19-23 (1965).

46. Chemical and physical development of the pineapple
fruit III. Nitrogenous and enzyme constituents.
Willis A. Gortner and Vernon L. Singleton. J. Food
Sci. 30(1), 24-9 (1965).

47.-56. Precédé de préparation d'antibiotique à partir
d'une souche de Streptomyces. Samuel Owen Thomas,
James Alfred Lowery, and Vernon LeRoy Singleton.
French Patent 1,197,627 April 24, 1956; also
patented in Argentina (111,765; 3/7/58); Belgium
(547,329; 4/26/56); Brazil (59,605; 5/3/61); Can-
ada (622,710; 6/27/61); Denmark (89,906; 4/21/
1956); India (57,098; 2/13/56); Japan (255,458;
6/9/59); Spain (228,089; 5/26/56); and Sweden
(171,552; 4/27/56).

57. Wine: An introduction for Americans. M. A. Amerine
and V. L. Singleton. University of California
Press, Berkeley. 357 p., 1965.

58. Colorimetry of total phenolics with phosphomolybdic-
phosphotungstic acid reagents. V. L. Singleton
and Joseph A. Rossi, Jr. Am. J. Encl. Viticult.
16(3), 144-58 (1965).

59. Identification of ellagic acid as a precipitate from loganberry wine. V. L. Singleton, George L. Marsh, and Monroe Coven. J. Agr. Food Chem. 14(1), 5-8 (1966).

60. The total phenolic content of grape berries during the maturation of several varieties. V. L. Singleton. Am. J. Enol. Viticult. 17(2), 126-34 (1966).

61. Density separations of wine grape berries and ripeness distribution. V. L. Singleton, C. S. Ough, and K. E. Nelson. Am. J. Enol. Viticult. 17(2), 95-105 (1966).

62. Paper chromatography of phenolic compounds from grapes, particularly seeds, and some variety-ripeness relationships. V. L. Singleton, Diana E. Draper, and Joseph A. Rossi, Jr. Am. J. Enol. Viticult. 17(3), 206-17 (1966).

63. Contributions of grape phenols to oxygen absorption and browning of wines. Joseph A. Rossi, Jr. and Vernon L. Singleton. Am. J. Enol. Viticult. 17 (4), 231-9 (1966).

64. Flavor effects and adsorptive properties of purified fractions of grape-seed phenols. Joseph A. Rossi, Jr. and Vernon L. Singleton. Am. J. Enol. Viticult. 17(4), 240-6 (1966).

65. Fining-phenolic relationships. Vernon L. Singleton. Wines & Vines 48(3), 23-6 (1967).

66. Adsorption of natural phenols from beer and wine. Vernon L. Singleton. Master Brewer's Assoc. Amer. Technical Quarterly 4(4), 245-53 (1967).

67. Some relationships between enzyme activities and phenolic components in banana fruit tissues. G. H. DeSwardt, E. C. Maxie, and V. L. Singleton. S. African J. Agr. Sci. 10, 641-50 (1967).

68. Wine: an introduction for Americans. M. A. Amerine and V. L. Singleton. University of California Press, Berkeley. 357 p., 1967. 3rd printing. Revised paperback edition.

69. Wine quality prediction from juice Brix/acid ratio and associated compositional changes for 'White Riesling' and 'Cabernet Sauvignon'. C. S. Ough and V. L. Singleton. Am. J. Enol. Viticult. 19 (3), 129-38 (1968).

70. Toxicity and related physiological activity of phenolic substances of plant origin. V. L. Singleton and F. H. Kratzer. J. Agr. Food Chem. 17(3), 497-512 (1969). Also separately printed in: Symposium on Natural Food Toxicants, 156th Meeting, Am. Chem. Soc., Sept. (1968). (129 p. total).

71. An estimate of the nonflavonoid phenols in wines. Thomas E. Kramling and V. L. Singleton. Am. J. Enol. Viticult. 20(2), 86-92 (1969).

72. Identification of three flavan-3-ols from grapes. Cathey Tsai Su and V. L. Singleton. Phytochemistry 8(8), 1553-8 (1969).

73. Phenolic substances in grapes and wine, and their significance. Vernon L. Singleton and Paul Esau. Advances in Food Research, Supplement 1, Academic Press, N. Y., 282 p., 1969.

74. Browning of wines. V. L. Singleton. Die Wynboer (No. 455), 13-4 (1969).

75. Wine industry. Vernon L. Singleton. Americana Annual. Encyclopedia Americana, p. 73, 1970.

76. An analysis of wine to indicate aging in wood or treatment with wood chips or tannic acid. V. L. Singleton, Anthoula Randopoulo Sullivan, and Cynthia Kramer. Am. J. Enol. Viticult. 22(3), 161-6 (1971).

77. A list of bibliographies and a selected list of publications that contain bibliographies on grapes, wines, and related subjects. Maynard A. Amerine and Vernon L. Singleton. University of California Agricultural Publications, Berkeley, 39 p., 1971.

78. Some aspects of the wooden container as a factor in wine maturation. V. L. Singleton. Proceedings of the 3rd International Oenological Symposium, Cape-town, South Africa. Mar. 6-10, 1972. Paper 9, p. 1-21 (1972).

79. Common plant phenols other than anthocyanins, contributions to coloration and discoloration. V. L. Singleton. In The Chemistry of Plant Pigments, C. O. Chichester, Editor. Adv. Food Res., Suppl. 3, p. 143-91 (1972).

80. Effects on red wine quality of removing juice before fermentation to simulate variation in berry size. V. L. Singleton. Am. J. Enol. Viticult. 23, 106-13 (1972).

81. South Africa symposium: a scholar's view. Vernon L. Singleton. Wines & Vines 54(5), 26-7; (6), 70-2 (1973).

82. Characterization of populations of grapes harvested for wine and compensation for population differences. V. L. Singleton, P. DeWet, and C. S. DuPlessis. Agroplantae 5, 1-12 (1973).

83. Catecholase activity in grape juice and its implications in winemaking. S. Traverso-Rueda and V. L. Singleton. Am. J. Enol. Viticult. 24(3), 103-9 (1973).

84. Plant phenolics. V. L. Singleton and F. H. Kratzer. Chapter 15. In Toxicants Occurring Naturally in Foods, 2d ed. National Academy of Science, Washington, D.C., p. 309-45, 1973. (624 p. total).

85. Role of polyphenols in aging of white wine and few contrasts with beer. Vernon L. Singleton. MBAA Technical Quarterly 11(2), 135-40 (1974).

86. The production of aldehydes as a result of oxidation of polyphenolic compounds and its relation to wine aging. H. L. Wildenradt and V. L. Singleton. Am. J. Enol. Viticult. 25(2), 119-26 (1974).

87. Analytical fractionation of the phenolic substances of grapes and wine and some practical uses of such analyses. Vernon L. Singleton. In Chemistry of Winemaking. A. Dinsmoor Webb, Editor. American Chemical Society, Washington, D.C. Adv. Chem. 137, 184-211 (1974).

88. Some aspects of the wooden container as a factor in wine maturation. Vernon L. Singleton. In Chemistry of Winemaking. A. Dinsmoor Webb, Editor. American Chemical Society, Washington, D.C. Adv. Chem. 137, 254-77 (1974). (A slightly revised republication of No. 78).

89. Protein stability in wine. Vernon L. Singleton. Proc. 7th Pennsylvania Wine Conference, Pennsylvania State University, University Park, PA, Dec. 5-6, 1974. p. 46-60 (1974).

(90). Relative distinctiveness of varietal wines esti-
mated by the ability of trained panelists to
name the variety correctly. Walter Winton, C.
S. Ough, and V. L. Singleton. Am. J. Enol.
Viticult. 26(2), 5-11 (1975).

✔ (91). Composition and sensory qualities of wines prepared
from white grapes by fermentation with and with-
out grape solids. V. L. Singleton, H. A. Sieber-
hagen, P. deWet, and C. J. van Wyk. Am. J. Enol.
Viticult. 26(2), 62-9 (1975).

✔ 92. Characterization and growth-depressing activity for
chickens of several natural phenolic materials.
F. H. Kratzer, V. L. Singleton, R. Kadirvel, G. V.
N. Rayudu. Poultry Science 54(6), 2124-7 (1975).

✔ 93. The future of spirits-added wines and their barrel
aging--one man's opinions. Vernon L. Singleton.
Proc. Second Ann. Wine Ind. Tech. Seminar, Fresno,
CA, Nov. 14, 1975. 15 p. (1975).

94. Wine flavor and phenolic substances. V. L. Singleton
and A. C. Noble. In Phenolic, Sulfur, and Nitro-
gen Compounds in Food Flavors. G. Charalambous
and I. Katz, Editors. American Chemical Society,
Symposium Series No. 26, 47-70 (1976).

✔ (95). Browning of white wines and an accelerated test for
browning capacity. V. L. Singleton and T. E.
Kramling. Am. J. Enol. Viticult. 27(4), 157-60
(1976).

96. Wine aging and its future. V. L. Singleton. The
First Walter and Carew Reynell Memorial Lecture,
Roseworthy Agricultural College, South Australia,
Australia, July 28, 1976.

97. Wine aging and its future. Vernon L. Singleton. Austral. Wine, Brew. Spirit Rev. 94(11), 18,20 (1976).

98. Modern trends in wine making. Vernon L. Singleton. In Wine Quality - Current Problems and Future Trends. F. W. Beech, A. G. H. Lea, and C. F. Timberlake, Editors. Long Ashton Res. Sta., Univ. of Bristol, Subject Day, Sept. 17, 1976. p. 1-9 (1976).

99. Total phenol analysis: automation and comparison with manual methods. Karen Slinkard and Vernon L. Singleton. Am. J. Enol. Viticult. 28(1), 49-55 (1977).

100. Wine aging and improvement. V. L. Singleton. Calif. Agr. 30(10), 24 (1976).

101. Dr. Vernon L. Singleton on the anomaly of aging. Vernon L. Singleton. Wines & Vines 58(8), 31-2 (1977).

102. Chromatography of natural phenolic cinnamate derivatives on Sephadex LH-20 and G-25. Vernon L. Singleton, Colin F. Timberlake, and Geoffrey C. Whiting. J. Chromatog. 140, 120-4 (1977).

103. Wine: an introduction. Maynard A. Amerine and Vernon L. Singleton. Second completely revised edition. University of California Press, Berkeley, 373 p., 1977.

104. High points and low on the way to the future. Vernon L. Singleton. Proc. Fourth Ann. Wine Ind. Seminar, Monterey, Calif., Dec. 3, 1977. 10 p. (1977).

105. Phenols (née tannins and pigments), important parts of wine. Vernon L. Singleton. Bull. Soc. Med. Friends Wine 20(1), 3,4,7,8 (1978).

106. The phenolic cinnamates of white grapes and wine. Vernon L. Singleton, Colin F. Timberlake, and Andrew G. H. Lea. J. Sci. Food Agr. 29, 403-10 (1978).

107. Wine and its aging. Vernon L. Singleton. In The Vineyard Almanac and Wine Gazetteer, Los Altos, Calif., p.37-41 (1978).

108. Phenolic cinnamates--cis-trans isomerism; phenolic cinnamates in white wine. V. L. Singleton, C. F. Timberlake, and A. G. H. Lea. Long Ashton Research Sta. Rept. 1977, 125-7 (1978).

109. Oxidation of wines. I. Young white wines periodically exposed to air. V. L. Singleton, Eugene Trousdale, and John Zaya. Am. J. Enol. Viticult. 30(1),49-54 (1979).

110. Recent developments in wine aging. Vernon L. Singleton. Proc. 5th Wine Industry Technical Seminar, Nov.25, 1978, Monterey,Calif., p. 31-7 (1979).

111. The nonflavonoid fraction of wine and its analysis. Thomas E. Myers and Vernon L. Singleton. Am. J. Enol. Viticult. 30(2), 98-102 (1979).

112. Wine maturation and aging. V. L. Singleton. Wine Media Day, Wine Institute, July 17, 1979, San Francisco CA, 4 p.

113. The procyanidins of white grapes and wines. Andrew G. H. Lea, Peter Bridle, Colin F. Timberlake, and Vernon L. Singleton. Am. J. Enol. Viticult. 30(4), 289-300 (1979).

114. Freezing of wine during shipment. Vernon L. Singleton and Roger B. Boulton. Wine Institute Summary (Wine Institute Transportation Manual), Sept. 14, 4 p. San Francisco, Calif. (1979).

115. James Fuqua Guymon. Vernon L. Singleton, A. Dinsmoor Webb and Roger Boulton. In In Memorium, University of California, p. 53-5 (Sept., 1979).

✓ (116). White table wine quality and polyphenol composition
     as affected by must SO2 content and pomace contact
     time. V. L. Singleton. John Zaya, and Eugene
     Trousdale. Amer. J. Enol. Vitic. 31(1), 14-20
     (1980).

X117. The technology of wine making. M. A. Amerine, H. W.
     Berg, R. E. Kunkee, C. S. Ough, V. L. Singleton,
     and A. D. Webb Fourth Ed. 794 p. Avi Publishing
     Co., Westport, Conn. (1980).

* (118). Bitterness and astringency of phenolic fractions in
     wine. Richard A. Arnold, Ann C. Noble, and Vernon
     L. Singleton. J. Agr. Food Chem. 28(3), 675-8
     (1980).

X119. A century of wine and grape research. Vernon L.
     Singleton, Harold W. Berg, and A. Dinsmoor Webb.
     Calif. Agr. 34(7), 4-5 (1980).

120. Why alcohol and calories are not added to food
     cooked with wine. Vernon L. Singleton. Wine
     Institute, public response mailings. 1 p. Dec. 15
     (1980).

121. Thoughts on the Ames test and carcinogenicity. V. L.
     Singleton. Wine Institute Press Release 5 p. June
     19 (1981).

X122. Naturally occurring food toxicants: phenolic
     substances of plant origin common in foods. Vernon
     L. Singleton. Adv. Food Res. 27, 149-242 (1981).

X123. Wine aging and phenolic oxidation -- a research
     generalist's view of oxidation in wine. Vernon L.
     Singleton. J. Inst. Enol. Viticult., Yamanashi
     Univ. 16, 47-60 (1981).

X124. Using wooden cooperage in the winery today. Vernon
     L. Singleton. Vinifera Wine Grower's J. 8(4), 227-
     38 (1981).

✓ 125. Grape and wine phenolics: background and prospects.
     V. L. Singleton. Proc. Symp. Univ. Cali. Davis,
     Grape and Wine Centennial, 1980, 215-27 (Pub.

126. Oxidation of wine. V. L. Singleton. International
     Symposium on Viticulture, Vinification, and the
     Treatment and Handling of Wine. Institute of
     Master's of Wine. Oxford University, England.
     Mar.30 - Apr. 1, paper 18, 14 pp. (1982).

127. The reactions between polyphenols and aldehydes and
     the influence of acetaldehyde on haze formation in
     beer. J. A. Delcour, P. Dondeyne, E. K. Trousdale,
     and Vernon L. Singleton. J. Inst. Brewing 88, 234-
     43 (1982).

DESCRIPTION OF PUBLICATIONS AND DETAILS OF RESPONSIBILITY

A complete list of the candidate's published writings through 1982 has been
presented. Those submitted as part of this Dissertation are numbers 24-26, 29,
33, 35, 39, 42, 59-64, 70-72, 74, 76, 80, 82-84, 86, 87, 91, 92, 95, 106, 109,
111, 116, and 125 on that listing and these numbers will be used in subsequent
comments.

The papers could be grouped in various ways, but it seemed more efficient and
perhaps helpful to the reader to leave them in chronological order and briefly
comment on each in order. As relationships seem important, cross reference by
number will be made.

Papers 24 and 25 were written by W.A. Gortner but taken from a report (17) of
research conceived, largely carried out, and originally written by VLS (est.
67%). The carotenoids of pineapple include epoxide forms that when exposed to
heat or acid, including the acid of the fruit cell vacuole, isomerize to fura-
noid forms with accompanying spectral changes. This finding was used to develop
a method of determining the degree of tissue disruption from bruising, for
example during different methods of transporting fresh fruit to market or the
cannery. This practical application of fundamental findings of fruit or wine
composition is a theme that follows throughout this research and particularly
has much in common with papers 59 and 83. Papers 24 and 25 are the only ones
included that stem from employment in Hawaii, 1954-1958.

Paper 26 is an adaptation and amplification of a technique which had not pre-
viously been published, but was learned during employment in antibiotic research
at a large pharmaceutical company. There we had used salting out of acetone as
an extraction procedure. Here we investigated a wide range of salts and solvents
and showed that ethanol could be salted to a second phase by ammonium sulphate
in, for example, dessert wines. This has proven quite useful in the laboratory
quantitatively to separate and concentrate flavors and pigments from wines,
leaving the highly polar sugars and acids behind in the aqueous salt solution.
It is capable of preparing concentrated wine for reconstitution for consumption,
but no commercial applications have been forthcoming to date. It has been de-
monstrated on a pilot scale, 1-2 hectoliters.

The research leading to paper 29 was conceived, partly conducted, and the paper written by VLS (est. 60%). C.S. Ough supplied the wines and co-operated throughout. This work proved that complexity of flavor was a positive quality important in wine and this must apply in other foods as well.

Paper 33 is one of several related to understanding, hastening or simulating aging reactions in wine. Irradiation with cobalt gamma rays produced extensive changes in wine. If sufficiently restrained, building on the results of paper 29, this treatment might contribute to increased aged quality in wine, but at appreciable levels the flavors were "off".

Papers 35 and 39 were planned and written (by VLS, est. 75%) to understand the benefits claimed by Russian workers for fortifications of wines in several portions rather than a single massive addition of brandy. As predicted, aldehydic brandy was reduced by continuing fermentation after the first portion, but for purer spirit there was no benefit. An unanticipated result was that aldehydic brandy increased the red color of port and this finding and its interpretation has led to other important research by ourselves (paper 86) and others.

Paper 42 (70% VLS) details effects of heating wines under O_2, N_2, H_2, or CO_2. Heating without any oxygen gave the same effects regardless of the other three gases and rapidly produced apparent age. The table wine under these inert gases gave increased bottle-bouquet in about the same degree as wines stored in a cool cellar for much longer time ($Q10$ about 2), but lost fruity aroma faster also. Suitable blending of a portion of non-fruity base wine treated about 30 days at 53°C in the complete absence of oxygen was recommended for commercial trial.

Ellagic acid (paper 59, VLS 80%) was identified as the cause of a precipitation with time in commercial loganberry wine. Mr. Coven brought the problem to our attention and Prof. Marsh was included because he was historically in charge of berry wine research.

Papers 60 (100% VLS), 61 and 62 were from research conceived, supervised, and written by VLS (61 est. 50%, 62 est. 50%). All three relate to more detail of phenolic substances, ripeness development, and variety comparisons. Paper 60 showed that there was a net biosynthesis of phenols per berry even though

the concentration fell during most of the ripening period. Paper 61 adapted a density flotation method to study ripeness and ripeness distribution within a single harvest date and therefore separate from variable weather influences. Different populations could have the same combined mean Brix or acid but quite different subpopulation groups, a factor of importance in comparing vineyards and vintages. Paper 62 showed that different phenolic compounds in grapes changed differently during ripening; for example, epicatechin gallate decreased during ripening.

Papers 63 and 64 resulted from work planned and written largely by VLS, but were conducted (66% contribution estimated) by Mr. Rossi and used by him for his M.S. thesis. They detail studies showing that the catechins contribute heavily to browning in musts and white wine, that different adsorbents have some selectivity toward different seed phenols, and that monomeric flavonoids tend to be bitter without astringency whereas oligomeric flavan tannins are very astringent as well as bitter in water and in wine. These papers are part of a set that represented the first useful fractionation and comparative study of grape seed phenols.

Papers 70, 84, and 92 represent two reviews of the roles of phenols as toxins to animals and as dietary constituents, plus an experimental study of the effect of feeding phenols to chicks. The two reviews were largely researched and written by VLS (est. 90%), but the contribution to paper 92 was largely in chemical analyses and their interpretation (est. 15%). These studies clarified and integrated the understanding of plant phenols as factors in animal health. Together with paper 122 (not submitted) they constitute landmarks in the field from a review viewpoint.

Papers 71, 76, and 87 involve development, verification and application of analytical methods for phenols. Paper 71 was part of Mr. Kramling's M.S. thesis, but was conceived and written by VLS (40%). It developed a new method of distinguishing quantitatively between flavonoids and non-flavonoid phenols. Verification of the quantitative aspects of this assay and total phenol content in relation to structure of many known phenols was the subject of paper 87 (VLS 100%). Paper 76 (VLS planned and wrote, est. 75%) proved that the phenols contributed to wine by wooden containers and corks were very predominantly non-flavonoid and applied their assay to showing the effect of wood storage on wine composition, solving a very practical problem.

Paper 72 represents M.S. thesis work of Mrs. Su and was initiated, supervized and the paper written by VLS (45%). The identities of (+)-catechin, (-)-epicatechin, and (-)-epicatechin gallate from grape seeds were proved by isolation and physical-chemical data. Previous work had been ambiguous and based almost solely on paper chromatography.

Paper 74 was based on work done while on leave in South Africa which showed that the flavonoid content was the primary indicator of browning capability (see also papers 63, 71 and 95).

Paper 76 was discussed with paper 71.

Paper 80 involved study of the effect of free-run juice removal on red wine color, composition and quality. The results indicated important improvement in quality with increased skin to juice proportion and simulated the favorable effect of smaller berry size.

Paper 82 extended the findings of paper 61 to a South African situation. VLS planned the work, did part of it and drafted the paper (est. 50%). It illustrated the value of density separations to characterize harvests, vineyards, and vintages of grapes. Contrary to many fruits, grapes are free of air and the density of the berry is slightly higher than that of its juice. Low Brix but large size berries indicate overcropping.

Catecholase activity increased several fold upon grape berry bruising in air but not in nitrogen atmosphere (Paper 83). This paper was a major part of Dr. Traverso's M.S. thesis (VLS est. 10%).

Paper 84, see paper 70 discussion.

Paper 86 was part of the Ph.D. thesis of Dr. Wildenradt, but was planned, supervised, and appreciably written by VLS (est. 45%). The findings are important because they explained for the first time the origin of acetaldehyde during wine oxidation. This is via coupled autoxidation with the wine's phenols. They co-produce a strong oxidant believed to be hydrogen peroxide. This, then, oxidizes the first ethanol molecule (or other susceptible substance) encountered.

Paper 87, see discussion of paper 71.

Paper 91 details research in South Africa dilating on the effect of clarification by settling of free run must and cool fermentation on producing higher quality and special estery fermentation bouquet (VLS 50% est.).

Paper 92, see discussion of paper 70.

Paper 95 describes the development and testing of a standardized test for the capacity of a white wine to brown (VLS est. 60%). Even when heated to about 50°C white wine do not brown appreciably in the absence of oxygen. When exposed to oxygen they brown more in the presence of higher flavonoid content or added catechin. This corroborates data of paper 63.

For paper 106 VLS did most of the work and wrote it (est. 60%). It verified by isolation and characterization the presence of caffeoyl tartaric acid and the analogous p-coumaric derivative rather than esters of quinic acid in grapes and wine. This cleared up contradictions in the literature.

Oxidation of young white table and sherry material wines was reported in paper 109 (planned, supervised and written by VLS, est. 40%). The exposure of wine to 60 ml of oxygen per liter is enough to convert it from table wine to sherry-like. White table wines improved, if any, only with small (four saturations or less) exposure to air.

Mr. Myers' M.S. thesis was the subject of paper 111, the work being initiated and supervised by VLS (est. 20%). The effort was to explain the components of the "non-flavonoid" content of white wines when determined by our Folin-Ciocalteu method (paper 71). New but unidentified components were separated and their contribution was appreciable.

Paper 116 reports work planned, supervised, partly done and written by VLS (50%) on the effects of oxidation, sulphur dioxide, and pomace contact upon phenol content, browning capacity, color, and quality of the white wines from four cultivars. Deliberate oxidation of the must minimized later browning capacity of the wines but quality was lowered. Total phenol increased about 3 mg/L/hr during pomace contact at 24°C and about 11 mg/L if SO_2 was raised from 50 to 100 mg/L during at least 3 hours pomace contact. These findings have practical value in relation to recommended commercial practice of wine making.

Paper 125, the final submission, is to a degree, the most appropriate final paper as it was prepared to introduce a session within the University of California's symposium commemorating 100 years of grape and wine research. Some critical review of work by others and by the candidate is presented.

In sum, the papers submitted are believed sufficient to indicate the scope and accomplishments of the candidate and it is hoped they are deemed worthy of the D.Sc. in Agriculture (Oenology) at the University of Stellenbosch.

Carotenoid Pigments of Pineapple Fruit. I. Acid-Catalyzed Isomerization of the Pigments [a]

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SUMMARY

The total carotenoid pigments of pineapple fruit contain a high proportion of epoxide groups which are readily isomerized to furanoid forms in an acid but not in an alkaline environment. This isomerization causes a characteristic hypsochromic shift in the absorption maxima of the pigment extract. The absorbance at 425 mμ remains relatively unchanged as isomerization proceeds, and thus can serve as a measure of the total carotenoid pigment regardless of its isomeric form. The sharp maximum at 466 mμ is lost as isomerization progresses. Thus, the ratio of absorbances at 466 and 425 mμ can serve as a measure of the extent of isomerization of the pigments.

The concentration of carotenoid pigments in the flesh of pineapple fruit varies over a wide range among pineapple varieties, distinguishing the golden-fleshed varieties such as Queen or Cayenne from the paler Red Spanish or Cabezona pineapple (2). Within a variety, the intensity of color has served as one measure in quality grading of the processed pineapple.

It has been known for many years that the absorption spectrum of the pigments extracted from canned pineapple differed from that of fresh pineapple. Unpublished work in 1941 by A. J. Haagen-Smit, J. G. Kirchner and A. G. R. Strickland at the California Institute of Technology, in 1944 by G. H. Ellis and his associates at the U. S. Plant, Soil and Nutrition Laboratory in Ithaca, and in 1953 by H. Y. Young in our laboratory showed that the absorption curve of the carotenoids of fresh pineapple is changed and shifted toward the ultraviolet when the tissue is heated. This change obviously has implications in the colorimetric determination of pineapple pigments.

EXPERIMENTAL METHODS

Field-ripe fruit of Hawaiian pineapple, *Ananas comosus* var. Cayenne, were used throughout. Ca-

[a] Approved by the Director as Technical Paper No. 271 of the Pineapple Research Institute of Hawaii.

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rotenoid pigments were extracted from the edible portions by blending 25 g of tissue with 50 ml of a 1:1 mixture of petroleum ether (BP 60–110° C) and 95% ethanol. The petroleum ether supernatant obtained by centrifugation was used for the absorption spectra measurements in the Beckman DK-2 recording spectrophotometer.

RESULTS AND DISCUSSION

THE SPECTRAL SHIFT

The absorption spectrum of an extract of fresh pigment shows an intense, sharp maximum at 466–467 mμ, a broader and slightly more intense peak at 438–439 mμ, a less intense peak at 415–418 mμ (a shoulder in less than optimal samples) and finally two weak, broad maxima at about 328 and 315 mμ. The isomerized (canned-type) pigment extracts show a weak shoulder at about 470 mμ, a moderately intense absorption maximum at 447–449 mμ, the most intense peak at 425–426 mμ, the next most intense at 401–403 mμ, the third most intense (shoulder) at about 382 mμ, and vague inflections below this wave length (Figure 1).

Since the isomerized pigment has lost the 466 mμ peak and yet retains its absorbance at other lower wave lengths, these changes readily lend themselves to spectrophotometric determination of the relative amounts of the unisomerized and isomerized pigments in an extract. However, the eye is less discriminating and to the human eye these spectral changes are hardly visible. The isomerism

results in a small shift in the shade of color from a slightly orange-yellow in fresh fruit to a somewhat more lemon-yellow color of nearly the same intensity in the isomerized pigments of canned fruit.
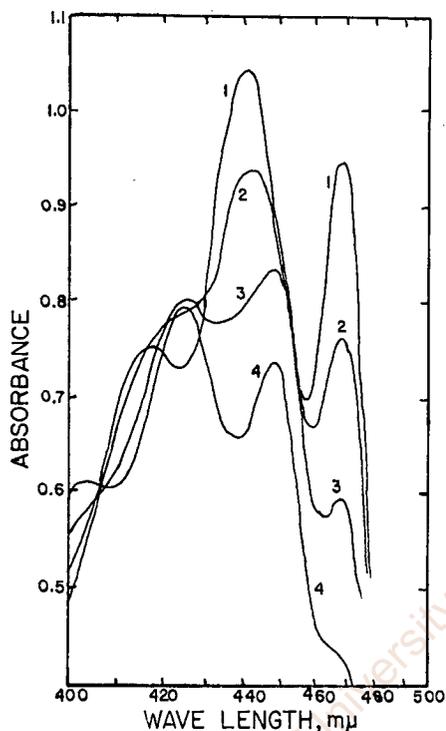

FIG. 1. Change in absorption spectrum of pineapple fruit carotenoids as isomerization progresses, showing especially the loss of the absorbance peak at 466 mμ, the lowered absorbance and shift to higher wave length of the peak at 438 mμ, and the retention of absorbance in the neighborhood of 425 mμ. A tissue homogenate was allowed to stand at 24° C for various periods before extraction of the pigments into petroleum ether. Curve 1 = after 3 min; curve 2 = 1 hr; curve 3 = 2 hr; curve 4 = 4 hr 22 min.

CHEMICAL NATURE OF THE ISOMERIZATION

Carotenoids of undisrupted fresh pineapple fruit tissue isomerize only very slowly, even when cut chunks are allowed to stand for some time. On the other hand, homogenates of the tissue soon exhibit a shifting in the absorption spectra of their extracts. This shift can be prevented by the addition of $CaCO_3$ or NaOH to neutralize the acid of the pineapple. This is illustrated by data in Table 1. Some isomerization occurred before the pH of the blended tissue was adjusted, but thereafter isomerization proceeded only under acid conditions. At the lowest pH normal for the fruit, isomerization was essentially complete after heating.

The type of isomerization that is well known for carotenoids and other polyenes is cis-trans isomerism. This is know to be catalyzed by acids, but is more readily produced by iodine or sunlight. Literature values (1) for the spectral shifts produced by iodine isomerization are of the order of a few millimicrons, whereas the shift caused by canning was considerably higher. Iodine and sunlight treatments or several days' standing at room temperature in the light caused some slight reduction in the absorption intensity of fresh pineapple carotenoid extracts, but did not produce the "canned-type" spectral shift.

There are other indications that the important isomerization is not one of cis-trans change. When the carotenoid extract from fresh pineapple flesh is shaken with strong hydrochloric acid, part of the yellow pigments are converted to a blue solid, soluble in methanol but insoluble in water or petroleum ether. When treated with alkali, the blue material again becomes yellow and soluble in petroleum ether. The absorption spectrum of the residual yellow solution after removal of the blue resembles that of the fresh pigments, whereas the spectrum of the pigments regenerated from the blue solid approximates the "canned-type" spectral shift.

This information coupled with chromatographic and other data characterizing the individual pigments, to be reported later, makes it clear that the spectral shift is caused

TABLE 1

PERCENTAGE ISOMERIZATION OF PINEAPPLE CAROTENOIDS AFTER TISSUE HOMOGENATES ADJUSTED TO VARIOUS pH LEVELS WERE HEATED 30 MINUTES AT 100° C BEFORE EXTRACTION

pH	3.40	4.20	4.60	4.90	5.70	6.05	6.50	7.04
% Isomerized	98	85	84	75	44	38	27	24

by the isomerization of one or more carotenoids containing epoxide groups into their furanoid rearrangement products (2, 3) in a reaction of the following type:

CH$_3$ CH$_3$ — CH=CH-C=etc. · H$^+$ ⟶ O CH$_3$ CH$_3$ CH$_3$ CH CH-C=etc. O CH$_3$ CH$_3$
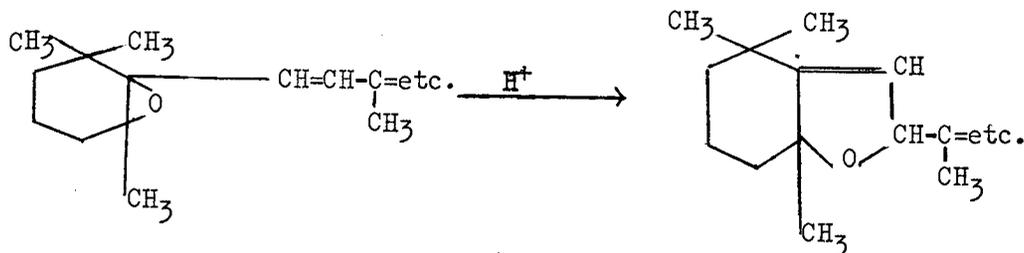
Epoxide pigments are widespread in nature, especially in ripening fruits.

The blue pigment formed with strong acids probably consists of oxonium salts, which would explain its solubility in methanol and not petroleum ether.

DETERMINATION OF TOTAL CAROTENOID PIGMENT AND THE DEGREE OF ISOMERIZATION

As seen in Figure 1, the unisomerized pigments show a strong peak at 466 mμ which becomes a low shoulder after isomerization. They also have a small absorption minimum at 425 mμ which is changed into the maximum absorbance when isomerization is complete. Absorbance determinations at these two wave lengths can serve as a measure of the degree of isomerization of the carotenoid pigments extracted from pineapple tissue.

A large number of individual determinations on fruit carefully selected and handled to avoid tissue breakdown, and extracted rapidly and in the presence of excess alkali to avoid isomerization, gave an average ratio of absorbances at 466 and 425 mμ of 1.38. This, then, characterizes the "fresh" or unisomerized pigments. Analyses on pigments of heated flesh homogenates or juice gave an average ratio of 0.40 for the absorbances at the two wave lengths.

These two ratios enable one to calculate the degree of isomerization of pigment in a given extract according to the formula:

$$\% \text{ unisomerized} = \frac{\dfrac{(\text{absorbance at } 466 \text{ m}\mu)}{(\text{absorbance at } 425 \text{ m}\mu)} - 0.40}{1.38 - 0.40} \times 100 = \frac{R - 0.40}{0.0098}$$

The spectra of the pigments before and after the acid-catalyzed isomerization also suggest a means of analyzing for total carotenoid pigment regardless of the isomeric form. This involves selection of an isosbestic-like wave length for the measurement.

A true isosbestic point can exist only when a single compound is being transformed into (or is in equilibrium with) another and they have different absorption spectra. Under these conditions, a sequential series of spectral absorption curves of samples with varying degrees of conversion will all pass through a point of constant absorbance, i.e., the isosbestic point. Although preliminary chromatography had shown that at least three carotenoids were in the natural pineapple mixture, it was hoped that one or more points of nearly constant absorbance could be found as the fresh-type pigment was converted to the canned-type. These pseudo-isosbestic wave lengths could then be used for a colorimetric determination which would give the same reading for fresh *or* canned yellow pigment.

As will be noted from Figure 1, the spectral curves on pineapple carotenoids at various stages of isomerization approach a common absorbance near 425 mμ and at 406 mμ. The 406 mμ point is not on a peak for either the isomerized or the unisomerized pigment spectrum, but rather is in a region of rapid change of absorbance for both. Thus a small wave-length difference could lead to a rather large change in the apparent pigment concentration in the neighborhood of 406 mμ.

Measuring pigment concentration at 425 mμ does not have this disadvantage, since this wave length coincides with a peak on the curve for isomerized pigment and is near a saddle on the spectrum of the unisomerized pigment. Thus, where a mixture of the isomeric forms is being analyzed for

total carotenoid, the absorbance at 425 mμ measured with a narrow band-pass spectrophotometer compared with an appropriate known β-carotene standard measured at the same wave length can give the "ppm carotene" equivalent with satisfactory accuracy.

EFFECT OF TIME AND EXTRACTION TECHNIQUE ON ISOMERIZATION

The marked effect of pH on isomerization of the carotenoid pigments of pineapple has already been referred to (Table 1).

TABLE 2

PERCENTAGE ISOMERIZATION OF PINEAPPLE CAROTENOIDS AFTER TISSUE HOMOGENATES WERE ALLOWED TO STAND FOR VARYING TIME AT 24° C BEFORE EXTRACTION

	Time delay before exten.	Per cent isomerized
Blended fruit tissue		
	3 min	8
	11 min	11
	1 hr	38
	2 hr	65
	4 hr 22 min	84
	6 hr	87
Blended with NaOH		
	3 min	5
	1 hr	4
Blended with solvent		
	0 min	9
Blended with NaOH + solvent	0 min	0

At room temperature, isomerization is detectable within a few minutes and proceeds for several hours at the pH of blended pineapple fruit (Table 2 and Fig. 1). An excess of alkali effectively stops isomerization of the carotenoids; no further important spectral change occurs upon standing.

A delay in extraction of the pigments after the fruit tissue is blended can lead to appreciable alteration in the spectrum. If the sampling method requires weighing an aliquot of blended tissue for pigment extraction, the blending should be done in the presence of sufficient alkali to maintain a pH above 7 if the pigments are desired in the isomeric forms present in the original fruit.

No further isomerization of the pigments of the type we are discussing occurs once the carotenoids are extracted into petroleum ether.

LITERATURE CITED

1. Karrer, P., and E. Jucker. 1950. *Carotenoids.* Elsevier Publishing Co., New York.

2. Magistad, O. C. 1935. Carotene and xanthophyll in pineapples. *Plant Physiol.,* **10,** 187.

3. Tsukida, E., and L. Zechmeister. 1938. The stereoisomerization of β-carotene epoxides and the simultaneous formation of furanoid oxides. *Arch. Biochem. Biophys.,* **74,** 408.

Carotenoid Pigments of Pineapple Fruit. II. Influence of Fruit Ripeness, Handling and Processing on Pigment Isomerization [a]

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SUMMARY

The acid-catalyzed isomerization of pineapple fruit carotenoid pigments is influenced by any condition leading to loss of integrity of the cells of the fruit tissue. The swollen lower half of fully ripe, yellow, translucent fruit often will contain an appreciable fraction of isomerized pigment. Any post-harvest handling of the fruit that causes bruising of the tissue will lead to pigment isomerization in the damaged areas. Canning completely isomerizes the carotenoid pigments. Frozen fruit contains a high proportion of isomerized carotenoids; after thawing, further change takes place until the spectrum is that of the isomerized or "canned" type pigment.

The first paper in this series (1) demonstrated that pineapple fruit carotenoids undergo isomerization when the tissue is disrupted and the cell-vacuole acid comes in contact with carotenoid-bearing plastids. This isomerization from epoxide to furanoid forms can be measured spectrophotometrically by determining the ratio of the absorbances at 466 and 425 mμ.

It was evident that bruising, freezing and thawing, canning, or even cell wall breakdown due to senescence could lead to this carotenoid isomerization. Accordingly, various of these factors associated with the harvesting and processing of pineapples have been studied for their influence on the carotenoid pigments of the fruit. While data have been obtained only with pineapple, the technique should be applicable to any acidic fruit or tissue with a high proportion of carotenoid epoxides.

EXPERIMENTAL METHODS

Fruit of Hawaiian pineapple, *Ananas comosus* var. Cayenne, were used throughout. Where a tissue homogenate was first prepared, it was generally made by blending large pieces of flesh (trimmed free of adhering shell) with 1.5–3.0 ml

of 10 N NaOH per 100 g tissue. Some of the experiments were run by blending the tissue with the alkali in the presence of the extracting solvent. The pigments were extracted by blending 25 g fruit tissue with 50 ml of a 1:1 mixture of petroleum ether (B.P. 60–110° C) and 95% ethanol. The centrifuged supernatant was used for absorbance measurements at 466 and 425 mμ in a Beckman DU or DK-2 spectrophotometer.

The proportion of unisomerized pigment in the mixture was calculated (1) from the ratio (R) of these two absorbances:

$$\% \text{ unisomerized} = \frac{R - 0.40}{0.0098}$$

RESULTS AND DISCUSSION

EFFECT OF RIPENESS

When the entire edible portion of a number of pineapple fruit varying in their ripeness characteristics was used for pigment analysis, the percentage of isomerized pigment found did not exceed 10% regardless of the degree of yellowness of the shell or the translucence of the fruit flesh. However, in an experiment in which various parts of the edible portion of sound fruit were examined, appreciable isomerization was noted in the swollen mid-sections of very ripe fruit. Two fully yellow and fully translucent fruit showed 8–11% pigment isomerization in the top third, 18–24% in the middle third, 7–17% in the bottom third, and 6–9% in the core. By contrast, two fully yellow but

TABLE 1

Mean Percentage Isomerization of Carotenoids in 25-Gram Wedges Cut from Various Sectors of Sound Pineapple Fruit Varying in Their Ripeness Characteristics

Ripeness		Quarter of fruit represented [1]			
Shell color	Translucence	Top	2nd	3rd	Bottom
Completely yellow	Opaque	6 (1)	0 (1)	0 (1)	
	Semi-opaque	0 (2)	2 (4)	0 (4)	0 (3)
	Intermediate	0 (1)	1 (3)	1 (3)	0 (2)
	Semi-translucent	0 (1)	4 (2)	4 (1)	4 (1)
	Translucent	3 (3)	15 (3)	16 (5)	24 (3)
Mostly yellow;	Opaque				2 (1)
some green near	Semi-opaque		2 (6)	0 (1)	0 (1)
top	Intermediate	6 (1)			
	Semi-translucent	1 (1)	0 (3)		
	Translucent	5 (1)		2 (2)	2 (1)
Three-fourths	Semi-opaque	14 (1)		2 (2)	
yellow	Semi-translucent				2 (1)
	Translucent		0 (2 core samples)		
Half yellow	Semi-opaque	6 (1)			
	Intermediate			0 (1)	

[1] Values in parentheses are the number of determinations entering into the mean value for isomerization.

semi-opaque fruit had only 1–3% isomerization in the top two-thirds of the fruit.

These preliminary indications that isomerization of the carotenoids may occur with certain stages of ripeness led to a more detailed study. These data are summarized in Table 1. They confirm earlier indications that appreciable isomerization occurs only in fully ripe and translucent fruit. The pineapple fruit ripens from the bottom fruitlets upward, with several days' difference in reaching ripeness between the bottom and top. Thus it is not surprising to find that the pigment isomers are found in the lower portions but not in the top part of the ripe fruit.

The data in Table 1 demonstrate that when a fruit is allowed to become fully ripe, the lower portion tends to become overripe. The result is a softening and breakdown of the cell walls, allowing the fruit acids to catalyze isomerization of the carotenoid pigments.

EFFECT OF HANDLING

The change in the spectrum of pineapple carotenoids that occurs with cell disruption suggested that isomerization might serve as a measure of bruising or tissue damage from post-harvest handling of the fruit. If one allows sufficient time for isomerization within the damaged tissue to be complete, the extensiveness of bruising sufficient to cause "leakage" of the cells should be measurable.

Table 2 shows some data obtained in one of the tests of the effectiveness of the per-

TABLE 2

Percentage Isomerization of Carotenoids in Various Parts of Pineapple Fruit Bruised by Dropping on Their Sides Onto Concrete and Allowed to Sit Overnight

	Distance dropped, cm	Per cent isomerized pigments			
		Bruised area	Adjacent to bruise	Remainder, bruise side	Opposite to impact
Translucent fruit	33	36	15	10	....
	65	45	8	10	8
	98	38	19	14	9
Semi-opaque fruit	33	29	1	3	5
	65	41	5	1	3
	65	37	1	7	2

centage of isomerized pigment as a bruising index. Isomerization was extensive within the area trimmed out as showing bruising, but was not appreciably higher in the adjoining tissue than in the unbruised opposite side of the fruit. The amount of tissue trimmed out as bruised was directly proportional to the distance the fruit was dropped, and greater for the more translucent fruit than for semi-opaque fruit. Thus the total amount of isomerized pigment also increased with severity of bruising. These and other data confirm that the percentage of isomerization of the pigments can serve as a good index of the severity of the conditions causing bruising.

There are conditions in the handling of pineapple fruit that lead to damage without extensive breakdown of the cells. Static pressure sufficient to flatten the fruit causes fruit loss by tearing the flesh apart with minimal cell wall disruption. Under these circumstances, pigment isomerization would not be expected to correlate with damage of fruit in handling. A semi-opaque fruit crushed by a 30-lb weight overnight showed no detectable isomerization; a translucent fruit showed only a normal 8% carotenoid isomers under similar conditions. Both fruit were severely damaged, however.

EFFECT OF PROCESSING

In the first paper in this series (1) data were presented showing that carotenoid isomerization in tissue homogenates required many hours to reach completion at room temperature, but was complete within 30

min at 100° C. Canning obviously leads to a shift to the isomerized form of the pineapple pigments.

The isomerization observed with the heating of fruit homogenates is also evident in canning of solid-pack items such as slices or chunks.

Freezing and thawing also leads to tissue breakdown. Pigment isomerization is greatly slowed down in the frozen tissue, and thus frozen pineapple chunks contain both forms of the carotenoids. However, as shown in Table 3, the major part of the pigment has been isomerized even before thawing of the frozen chunks. While this does limit the potentialities of using pigment isomerization as a thawing indicator, the pigment data did show a variability in samples at the retail level, indicating that some of the packages had been mishandled either by being allowed to thaw or by being stored too long.

The somewhat erratic data are probably due to sampling, since only 25-g samples were removed at each period. Somewhat different acidity levels in the frozen chunks could account for the observed differences in rates of isomerization after thawing seen in Table 3.

LITERATURE CITED

[1] Singleton, V. L., Willis A. Gortner, and H. Y. Young. Carotenoid pigments of pineapple fruit. I. Acid-catalyzed isomerization of the pigments. *Food Research* (this issue).

TABLE 3

PERCENTAGE ISOMERIZATION OF CAROTENOIDS IN COMMERCIAL FROZEN PINEAPPLE CHUNKS AND IN THE CHUNKS FOLLOWING THAWING

	Frozen	Thawed and held at 24° C			
		2.5 hr	3.5 hr	5 hr	23 hr
Samples from packer's warehouse					
A	79	78	96	86	....
B	84	96	98	100	100
C	78	78	78	92	93
D	88	81	83	86	100
Samples from grocer's shelf					
E	89	95	95	98	100
F	75	78	84	78	....
G	100	100	100	100	100
H	100	100	100	100	100

AN EXTRACTION TECHNIQUE FOR RECOVERY OF FLAVORS, PIGMENTS, AND OTHER CONSTITUENTS FROM WINES AND OTHER AQUEOUS SOLUTIONS[1]

V. L. SINGLETON[2]

In work with wines and other aqueous-organic mixtures a recurrent problem is the collection of a group of constituents such as pigments, volatile or bitter flavors, etc. One may wish to concentrate them from the relatively dilute natural solution, to purify them by separation from other constituents, or to collect them into a menstruum more amenable to further treatment. Extraction of the wine or similar aqueous solution with an immiscible solvent is often a convenient method of reaching these objectives if the desired constituents are readily extracted.

One may be concerned, however, with relatively polar compounds that will not be extracted efficiently with butanol or any common solvent giving a two-phase system with water. Particularly if concentration of a group of constituents is a major objective or one is concerned about quantitative recovery with minimum danger of fractional loss of any member of the group of desired constituents, distribution coefficients highly favoring the solvent are required but may not be provided by available immiscible solvents.

An alternative is to change the system by adding an additional component (acid, base, solute, or solvent) so that the desired substances are more readily extracted, i.e., the distribution coefficients of the desired components (solvent/water) are increased to an effective level. The fact is well known that compounds may be salted-out of aqueous solution either as a separate phase or into extracting solvents. Add-

ing salts to the aqueous phase of an ordinary extraction system to increase its effectiveness is common, though the application of the technique is usually empirical.

An obvious extension of this procedure is suggested by the fact that solvents miscible with water may not be miscible with concentrated aqueous solutions and the solvent itself may be salted out. This would result in a system in which the distribution coefficients of relatively polar compounds should be much greater than those in a butanol-water system, increased both by the decreased solubility of the compounds in the aqueous phase and by the greater extraction and solvent power of the water-miscible solvent than that of the immiscible. General works on solvent extraction refer to this idea only briefly, if at all (14, 16, 17). The scattered experimental references to an extraction system of this type (9, 15) are a part of other research, and the general applicability and conditions are not reported. Detailed study of extraction systems of this general type has been reported by Albertsson, Porter, Craig, and others (1, 3, 4, 11), but their objective was systems with a high water content in both phases (near the plait point) for partition or countercurrent distribution studies in which a relatively low distribution coefficient is desirable.

This study was made to obtain more data on the usefulness of water-miscible-solvent/concentrated-aqueous-solution systems in providing very high partition coefficients, thereby producing concentrated extracts of water-soluble flavors, pigments, etc., in solvents more useful than water for further processing. The aim was to determine which were the most powerful salting-out agents among common laboratory reagents, what

was the most-polar of common water-miscible solvents that promised usefulness in such systems, and what was the composition and usefulness of one promising system. It was expected that extracted materials would be purified of contamination with such compounds as sugars but would be contaminated with salts, and therefore purification was considered less important than concentration, quantitative extraction, and relatively water-free solutions.

EXPERIMENTAL PROCEDURE

The salts (and other solutes) and solvents used were commercial analytical reagent quality except 2-methoxyethanol and formamide, which were practical grade. The water, ethanol, methanol, acetone, 2-methoxyethanol, n-butanol, n-propanol, and i-propanol were distilled, and a center fraction with a proper boiling point was used. Temperatures were 25 ± 1°C unless otherwise noted. Saturated solutions were prepared by frequent shaking of a mixture of solute and water (or other solvent mixture) until the formation of solution "schlieren" at the solid-solution interface could no longer be observed (12 hours or more). Apparent pH was determined with a Beckman GS pH meter equipped with standard glass and calomel electrodes.

Miscibility was estimated by adding 3 drops of the solvent to 3 drops of the saturated aqueous solution. If precipitation of the solute obscured whether or not two liquid phases were formed, dropwise additions of water and solvent were made slowly, with shaking to ensure equilibrium, until enough precipitate dissolved to permit a decision. For each solute, retests were made of the most polar solvent that gave two phases, the least polar solvent that gave one phase, and any results that seemed anomalous. Slightly larger amounts were used and the solutions were faintly colored with eosin-y to help show the formation of a second liquid phase.

Analyses were performed by determination of the solids content of an aliquot dried to constant weight at 80° in air or at 25 ± 1°C *in vacuo* over concentrated sulfuric acid. Calculations of solubility, apparent distribution coefficients, etc., were based on solids determinations upon samples and blanks handled identically to minimize the effects of partial loss of water of hydration, etc.

The phase diagram was prepared from data obtained in the usual way by titrating aqueous salt solutions with ethanol until a faint turbidity showed the formation of a second liquid phase (8). In experiments involving minimal saturation, the solutes were added in small increments so that, at saturation, no more than a few small crystals of excess solid salt were present. Time was allowed to counter heating or cooling effects from the mixing of solvents or solutes. Solutions being used for analysis were centrifuged in closed tubes to prevent contamination of one phase with another. The results were obtained very carefully, commensurate with the objective, but there was no intention of obtaining the ultimate physical-chemical parameters of these systems.

RESULTS

In table 1 a plus sign indicates the solvents that gave two liquid phases when added to the saturated aqueous phases of the various solutes, and a minus sign indicates those with a single liquid phase.

It appears from these data that methanol is the most-polar common solvent that can be salted out of water solution under practical conditions for solvent extraction. Ethanol was selected for further study since the range of possible salting-out agents, general availability, boiling point, etc., seemed to offer greater usefulness than 2-methoxyethanol or methanol. Of course, ethanol is particularly convenient in work with wine since it is already present. For reasons discussed below, ammonium sulfate and sodium dihydrogen phosphate were the salts selected for further study.

Figure 1 shows the solubility of sodium dihydrogen phosphate (added as the monohydrate) and ammonium sulfate in aqueous ethanol solutions. It can be seen that sodium dihydrogen phosphate offers no particular advantage over ammonium sulphate in terms of solubility in ethanol; neither is very soluble. Both these salts were usable over a fairly wide pH range, as shown by the fact that saturated solutions of sodium

dihydrogen phosphate adjusted with concentrated sodium hydroxide to pH 7.0, 6.3, and 4.7 and mixed with an equal volume of 95 percent ethanol, gave nearly the same relative volumes of two phases as did the original pH 3.5 solution. Similarly, saturated ammonium sulfate adjusted with concentrated solutions of sulfuric acid and ammonium hydroxide to pH 3.0, 6.3, and 7.0 and mixed with an equal volume of 95 per cent ethanol, gave nearly the same relative volumes of each phase as did the original pH 5.1 solution.

In view of these facts plus the fact that

TABLE I

Formation of Two Liquid Phases (+) as Observed When the Solvent is Mixed with a Saturated Aqueous Solution of the Solute (at 25 ± 1°C.)

SOLUTES	EtOAc	n-BuOH	n-PrOH	Pyridine	Acetone	i-PrOH	EtOH	MeOEtOH	MeOH	HO(CH₂)₂OH	glycerol	formamide	HOAc	Lactic Acid	Apparent pH (of satd. aq. soln.)	[a] g/100g aq. soln. at 25°C. (except as noted)
$K_2CO_3 \cdot 1\frac{1}{2} H_2O$	+	+	+	+	+	+	+	+	+	—	—	——	—		12.5	52.9
$NaH_2PO_4 \cdot H_2O$	+	+	+	+	+	+	+	+	+	—	—	—	——	—	3.5	—
$Na_2CO_3 \cdot 10\ H_2O$	+	+	+	+	+	+	+	+	+	—	—	—	——	—	11.2	22.6
$KF \cdot 2H_2O$	+	+	+	+	+	+	+	+	⊕	—	—	—	——	—	11.7	48.2 (18°)
$(NH_4)_2SO_4$	+	+	+	+	+	±	+			—	—	—	—	—	5.1	42.6 (20°)
$MgSO_4 \cdot 7\ H_2O$	+	+	+	+	+	+	±	—		—	—	—	—	—	6.2	55.3
Na_3 Citrate $\cdot 5H_2O$	+	+	+	+	+	+	±			—	—	—	—	—	8.1	48.1
$(NH_4)_2H$ Citrate	+	+	+	+	+	+	±			—	—	—	—	—	5.0	48.7
$(NH_4)_2HPO_4$	+	+	+	+	+	+				—	—	—	—	—	7.9	56.2 (14.5°)
$KHCO_3$	+	+	+	+	+	+				—	—	—	—	—	9.2	26.6
$NaCl$	+	±	+	+	+	+				—	—	—	—	—	6.6	26.5
$NaOAc$	+	+	+	+	+	+				—	—	—	—	—	7.1	33.6
Na_2SO_4	±	+	+	+	+	+				—	—	—	—	—	4.6	21.8
$CaCl_2 \cdot 6 \cdot H_2O$	+	+	+	+	±	+				—	—	—	—	—	3.6	46.1
KCl	+	+	+	±	—	+				—	—	—	—	—	6.5	26.5
$K_2C_2O_4 \cdot H_2O$	+	+	+	+	—					—	—	—	—	—	7.7	28.3
KH_2PO_4	+	+	+	+	—					—	—	—	—	—	4.1	—
$(NH_4)_2CO_3$	+	+	+	+	—					—	—	—	—	—	8.8	20
NH_4NO_3	+	+	+	+	+					—	—	—	—	—	5.9	68.3
NH_4OAc	+	+	—	+	+					—	—	—	—	—	7.6	—
$Mg(OAc)_2$	+	+	—	+	±					—	—	—	—	—	7.3	—
$KOAc$	+	—	—	+	+					—	—	—	—	—	9.3	68.7
$NaHCO_3$	+	+	+	—						—	—	—	—	—	8.8	—
glucose $\cdot H_2O$	+	+	+	—						—	—	—	—	—	6.2	49.5
NH_4Cl	+	+	+	—						—	—	—	—	—	5.5	26.3 (15°)
Na_2HPO_4	+	+	—	±						—	—	—	—	—	8.8	4.2 (17°)
H_2O	+	+	—							—	—	—	—	—	6.8	—
$Na_2B_4O_7 \cdot 10\ H_2O$	+	+	—							—	—	—	—	—	9.2	±
$CaCO_3$	+	+	—							—	—	—	—	—	9.1	—
$CaSO_4 \cdot 2\ H_2O$	+	—								—	—	—	—	—	7.4	0.2
$(NH_4)_2C_2O_4 \cdot H_2O$	+	—								—	—	—	—	—	6.7	5.0
$Na_2C_2O_4$	+	+	—							—	—	—	—	—	9.0	3.5
K_2SO_4	+	+	—							—	—	—	—	—	7.4	10.8
KNO_3	+	+	—							—	—	—	—	—	6.9	28.0
$Mg(NO_3)_2 \cdot 6\ H_2O$	+	±	—							—	—	—	—	—	5.0	57.3 (18°)
$Ca(OAc)_2$	+	+	—							—	—	—	—	—	7.2	—
Tartaric Acid	+	+	—							—	—	—	—	—	0.6	58.5 (15°)
Citric Acid $\cdot H_2O$	±	—								—	—	—	—	—	0.7	67.5

[a]From the Merck Index, 7th Ed., Merck & Co., Rahway, N. J., 1960
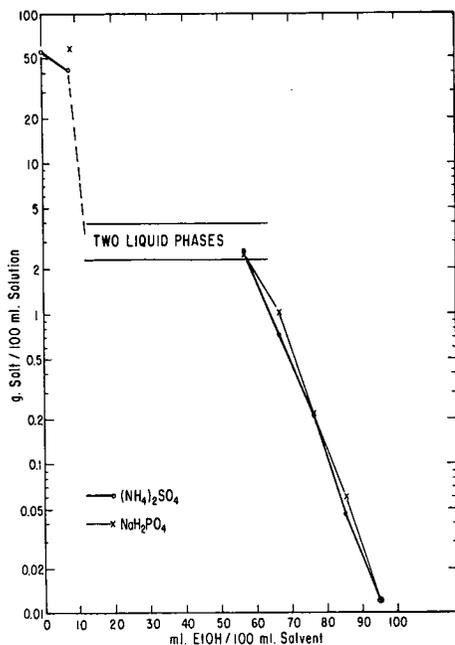
Figure 1. Solubility of (NH₄)₂SO₄ and Na₂PO₄ in Aqueous Ethanol Solutions at 25°C.

small crystals of salt and a faint turbidity from formation of a second liquid phase. The composition of this solution coincides with that of solution B (Figure 2), thus representing an independent check on the composition of this phase at maximum-salt minimum-ethanol composition. Point A was taken as the composition that, upon further addition of ethanol, deposited crystalline salt rather than a second liquid phase. The AB tie-line represents the composition of the system giving two liquid phases and completely saturated with ammonium sulfate. It gives the ethanol phase richest in ethanol and poorest in salt, that is, solution B.

The compositions of the AB pair at 25°C as determined by the several experiments were: aqueous phase—35.5 per cent (w/w) ammonium sulphate, 41.6 per cent water, and 56.1 per cent ethanol. A solution of each of these compositions was prepared, and 50 ml of each were mixed. They immediately separated into 50 ml layers, indicating the correctness of these compositions.

The amount of each component required for a given experiment is readily calculated from the data given. As an easily remembered approximation the following has proven accurate enough for most ex-

sodium dihydroxide phosphate was much slower to saturate aqueous solutions and was, of course, relatively highly buffered, ammonium sulfate appeared to be the salt of choice for more detailed study.

Figure 2 shows the resultant curve when various concentrations of aqueous alcohol were just saturated with ammonium sulfate and the proportion of the total volume, that the ethanol phase was, was determined. This offered a simple means of estimating the composition of the two phases. Points A and B on this curve respectively represent the ethanol and water phase compositions, which form mutually saturated immiscible layers when saturated with salt. The solids contained by such solutions were determined (see also figure 1).

Figure 3 gives the phase diagram of this ternary system. Point B was determined by starting with a saturated aqueous solution of ammonium sulfate and adding ethanol or water dropwise until there were a few


Figure 2. Estimation of Composition at Points of Transition to a Single Liquid Phase in the Ethanol-Saturated-Aqueous-(NH₄)₂SO₄ System at 25°C.
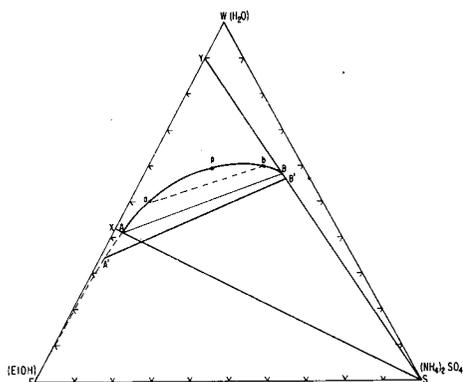
Figure 3. Phase Diagram at 25°C. of the Ternary System Ethanol-Water-Ammonium Sulfate (A'B' at 50°C.).

traction experiments: for each volume desired in the ethanol phase—add ⅔ volume of ethanol and 2.5 g of ammonium sulfate for each 100 ml; subtract ⅓ of the desired ethanol phase volume from the volume of water to be extracted and for each 100 ml of the remainder, add 64 g ammonium sulfate and 15 ml ethanol. For example, if 100 ml of water solution is to be extracted and the ethanol phase should be about 50 ml, about 55 g ammonium sulfate and 45 ml of ehanol will be required. One may use 95 per cent ethanol, of course, if allowance is made for the water content.

By experimentation and from the literature, data have been obtained regarding the effect of temperature upon the ethanol-ammonium sulfate-water system. The effects in the usual room-temperature range are not great, but are such that it is preferable to work at the warmer temperatures. The lower critical temperature of demixing has been reported to be 8°C (18), but the separation of two liquid phases has also been reported at 0°C (13). In any case, it is low enough that extractions may be carried out at all usual laboratory temperatures.

It was found that there was more water, and consequently more salt (about 1 percent more), in the ethanol layer at 20°C than at 25°C. This is probably the result of the

fact that at warmer temperatures more salt is required to saturate the aqueous layer and it then competes more strongly for the water in the ethanol phase. The A'B' tie-line shown in Figure 3, taken from published data on this system at 50°C (13), illustrates a continuation of this effect even at this relatively elevated temperature. It is obviously preferable to conduct extractions at a relatively warm temperature (taking care that enough ammonium sulfate is present to saturate the system).

The apparent distribution coefficients were determined for a series of organic substances in the system n-butanol/water, n-butanol/saturated ammonium sulfate, and ethanol/saturated ammonium sulfate. The results are shown in table 2.

DISCUSSION

It is clear that the ethanol system offers real advantages in terms of increased distribution coefficient, and thus a greater concentration and efficiency of extraction of relatively polar substances than with immiscible solvent systems. Separation of polar compounds from even more polar compounds, such as represented by glycine and glucose, can still be rather good. It is interesting to note (Table 2) that the distribution coefficients of ionic or extremely polar compounds were decreased in the butanol-salt system as compared to the butanol-water system. Presumably this is because the salt solution competes for the water and decreases the water content of the butanol layer. This helps explain the much greater extraction power of ethanol than butanol: it is not only a more powerful solvent for polar compounds but also contains a higher proportion of water even in the presence of saturated salt solutions. Since distribution coefficients of typical compounds were near one in the ethanol system, this system should be applicable for countercurrent distribution of sugars and other very polar compounds.

Considering the data shown and the further possibilities of pH adjustment, countercurrent extraction, preliminary extraction, etc., it is felt that the usefulness of this system and the technique in general have been verified. Systems other than the ethanol-ammonium

TABLE 2

Relative Extraction of a Series of Organic Solutes from Water as Influenced
by Salting-out the Solute and the Solvent

Apparent Distribution Coefficient[a]

	n-BuOH/H$_2$O	n-BuOH/satd.(NH$_4$)$_2$SO$_4$	EtOH/satd.(NH$_4$)$_2$SO$_4$
glycine	0.01	0.003	0.09
glucose	0.03	0.004	0.6
tartaric acid	0.2	0.02	0.5
caffeine	0.8	46	>100
succinic acid	0.9	1.9	>100
pyrogallol	1.8	5.7	>100
gallic acid	4.0	3.6	>100
d-catechin	22	>100	>100
tannin (NF)	>100	>100	>100

[a]As measured by the concentration of solids in the solvent phase corrected for the solids content
of a blank determination divided by the aqueous phase solids concentration determined by difference
from the solids content of the original aqueous solution.

sulfate-water system may be applied in similar fashion and may be preferable in particular situations. The data in table 1 can be used as a guide in selecting or designing other systems. It should be noted that the testing of a system at a higher temperature will give a two-liquid-phase system if it has a critical temperature of de-mixing higher than 25 ± 1°C (18). A false or unstable positive might have resulted if the solvent gave a supersaturated solution from which crystallization was slow. Finally, although the conditions were chosen to minimize this possibility, the particular ternary compositions chosen for testing might have fallen outside the range of composition capable of giving two liquid phases with a given solute-solvent-water system. However, the data in table 1 agree well with available published results, and, since a solute capable of salting out methanol would be expected to salt-out ethanol and less polar solvents also, the data reinforce each other.

The few solutes (Table 1) other than salts that were tried were relatively poor agents for "salting-out" miscible solvents. It appears that the better agents for salting-out solvents are often the salts that have anions capable of multiple hydrogen bonding with water molecules (such as carbonate, phosphate, sulfate, and fluoride) combined with cations that allow a high solubility in water (and in the case of ammonium ion participate in hydrogen bonding). It is interesting to note the similarity of the results of these tests to the results of experiments intended to determine which salts are better for salting-out proteins and other compounds (5, 8, 12), dehydrating moist solvents, or decreasing vapor pressure (6, 7, 10). Also, similar are the solvent series in approximate order of "polarity" as determined by dielectric constant and Trappe's elutropic series (2, 17). It appears that a few tests in comparison with a series of salts and solvents on this list would be a quick and useful aid in deciding the appropriate place in such series for additional solutes and solvents.

Although ethanol seems to offer a good compromise between available salting-out agents on the one hand and high polarity and solvent power on the other, one might prefer to work with acetone or other solvents under some circumstances. Even in such cases, there appears to be no reason to prefer a salting-out agent of low potency, other factors being equal. The salt to be used must form a stable two-liquid phase system at room temperature and be relatively unreactive. It should be inexpensive, easily available, non-toxic, and capable of use over an adequate pH

range. The salt is preferred that has a low solubility in the solvent phase and dissolves to saturation or crystallizes rapidly from supersaturated solutions. Since polyhydrates are often slow to dissolve or crystallize near saturation and since their phase diagrams are more complicated, salts crystallizing without or with less water of hydration are preferable, other things being equal.

. The salts in table I that give promise of usefulness by forming two layers with ethanol are ammonium sulfate, sodium dihydrogen phosphate, sodium carbonate, potassium carbonate, potassium fluoride, magnesium sulfate, trisodium citrate, and diammonium citrate. It was found that the two-liquid-phase systems formed by adding ethanol to the saturated aqueous solutions of the latter three salts were metastable, and, by gradually depositing salt, reverted to a single liquid phase. Saturation of about 50 per cent aqueous ethanol with these three salts did not lead to de-mixing of the liquid phase at room temperature. They therefore were not studied further. The two carbonates were very effective agents for salting-out, but they are, of course, quite alkaline, and the respective bicarbonates were inefficient in salting-out solvents. Wines and other natural products of interest contain acids, sugars, esters, etc., that would react with or be degraded by these salts. Potassium fluoride is alkaline, toxic, and relatively expensive. For these and the reasons given before, ammonium sulfate appeared to be the salt most useful among those tested.

Considering the objectives of this research and the advantage of the ethanol-ammonium sulfate-water system, the compositions represented by points A and B in figure 3 are those that should be most useful. They should give the highest possible distribution coefficient favoring the ethanol phase for any fourth substance that is more soluble in ethanol than in water saturated with salt.

It is recognized, of course, that the addition of other components invalidates strict application of data obtained with the pure ternary system. In practice, however, the composition and phase separations predicted from the ternary diagram have proved reliable in all cases investigated.

Projection of the AB line on the water-ethanol axis from the salt point (points x and y, figure 3) gives the maximum and minimum ethanol contents of a water solution that, when saturated with ammonium sulfate, will just begin to form a second liquid phase. Note that the presence of excess salt (ensuring saturation) after addition to any aqueous ethanol solution between x and y will ensure that the alcohol phase will coincide with composition A, and therefore have the desired properties. Of course, the relative volumes of the two phases will depend on the nearness to x or y of the composition of the original solution.

If the ammonium sulfate present is less than the maximum soluble amount, the composition will fall in area APB and the two liquid phases will be a tie-line pair, such as a-b (schematic), whose compositions approach each other, and distribution coefficients of other components in the two phases will therefore approach 1. Such mixtures may prove useful for partition chromatography or countercurrent distribution. The plait point, P, representing the composition of disappearing compositional difference in the two liquid phases, was determined only approximately. Systems with too much or too little ethanol to fall between x and y will have only one liquid phase, even when saturated with ammonium sulfate.

Systems of the ethanol-ammonium sulfate-water type have also some manipulative advantages; for example, the high surface tension and density of the aqueous phase compared to those of the solvent phase (A about 0.89 g/ml, B about 1.16 g/ml at 25°C for the ethanol-ammonium sulfate-water system) are such that the phases usually separate quickly and emulsification is seldom troublesome. Because of the high solids in the aqueous phase, however, one must be careful to avoid contamination of the alcohol phase by droplets of the aqueous. Polymeric substances such as proteins and gums often precipitate in the interface, being soluble in neither layer, and can be recovered by filtration if desired.

It is interesting to note that the salting-

out of a second (ethanol) phase from a wine by ammonium sulfate, followed by removal of the salt from the ethanol phase (by drying, ion exchange, etc.) can give a consumable solution suitable for taste testing or further flavor studies.

SUMMARY

The forcing of water-miscible solvents from aqueous solution by saturation of the water with salts and other solutes and the use of such systems in extraction of solutions similar to wines has been investigated. Ethanol was the solvent of choice among those tried. Ammonium sulfate was the salting-out agent most generally useful among the 38 tested. The phase diagram of the ethanol aqueous-ammonium sulfate system was prepared and the composition of the two liquid phases determined for the mixture that would have the least salt and the most ethanol in the solvent phase. A comparison of this system with butanol-water and butanol-ammonium sulfate systems showed that distribution coefficients of relatively polar compounds favored the solvent layer for the ethanol system much more than for the butanol systems. The ethanol-water-ammonium sulfate and similar systems offer considerable promise in special situations as a means of extraction and concentration of volatile flavors, bitter flavors, tannins, pigments, etc. and fractionation of them from sugars, acids, etc. as found in wines and similar solutions.

LITERATURE CITED

1. Albertsson, P. A. Particle fractionation in liquid two-phase systems. Composition of some phase systems and the behavior of some model particles in them. Application to the isolation of cell walls from microörganisms. Biochem. et Biophys. Acta 27:378-95 (1958).

2. Cassidy, H. G. Technique of Organic Chemistry, Vol. 5, Interscience Publishers Inc., New York (1951).

3. Colowick, S. P. and N. O. Kaplan. Methods in Enzymology, Vol. I, p. 98-112, Academic Press Inc., New York (1955).

4. Craig, L. C., C. Golumbic, H. Mighton, and E. Titus. Identification of small amounts of organic compounds by distribution studies. III. The use of buffers in counter-current distribution. J. Biol. Chem. 161:321-32 (1945).

5. Dobry-Duclaux, A. Über Lyotropie. Chem. Ztg. 76:805-9 (1952).

6. Foote, H. W. and S. R. Scholes. The vapor pressure of hydrates, determined from their equilibria with aqueous alcohol. J. Am. Chem. Soc. 33:1309-26 (1911).

7. Frankforter, G. B. and S. Temple. Equilibria in the systems of the higher alcohols, water and salts. J. Am. Chem. Soc. 37:2697-716 (1915).

8. Glasstone, S. Textbook of Physical Chemistry, 2nd Ed., p. 790-814, 1254, D. Van Nostrand Co., New York (1946).

9. Herbert, D. and J. Pinsent. Crystalline bacterial catalase. Biochem. J. 43:193-202 (1948).

10. Meissner, H. P. and C. A. Stokes. Solvent dehydration by salting out-prediction of maximum degree of dehydration. Ind. Eng. Chem. 36:816-20 (1944).

11. Porter, R. R. Partition chromatography of insulin and other proteins. Biochem. J. 53:320-8 (1953).

12. Schmidt, C. L. A. The Chemistry of the Amino Acids and Proteins, 2nd Ed., C. C. Thomas, Springfield, Ill. (1945).

13. Seidell, A. Solubilities of Inorganic and Metal Organic Compounds, 3rd Ed., Vol. I, p. 1130. D. Van Nostrand Co., New York (1940).

14. Seidell, A. and W. F. Linke. Solubilities of Inorganic and Organic Compounds, Supplement to the 3rd Ed., p. 828, D. Van Nostrand Co., New York (1952).

15. Thomas, S. O., J. A. Lowery, and V. L. Singleton. Nucleocidin and the process of obtaining the same. U. S. Patent 2,914,525 (Nov. 24, 1959).

16. Treybal, R. E. Liquid Extraction, p. 2, 19-20, McGraw-Hill Co., New York (1951).

17. Weissberger, A. Technique of Organic Chemistry, Vol. 3, Chapter 4, p. 299, Interscience Publishers Inc., New York (1950).

18. Zernike, J. Chemical Phase Theory, p. 309-325, Ae. E. Kluwer, Antwerp (1955).

Complexity of Flavor and Blending of Wines

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SUMMARY

Thirty-four pairs of similar, commercially acceptable wines and a freshly prepared 50-50 blend of each pair were scored for quality by a small expert panel. The composite scores of the blends did not differ to even a low degree of significance from the score of the highest-scoring member of the pair. The scores of the blends were very highly significantly better than the scores for the low-scoring wines or the mean of the low- and high-scoring wine in each pair. In 7 cases among the 34 the blend's composite score was higher than that of the highest-scoring wine, and in no case was it lower than that of lowest-scoring wine of the pair. Increased complexity of flavor is believed to be a major factor in the favorable effect of blending shown in this study; other possible effects are discussed.

Complexity has long been considered a desirable factor in the quality of most flavorsome or odorous products. In cookery or perfumery, efforts are made to improve the product by adding many ingredients in amounts small enough to influence flavor or odor without being individually obvious. In wines, flavor complexity is considered very important to high quality and is believed to be one of the primary effects produced by proper aging. Although much blending of different lots of wine is done commercially, largely to improve or standardize the average final product quality, this blending remains largely an art. So far as the authors have been able to determine, there are no published studies on any product that demonstrate the value of a complex flavor or estimate the value of blending in producing increased complexity. Of course, correcting a frank deficiency or meeting uniformity standards by blending is an obviously similar problem and one relatively easily solved, but can blending or flavor modification within the limits of a single commercially acceptable product be expected to improve it, and, if so, how much?

As a part of a research program upon the aging of wines, experimental and less subjective evidence was sought testing the validity and estimating the magnitude of the presumed favorable effect of flavor complexity on wine quality.

EXPERIMENTAL METHODS

Pairs of wines were sought that were similar as to type, scored similarly as to quality and within the commercially acceptable range, and yet appeared to have different flavor qualities. The wines from which selections were made were the 1960 vintage produced in the University winery by standard production methods. Most of the grapes were from the University vineyards at Davis or Oakville, and the wine lots were each from a single grape variety. The wines were presented to a small (10-member) panel whose members had had considerable experience as sensory panelists and were familiar with wines. The manner of presentation and scoring was essentially as reported by Ough and Baker (1961), except that 10 samples were tasted each day and these were grouped as to type (and usually vineyard region), e.g., dry red table wines one day, red dessert wines the next, dry white table wines the third, etc. The wines were first scored during November–January, 1960–61, about three months after vintage. The average scores and comments were used to choose from among a given daily set of ten wine lots, two that had the most similar scores but the most different comments. These two lots, plus a sample prepared by blending equal volumes of these two, were retasted about five months later as part of a continuing panel evaluation of the 1960 vintage (during April–June, 1961).

The usual chemical analyses for alcohol, total acid, etc., were completed on each wine shortly after the first tasting. The wine lots were from 5 to 50 gallons each. The dry white wines were held in glass, cork-stoppered containers throughout the experiment with the exception of wine No. 3 (Table 1) which was in a 25-gallon previously used oak barrel until it was bottled on February 2, 1961. The other wines were in previously used oak cooperage throughout the experiment with the exception of wine Nos. (Table 1) 38, 41, 42, 45, 47, 48, 49, 50, 51, 56, 57, 58, and 66, which were in glass, cork-stoppered containers,

Table 1. Origin and analyses of the selected wine pairs.

Wine no.	Grape variety [a]	Total acidity (g tartaric/ 100 ml)	Volatile acidity (g HOAc/ 100 ml)	pH	Extract (g solid/ 100 ml)	Sugar (g glucose/ 100 ml)	Ethanol (% v/v)	Tannin (g tannic acid/ 100 ml)	Color (arbitrary units)[b]	First tasting Mean score	Second tasting Comments	Second tasting Mean score
Dry white table wines												
1	Sylvaner	0.76	0.027	3.11	2.6	0.1	12.8	0.02	7.0	12.7	rich, med. distinctive	15.4
2	Sylvaner	0.98	0.028	3.13	2.4	0.1	12.6	0.02	7.3	13.7	fresh, lo dist.	14.3
blend											fresh, full med. dist.	15.3
3	Pinot blanc	0.62	0.022	3.35	2.2	0.1	10.6	0.03	7.0	14.0	hi dist., slt. stemmy	14.0
4	French Colombard	1.03	0.022	2.93	2.9	0.2	10.3	0.02	7.2	13.6	very woody, tart, spicy	13.9
blend											hi dist., tart, slt. wood	15.8
5	Chenin blanc	0.73	0.016	3.35	2.4	0.1	12.0	0.02	5.6	13.2	fruity, young	14.6
6	Aligote	0.63	0.024	3.24	2.3	0.2	13.6	0.03	5.0	13.9	lo dist., slt. weedy, bitter	13.6
blend											fruity, lo dist.	14.3
7	Sauvignon blanc	0.80	0.020	3.12	2.8	0.1	13.7	0.02	6.9	15.8	med. dist., slt. fruity	16.0
8	Sauvignon blanc	0.81	0.013	3.17	2.9	0.1	12.4	0.02	3.6	15.6	med. dist., Sauv. blanc, tart	15.9
blend											med. dist., spicy, aromatic	15.9
9	White Riesling	0.70	0.016	3.19	2.5	0.2	13.0	0.03	7.7	14.2	weedy, overripe Riesling	15.1
10	Chenin blanc	0.85	0.019	3.13	2.6	0.1	10.9	0.02	3.3	13.9	acidulous	14.3
blend											fruity, distinctive, slt. oxid.	14.4
11	Aligote	0.58	0.032	3.33	2.4	0.3	13.3	0.02	2.6	16.6	med. dist., fruity, rich	16.4
12	Aligote	0.63	0.022	3.19	2.4	0.2	13.7	0.03	2.9	15.1	med. dist., clean, slt. thin	15.3
blend											med. dist., fruity, fragrant	15.8
13	Chenin blanc	0.66	0.012	3.32	2.4	0.1	10.9	0.02	2.6	14.2	med. dist., slt. oxidized	15.1
14	Clairette blanche	0.49	0.022	3.52	2.6	0.1	12.9	0.02	10.7	13.8	lo dist., slt. meaty, thin	14.1
blend											lo dist., clean, balanced	14.9
15	French Colombard	0.79	0.016	3.15	2.6	0.1	12.2	0.02	3.3	11.6	fruity, odd aftertaste	14.9
16	Sémillon	0.54	0.018	3.68	4.0	0.1	13.6	0.03	7.1	12.4	med. dist., full	14.1
blend											med. dist., clean, SO$_2$	14.8
17	White Riesling	0.76	0.018	3.19	3.3	0.6	13.7	0.03	3.4	16.7	hi dist., full	16.9
18	Emerald Riesling	0.87	0.025	2.90	2.8	0.1	13.2	0.03	3.8	15.7	hi dist., rich	15.6
blend											hi dist., fruity, fresh	17.3

Table 1. Origin and analyses of the selected wine pairs (cont'd).

Wine no.	Grape variety [a]	Total acidity (g tartaric/100 ml)	Volatile acidity (g HOAc/100 ml)	pH	Extract (g solid/100 ml)	Sugar (g glucose/100 ml)	Ethanol (% v/v)	Tannin (g tannic acid/100 ml)	Color (arbitrary units) [b]	First tasting Mean score	Second tasting Comments	Second tasting Mean score
19	St. Emilion	0.54	0.018	3.58	2.8	0.3	13.5	0.03	16.7	14.8	vinous, soft	13.5
20 blend	St. Emilion	0.58	0.018	3.71	3.0	0.3	14.3	0.03	4.6	14.5	vinous, thin, slt. flat	13.3
											vinous, slt. flat	13.5
21	St. Emilion	0.61	0.016	3.61	2.8	0.1	14.9	0.03	3.6	13.5	med. dist., clean	13.8
22 blend	St. Emilion	0.65	0.015	3.34	2.7	0.1	11.9	0.03	4.0	13.1	vinous, slt. bitter	13.4
											vinous, fresh, balanced	14.6
23	St. Emilion	0.57	0.016	3.49	3.0	0.2	13.1	0.04	3.7	14.1	med. dist., slt. bitter	14.0
24 blend	St. Emilion	0.54	0.028	3.54	3.1	0.6	12.6	0.03	3.3	14.5	med. dist., bitter	13.5
											med. dist., tart	13.8
25	St. Emilion	0.54	0.016	3.63	2.8	0.2	14.2	0.03	5.6	13.0	med. dist., full	13.1
26 blend	St. Emilion	0.52	0.018	3.78	3.1	0.2	13.5	0.03	5.3	11.9	med. dist., slt. skunky	11.9
											med. dist., slt. bitter	13.0
27	Folle blanche	0.65	0.040	3.18	2.9	0.2	9.9	0.03	5.6	13.5	med. dist., tart	12.2
28 blend	Flame Tokay	0.43	0.026	3.88	2.5	0.1	11.2	0.02	5.6	13.9	med. dist., fruity, slt. off	11.3
											med. dist., fruity, tart	13.3
29	French Colombard	0.84	0.023	3.00	2.9	0.2	12.5	0.03	7.7	15.1	lo dist., slt. bitter, tart	15.1
30 blend	Furmint	0.65	0.018	3.78	3.2	0.2	12.9	0.03	4.6	14.0	med. dist., overripe grapes	13.7
											med. dist.	14.0

Dry red table wines

Wine no.	Grape variety [a]	Total acidity (g tartaric/100 ml)	Volatile acidity (g HOAc/100 ml)	pH	Extract (g solid/100 ml)	Sugar (g glucose/100 ml)	Ethanol (% v/v)	Tannin (g tannic acid/100 ml)	Color (arbitrary units) [b]	First tasting Mean score	Second tasting Comments	Second tasting Mean score
31	Pinot noir	0.72	0.017	3.47	3.2	0.1	11.4	0.20	167	14.5	distinctive Pinot	14.6
32 blend	Pinot Pernand	0.58	0.022	3.62	3.4	0.1	13.4	0.12	111	13.8	sla. oxidized, malolactic	11.8
											Pinot, malolactic, wood	13.8
33	Gamay Burgogne	0.72	0.031	3.32	3.2	0.1	10.5	0.05	31	13.3	med. dist., fruity, rose, wood	12.4
34 blend	Valdepenas	0.71	0.022	3.79	3.4	0.1	11.9	0.14	117	14.7	lo dist., oxidized, med. red	11.9
											complex, wood	14.2
35	Meunier	0.72	0.017	3.66	3.3	0.2	13.5	0.15	110	13.4	wood, slt. flat, fruity	14.4
36 blend	Gamay Beaujolais	0.71	0.017	3.67	3.1	0.3	14.4	0.14	100	13.1	oxidized, odd flavor	11.4
											wood, slt. Pinot, slt. flat	14.3

Table 1. Origin and analyses of the selected wine pairs (cont'd).

Wine no.	Grape variety [a]	Total acidity (g tartaric/ 100 ml)	Volatile acidity (g HOAc/ 100 ml)	pH	Extract (g solid/ 100 ml)	Sugar (g glucose/ 100 ml)	Ethanol (% v/v)	Tannin (g tannic acid/ 100 ml)	Color (arbitrary units)[b]	First tasting Mean score	Second tasting Comments	Second tasting Mean score
37	Selection 37	0.70	0.019	3.49	3.3	0.2	12.6	0.15	200	14.7	wood, rich	15.6
38	Selection 545	0.86	0.025	3.58	3.5	0.3	13.8	0.35	500	14.9	fruity, slt. bitter	15.1
blend											slt. wood, rich tannic	15.6
39	Malbec	0.49	0.038	4.13	3.3	0.1	11.6	0.14	220	12.1	hi dist., some bouquet	15.8
40	Cabernet Sauvignon	0.40	0.050	3.26	3.4	0.1	10.0	0.09	312	12.0	med. dist., malolactic, horsey	12.3
blend											hi dist., malolactic, rich	15.0
41	Selection 682	1.06	0.020	3.08	3.4	0.9	13.4	0.27	500	15.0	berry, hard and rough	14.6
42	Selection 547	1.03	0.019	3.12	3.4	0.2	11.6	0.33	500	14.4	full, fruity, acid	14.0
blend											fruity, bitter, tannic	14.4
43	Ruby Cabernet	0.86	0.015	3.47	3.4	0.1	12.6	0.26	500	15.9	weedy, Ruby Cabernet	16.2
44	Cabernet Sauvignon	0.53	0.028	3.78	3.1	0.2	13.5	0.18	334	16.2	wood, Cabernet type	15.9
blend											hi dist. Cabernet, wood, rich	16.9
45	Tannat	0.93	0.025	3.61	3.8	0.1	13.3	0.33	500	14.9	tannic, young, med. dist.	15.6
46	Petit Verdot	0.73	0.033	3.88	3.1	0.2	13.2	0.21	333	14.6	wood, rich, malolactic	14.2
blend											fruity, young, harsh note	14.6
47	Selection M63-18	0.89	0.012	3.56	3.7	0.2	10.9	0.16	111	13.6	lo dist., fruity	13.5
48	Selection M60-29	0.72	0.018	3.71	3.4	0.1	12.9	0.14	167	14.5	lo dist., harsh	11.9
blend											med. dist., slt. bitter, slt. oxid.	12.6
49	Selection M59-21	0.58	0.036	3.84	3.4	0.1	11.6	0.17	111	13.9	bacterial, slt. off, slt. metallic	12.6
50	Selection M58-54	0.49	0.051	4.26	4.1	0.1	12.4	0.19	167	12.7	bacterial, bitter, slt. ropey	10.6
blend											slt. off, lactic sour	12.6
51	Ruby Cabernet	0.79	0.020	3.67	3.5	0.1	13.1	0.23	500	15.3	hi dist., Ruby Cabernet	16.0
52	Ruby Cabernet	0.79	0.017	3.52	3.1	0.1	12.4	0.63	500	15.6	fruity, wood	15.3
blend											med. dist. Cabernet	15.5
53	Zinfandel	0.75	0.017	3.65	3.6	0.1	14.4	0.18	250	14.6	vinous, bitter	14.0
54	Calzin	0.82	0.030	3.29	3.8	0.5	14.9	0.48	333	13.9	dessertish, tannic	12.4
blend											vinous, tannic, bitter	13.6

Table 1. Origin and analyses of the selected wine pairs (cont'd).

Wine no.	Grape variety [a]	Total acidity (g tartaric/ 100 ml)	Volatile acidity (g HOAc/ 100 ml)	pH	Extract (g solid/ 100 ml)	Sugar (g glucose/ 100 ml)	Ethanol (% v/v)	Tannin (g tannic acid/ 100 ml)	Color (arbitrary units) [b]	First tasting Mean score	Second tasting Comments	Second tasting Mean score
55	Barbera	0.95	0.023	3.22	3.4	0.2	13.3	0.17	500	13.8	med. dist., wood	15.7
56	Carignane	0.51	0.018	3.69	3.2	0.1	12.7	0.12	125	14.3	vinous, slt. harsh	13.5
blend											lo dist., wood	14.2

White sweet fortified wines

Wine no.	Grape variety [a]	Total acidity	Volatile acidity	pH	Extract	Sugar	Ethanol	Tannin	Color	First tasting	Comments	Mean score
57	Early Muscat	0.43	0.020	3.98	15.4	11.0	19-20	0.03	8.4	15.8	hi dist., muscat, hot, heavy	16.9
58	July Muscat	0.28	0.016	4.21	17.2	13.4	19-20	0.03	8.3	15.6	fruity, slt. off	14.6
blend											hi dist. muscat, slt. hot, heavy	16 2
59	Fernão Pires	0.39	0.021	4.00	17.6	12.0	19.3	0.03	5.3	14.9	dessert, fruity, slt. hot	14.3
60	Mission	0.37	0.032	3.58	16.4	9.7	19-20	0.05	3.3	14.6	lo dist., slt. hot	13.7
blend											dessert, slt. oxidized, slt. hot	14.6

Red sweet fortified wines

Wine no.	Grape variety [a]	Total acidity	Volatile acidity	pH	Extract	Sugar	Ethanol	Tannin	Color	First tasting	Comments	Mean score
61	Royalty	0.48	0.014	3.92	16.0	13.2	19.6	0.28	500	16.1	med. dist., unusual fruity	16.2
62	Tinta Madeira	0.64	0.018	3.65	14.6	10.3	19-20	0.09	142	15.4	prunish, med. dist.	15.7
blend											med. dist., leafy, smooth	16.0
63	Zinfandel	0.42	0.016	3.77	14.2	10.9	19-20	0.11	125	13.1	slt. fusel, slt. hot	14.2
64	Muscat Hamburg	0.44	0.012	3.99	14.2	7.9	19.3	0.06	40	14.0	fruity, smooth	13.5
blend											slt. muscat, med. dist.	14.2
65	Teraldico	0.50	0.014	4.27	11.5	8.3	19-20	0.13	250	15.1	med. dist., smooth	15.4
66	Tinta Madeira	0.44	0.014	3.95	9.0	6.4	20.7	0.11	125	14.9	med. dist., slt. hot	14.9
blend											lo dist., smooth	14.9
67	Royalty	0.61	0.014	3.79	8.4	6.0	19.3	0.33	500	15.5	med. dist., bitter, medicinal	15.3
68	Tinta Madeira	0.56	0.022	3.98	15.2	7.5	19.6	0.16	200	14.9	med. dist., hot, rich	14.3
blend											med. dist., young, hot	15.3

[a] All wines from Davis vineyard except wines 37, 38, 41, 42, 54, and 55, from Oakville vineyard; wines 51 and 52, from a Napa Valley vineyard; wines 56 and 66, from a Madera vineyard; and wine 67, from a Stockton vineyard.

[b] Duboscq, color increases as values increase.

and wine 52, which was in wood, but was bottled about two months before its second tasting.

The second tasting included as a daily set the same ten wines as before plus the blended sample. The blend was prepared immediately before presentation of the samples to the panel. It was coded and randomized as to sequence, as were the other samples, so that the panelists were not aware of any special treatment and rated it as one of a group of eleven similar but unrelated individual wines. The scores recorded by each taster for each wine were based on the scale: 20–17, wines with some superior characteristics and no marked defects; 16–13, standard wines; 12–9, commercially acceptable wines with noticeable defects; 8–5, below commercial acceptance; and 4–1, completely spoiled. A minimum of six panelists tasted each day for which any results are recorded here. The flavor comments were those selected as most valid or revealing from the two or three panelists who included comments on their score sheets most frequently.

RESULTS AND DISCUSSION

Table 1 lists the origin and composition of the wine pairs chosen, the mean scores received in both tastings, and comments from the second tasting. If one compares the minimum detectable differences (p = 0.05, triangular test) in wine published by Hinreiner *et al.* (1955) with the analyses of each pair of wines, it is found that the following probably detectable concentration differences exist: sugar, pairs 57–8, 59–60, 61–2, 63–4, 65–6, and 67–8; alcohol, none; tannin, pairs 37–8, 51–2, 53–4, 61–2, and 67–8; and acid, pairs 1–2, 3–4, 9–10, 13–14, 15–16, 27–8, 29–30, 37–8, 43–4, 45–6, 47–8, 55–6, 57–8, and 61–2. Based on laboratory experience, the color differences large enough to have had a probable effect on scoring were in pairs 13–14, 19–20, 33–4, and 63–4. Eliminating duplicates, this totals 22, and thus 12 pairs of wines have no flavor or color differences that can be considered detectable on the basis of the available analytical and sensory information. Examination of mean scores of the pairs that do show analytical differences large enough to be sensorily significant (second tasting, Table 1) shows, for sugar, that the wine with lower sugar received the highest score in two cases, and the reverse was true in four cases. Only sweet wines are involved, and the differences are large enough that the

blend would also be detectably different from the wines in only one or perhaps two cases of the six.

Similarly with tannin, only red wines are involved, and in the three dry-wine pairs the lower tannin got the higher score, but in the two sweet-wine pairs the higher tannin scored higher. In only two cases is the difference large enough that the blend would be expected to be detectably different from either of the wines based on tannin alone. Comparison of the tannin content and scores of several pairs of wines having high tannin both with and without sufficient difference to be a factor in scoring of the pair shows several wines having high scores and yet high tannin. Thus it seems impossible to correlate quality judgment with tannin content within this series of wines.

Total acidity calculated as tartaric gives even less clear relationships with quality score. In nine cases the high-acid wine was scored higher, but in five cases the low-acid wine got the higher score, and in only three cases would one expect the blend to differ detectably in acid from either of the wines. Wines with unusually high or low acid content received equally high scores in many cases. With color, the relation to quality score was exactly split among the four cases of sizable differences (one each favoring high and low color within the white wines and similarly within the reds). Several other color differences were probably large enough to be detectable but were within the commercial range and did not appear to correlate with quality score.

Considering the facts just mentioned, the fact that the wines often scored differently in the two tastings, and that the estimated detectable differences are based upon immediate cross-comparison in a triangular test whereas the scores here were obtained by judging a single wine at a time within a standard scoring system, it seems correct to conclude that in nearly all cases the crucial factors in quality scoring are not explained by simple differences in taste related to usual analytical values available for wines. This is in line with the results Baker and Amerine (1953) reported; even an estimating equation including several analytical determinations did not predict quality score

reliably. The authors therefore conclude that the quality score of a blend, if it is higher than one would expect from the scores of wines entering the blend, cannot be attributed (at least within this series of wines) to the simple supplementation of deficiencies. Although the blend of two wines with objectionably high and low acidities, etc., would probably result in an improved wine, our efforts to minimize this type of effect—by selection of pairs of wines for blending, and comparison scoring on only commercially acceptable, carefully matched pairs—appear to have been successful.

The data in Table 1 are so arranged that the wine receiving the highest mean score in the second tasting was numbered odd, and the even-numbered wines were those scoring lower. It may be worth noting in passing that this same relative order within each pair prevailed in 22 cases and was reversed in 12 cases in the first tasting. This suggests that relative quality usually appeared early and was fairly consistent in these wines. Since about five months intervened between the two tastings of each pair of wines, considerable development of these wines did occur. The mean scores of 32 wines increased, 32 decreased, and 4 remained the same in the second tasting as

compared to the first, with a mean decrease of 0.1 score unit in the second tasting. This suggests that the panelists were either scoring in the first tasting partly on the basis of potential quality or that the temporary faults of the young wines such as lack of clarity and yeastiness were given less severe score deductions than later-appearing permanent defects such as oxidized flavors.

Although individual panelists did occasionally score a blend lower than either constituent wine, in no case (Table 1) did a blend receive a lower mean score by the whole panel than the lowest-scoring wine of the pair. In seven cases the blend received a higher mean score than either wine. A statistical evaluation of these results, considering the mean scores only, with no breakdown by tasters, etc., showed (Table 2) that the mean scores of the blends did not differ from those of the highest-scoring of the pair of wines blended, even to the lowest level of significance. However, the blend's scores were very highly significantly better than the average of the scores of the two constituent wines, and very highly significantly better than that of the lowest-scoring of the pair.

An analysis of variance of the scores given individually by five panelists who tasted most regularly (31 of these 34 pairs

Table 2. Tests for significance of the difference between the composite score for the blend and the composite scores for the wines in the blend.

	Difference of scores		
	Blend from higher wine	Blend from lower wine	Blend from mean of pair
(Student's t test, paired variates, null hypothesis, 33 degrees of freedom)			
Average deviation, $\overline{D}$	−.124	+1.00	+.438
Sums of squares of sample deviation minus average deviation, $\Sigma(D-\overline{D})^2$	17.401	22.02	11.176
$S_D = \sqrt{\dfrac{(D-\overline{D})^2}{33}}$	.726	.817	.582
$S_{\overline{D}} = \dfrac{S_D}{\sqrt{34}}$	.126	.142	.101
$t = \dfrac{\overline{D}-O}{S_{\overline{D}}}$	.981	7.03	4.32
Significance, p	zero	>.001	>.001

Mean Score: All samples 14.39, all blends 14.68, all higher of pairs 14.81, all lower of pairs 13.68, mean of pair means 14.24.

of wines) gave the results shown in Table 3. A highly significantly higher score was found for the blend than for the mean score of the two wines. Of note among the interactions is that treatment didn't interact with wines or tasters. The taster × wine interaction is normal, as are the taster and wine significant variances. These data agree with the previous conclusion that a 50-50 blend of wines chosen as these were receives a better quality score than might be expected from the scores of the two wines. Considering that the analysis of variance shows such a high degree of variability with respect to difference between individual wines, scores given by individual tasters, and the reaction of individual tasters to different wines, the high significance level for the treatment is especially noteworthy. These data, then, experimentally confirm the value of blending wines for improved quality. If the blending of two standard-quality wines seldom gives a product as bad as the poorer, usually gives one (at 50-50 blend level) as good as the better one, and sometimes gives one better than either, blending appears capable of useful, if still rather empirical, extension. Much blending, as now practiced commercially, appears to be based on firmer footing, and probably is of more value than previously suspected.

The reasons for the improvement of a blend over its constituents cannot be stated with certainty, because our understanding of the composition-quality interrelationships in wines is far from complete. At least in some cases, however, the evidence points to an increase in complexity as a major contrib-

utor to the quality increase. By this is implied the addition of flavors whose absence cannot be considered a serious deficiency but whose presence in the proper amount contributes favorably. A further implication is that a flavor that may be undesirable when recognizably strong, may be a contributor to complexity and therefore not undesirable if below the recognition threshold in the blend. This may help explain the data reported by Kramer and Ditman (1956), who showed that cantaloupes treated with an insecticide were scored significantly better than untreated cantaloupes.

Still another factor may be the fact that differences sensed as if on a unitized scale are actually a geometric rather than linear function of the stimulus (Stevens, 1961). It thus appears that a flavorsome constituent sensed as a given strength in one wine, may not seem much weaker when diluted one-half with a wine lacking this constituent. For example, a simple solution containing one aromatic ester compared with that of a different ester both near minimum detectable levels may appear less odorous, less complex, and less satisfactory as odorants than a blend of equal amounts of both solutions.

ACKNOWLEDGMENT

REFERENCES

Baker, G. A., and M. A. Amerine. 1953. Organoleptic ratings of wines estimated from analytical data. *Food Research* **18**, 381.

Hinreiner, E., F. Filipello, H. W. Berg, and A. D. Webb. 1955. Evaluation of thresholds and minimum difference concentrations for various constituents of wines. IV. Detectable differences in wine. *Food Technol.* **9**, 489.

Kramer, A., and L. P. Ditman. 1956. A simplified variables taste panel method for detecting flavor changes in vegetables treated with pesticides. *Food Technol.* **10**, 155.

Ough, C. S., and G. A. Baker. 1961. Small panel sensory evaluations of wines by scoring. *Hilgardia* **30**, 587.

Stevens, S. S. 1961. To honor Fechner and repeal his law. *Science* **133**, 80.

Table 3. Analysis of variance. Treatment is a comparison of mean score of two wines and the score of blend of both.

Source	df	ss	ms	F
Total	309	962.80		
Tasters	4	109.91	27.47	24.30 **
Treatment	1	9.85	9.85	8.71 **
Wines	30	360.47	12.01	10.62 **
Treatment × wines	30	38.69	1.28	1.13
Tasters × treatment	4	4.87	1.22	1.07
Tasters × wines	120	313.12	2.60	2.30 **
Error (residual)	120	135.74	1.13	

** Significant to 1% level.

Identification of Ellagic Acid as a Precipitate from Loganberry Wine

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A light-colored crystalline precipitate sometimes forms slowly in loganberry wine and may reduce the wine's acceptability after bottling. The significant constituent of this precipitate has been isolated and rigorously identified as free ellagic acid by ultraviolet and infrared absorption spectra, derivative preparation, and paper chromatography in comparison with synthesized ellagic acid. The ultraviolet absorption of ellagic acid in ethanol shifts gradually with the addition of small portions of anhydrous sodium acetate from an intense maximum at 255 to 256 mμ to an equally intense maximum at 286 to 288 mμ and the loss of a secondary maximum at 366 to 367 mμ. Isosbestic points near 267, 348, and 376 mμ were found. Although ellagic acid has been reported in several fruits, including grapes and raspberries (a parent of loganberries), it apparently has not been previously identified as a source of troublesome turbidity in wine.

SOME lots of commercial loganberry wine have deposited a fine, light-colored precipitate with time. This precipitate may continue to form during bottle aging and merchandizing of initially clear wine and result in poor appearance and decreased acceptability of the wine. Loganberry wine is often sold in colorless, flat-bottomed bottles and this precipitate is a light purplish gray which contrasts with the red wine and is very noticeable on the bottom of the bottle. When disturbed, the precipitate is readily suspended in the wine and the wine remains turbid for several hours before it again can be decanted relatively clear.

Because of high natural acidity, loganberries are usually relatively highly ameliorated with water and sugar, up to 60% of the total volume being permitted by federal regulation, in contrast to the 35% maximum permitted with many other fruits and the typical zero additions with ripe wine grapes. This precipitate therefore appeared to be a substance with unusually low solubility. The usual tests for the identification of precipitates in wine indicated that this was not any of the substances previously encountered. It therefore was of interest to study this condition and to identify the responsible substance.

Materials and Methods

The wines were commercial lots prepared from frozen loganberries by methods essentially typical for berry wines. The berries were allowed to thaw, 125 p.p.m. of SO$_2$ and yeast inoculum were added, and the fermentation was conducted at 65° to 75° F. for about 7 days. The free-run fluid was drawn off through the false bottom of the fermentor, and the residual pomace was leached with the ameliorating sugar solution in three portions. The combination of separated wine and ameliorant adjusted to be equivalent to an original Brix of 23° was fermented further to about 14% alcohol and then treated with SO$_2$ to arrest the fermentation. The wine was fined with bentonite and Sparkolloid (trade-mark) at 2 pounds each per 1000 gallons and racked after 2 weeks. After about 1 month of aging the wine should be ready for final filtration and bottling, but the unstable lots continued to deposit the precipitate even if filtered and bottled.

The amount of this precipitate present in a single bottle of unstable wine was estimated by filtration of portions of a 4-year-old sample presumed to have completed precipitation. The supernatant was used to correct for filterable, suspended amorphous material, and the precipitate was dried and weighed after collection on a membrane filter of 0.45-micron pore diameter and washing twice with 5 ml. of cold water. A larger sample (about 10 grams) of precipitate was collected from a tank of the unstable loganberry wine, washed with 12% ethanol plus 1% acetic acid in water, and dried in a desiccator.

The precipitate was studied visually with a polarizing microscope. Paper chromatograms were prepared by spotting about 10 μg. of the solid to be studied from pyridine solution onto Whatman No. 1 paper and developing (after 1/2 to 2 hours of equilibration in the vapor of the solvent) at 25° ± 2° C., descending mode, with the appropriate solvents. The developed chromatograms were studied under ultraviolet light, and ultraviolet light in the presence of ammonia vapor. Chromatograms were sprayed with a freshly prepared aqueous solution of equal volumes of 1% FeCl$_3$ and 1% K$_3$Fe(CN)$_6$ or 0.05% Fast Black K salt (12) in water followed by 20% Na$_2$CO$_3$.

Ellagic acid was synthesized from gallic acid by autoxidation of methyl gallate in aqueous ammonia (4, 7).

The synthetic ellagic acid and the wine precipitate were purified by decoloration with activated carbon and recrystallization several times from hot pyridine. The tetracarbethoxy derivative of ellagic acid and the similar derivative of the isolated substance were prepared from the sodium phenolates with ethyl chloroformate (2, 13).

The purified samples used for spectral analysis were treated with 10% sulfuric acid to remove residual pyridine, washed with water and acetone, and dried at 80° C. Ultraviolet spectra were recorded in absolute ethanol (8, 9) with a Bausch and Lomb 505 spectrophotometer. Infrared spectra were determined by the KBr disk technique. Other details of the methods employed were standard procedures or are not considered critical to reproducing the work.

Results and Discussion

Appearance and Amount of Precipitate. The precipitate collected from bottles of unstable loganberry wine was composed of a high proportion of very small birefringent crystals. They appeared colorless in reflected or non-polarized transmitted light under the microscope, but the noncrystalline portion of the precipitate was generally red or amber. The crystals appeared to be rectangular or blunt-ended needles. They occurred individually rather than as rosettes or large masses. Although a few slightly larger crystals were present in the sediment from large tanks, the crystals were mostly about 0.5 micron wide and 1 to 10 microns long.

The amount of the dry crystalline precipitate collectable from an unstable bottled wine was 86.3 mg. per liter. This amount was sufficient to produce the ill effects on appearance already described, but additional precipitate probably had been removed by treatments prior to bottling.

	EtOH					EtOH + NaOAc		Isosbestic Points, Mμ
Substance	λ_{max}, mμ	log ε	λ_{max}, mμ	log ε	Shoulders, mμ	λ_{max}, mμ	log ε	
Ellagic acid (synthesized)	255	4.70	366	4.00		277	4.54	267, 347, 376
Ellagic acid (8, 9)	255	4.77	366	4.13	293, 353	287	4.78	
Isolate from wine precipitate	256	4.70	366	4.02	292, 354	288	4.68	267, 348, 377
Wine precipitate	256	4.66	367	4.00	289, 354	286	4.65	

Isolation of Crystalline Substance. The wine precipitate was not completely soluble in hot pyridine. The insoluble residue was 6.3 and 8.6% of the original weight in two experiments. The solid which crystallized from the cooled pyridine, when dried to constant weight at 80° C., represented 52.3 and 52.6% of the amount of solid originally dissolved. Another experiment conducted by dissolving a further portion of the precipitate from loganberry wine in the dark red-brown pyridine mother liquor recovered from one of the above experiments gave 8.2% insolubles and an 84.2% recovery of recrystallized solid. Thus it appears that the major part of the wine precipitate can be recovered by recrystallization from pyridine.

The crystals, particularly after treatment in hot pyridine with decolorizing carbon and further recrystallization, were pale yellow needles, which upon standing in air or heating lost pyridine of crystallization and became more intensely yellow. The ellagic acid synthesized from gallic acid behaved similarly, except that removal of extraneous colored material was much less difficult. Neither the isolated substance nor ellagic acid melted at temperatures at the limit of the available apparatus (ellagic acid melting point > 360° C.).

The recrystallized substance and the crude wine precipitate itself as well as synthetic ellagic acid could be sublimed as a bright yellow crystalline solid, but high vacuum and high temperature were required (an oil bath at 250° C. was insufficient, but a micro gas burner was effective). Jurd (9) has reported that ellagic acid can be so sublimed directly from an ellagitannin without preliminary hydrolysis.

Qualitative and Color Reactions. The isolated crystalline material and synthetic ellagic acid gave identical reactions in a series of qualitative tests. Sodium bicarbonate solution had no immediate effect, but 10% sodium carbonate or sodium hydroxide gave a slow reaction to produce a yellow solution indicating weakly acidic (phenolic) groups without free carboxyls. The Greissmeyer test for ellagic acid (9)—a bright, transient red with fuming nitric acid and dilution with water—was positive. Ferric chloride solution produced a blue-black phenol color test. These tests were also positive with the crystals present in the original wine precipitate.

Spots on paper gave a dull purple on a
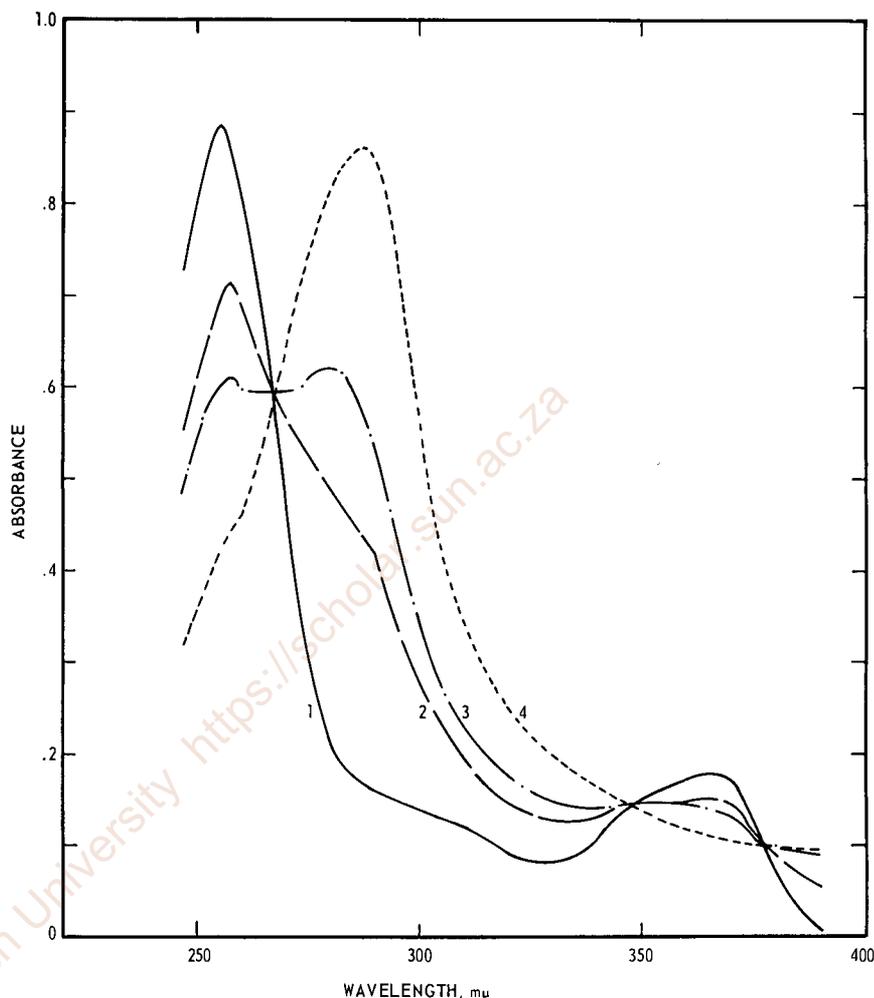
Figure 1. Ultraviolet absorption spectra of crystalline substance isolated from loganberry wine precipitate

1. In absolute ethanol
2, 3, 4. Absolute alcohol with increasing additions of very small amounts of anhydrous sodium acetate

pink background with Fast Black K followed by sodium carbonate. Blue spots were produced with ferric chloride-ferricyanide spray. Ultraviolet light gave a faint blue-white fluorescence converted to bright yellow when the paper was fumed with ammonia.

Spectra. The ultraviolet absorption spectrum of the synthetic ellagic acid in absolute ethanol agreed very well with that reported by Jurd (8, 9) and that of the isolated crystalline substance was essentially identical (Table I, curve 1 in Figure 1). The solution of the impure wine precipitate directly in ethanol without any heating gave a very similar absorption spectrum with only small amounts of impurities indicated by very slight shifts and decreases in absorbance at the maxima (Table I) or increases in absorbance at other wavelengths.

The saturation of these solutions by addition of anhydrous sodium acetate to the cuvettes, however, produced a bright yellow gelatinous precipitate. Addition of anhydrous sodium acetate in very small amounts to a fresh portion of each solution produced a sequence of changes, an intermediate stage of which was similar to that reported by Jurd (8–10) (Table I, Figure 1). Addition of sufficient (about 0.3 mg. per ml.) sodium acetate to produce apparent completion of the shift of the 256-mμ maximum to one at 288 mμ of essentially identical molar absorbance probably corresponds to the conversion of the two more acidic phenolic groups para to the carbonyl function into the sodium salts (10, 14) without sufficient conversion of the other two to produce insolubility. The apparent discrepancy between these re-
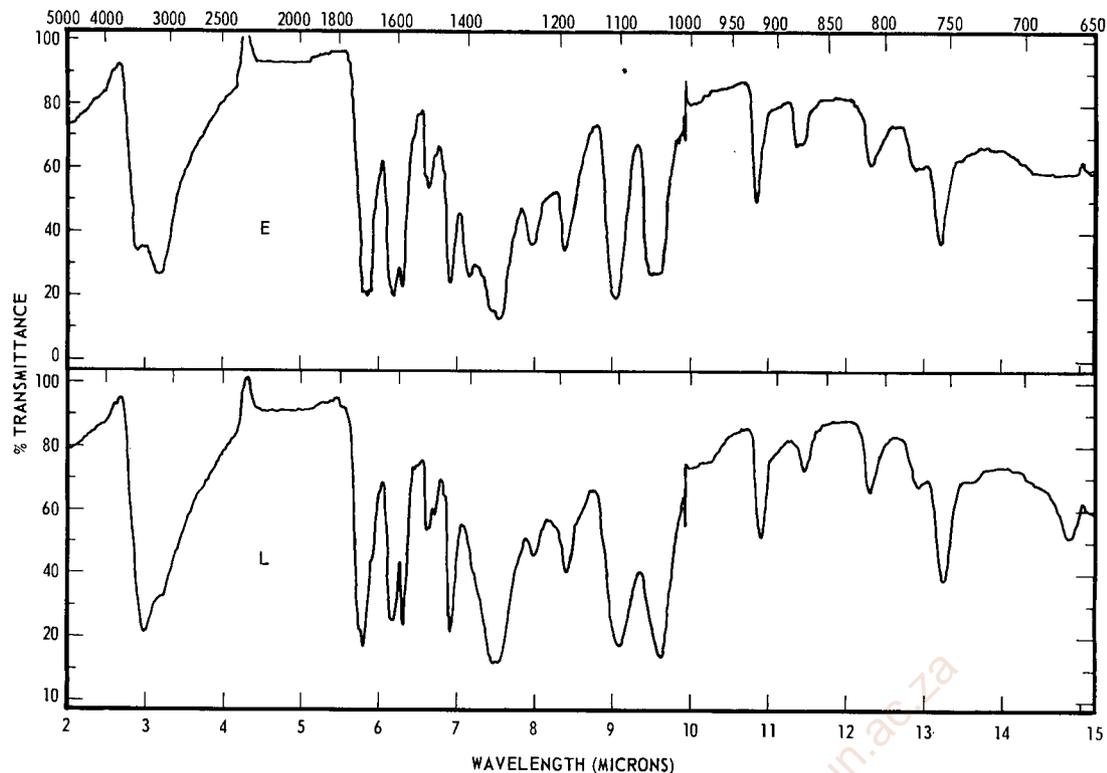
Figure 2. Infrared absorption spectra

E. Synthetic ellagic acid
L. Crystalline substance isolated from loganberry wine precipitate
0.5% in KBr disks

sults and the earlier reports (3, 8–10, 14) may depend upon the condition of the solvent used. Ours was freshly opened commercial absolute ethanol and no attempt was made to dry it specially.

In any case, the value of the shift produced by sodium acetate recommended by Jurd as a means of identification of ellagic acid is verified by our data and the isosbestic points disclosed by sequential small additions should serve as additional useful features for such identification. The identity of ellagic acid and the crystalline substance present in and isolated from loganberry wine precipitates seems highly probable from these spectra.

The infrared spectra of the isolated substance and that of synthesized ellagic acid are shown in Figure 2. There is very close agreement between them, since at least 15 features are nearly identical in absorbance and frequency. Differences do appear, notably at frequencies of about 3300, 1400, and 680 cm.$^{-1}$ These are attributed to differences in crystal form, hydrogen bonding, or possibly small compositional differences such as presence of different amounts of residual solvent of crystallization. Ellagic acid and similar compounds are notorious for crystallizing in more than one form and as solvates and hydrates. These infrared spectra are considered to be confirmatory evidence of the identity of the isolate with ellagic acid.

Table II. Paper Chromatography of Ellagic Acid and Wine Precipitate

(R_f values)

	Solvent A	Solvent B	Solvent C	Solvent D
1. Ellagic acid (synthesized)	0.39	0.74	0.00–0.03	0.83
2. Isolate from wine precipitate	0.38	0.74	0.00–0.03	0.83
3. Mixture of 1 and 2	0.39	0.74	0.00–0.03	0.80
4. Wine precipitate	0.40, 0.48, 0.69	0.75	0.00–0.03	0.83

All spots detectable with FeCl$_3$–K$_3$Fe(CN)$_6$. Solvent A = concd. HCl–HOAc–H$_2$O (3:30:10); B = dimethylformamide adjusted to pH 3.5 with formic acid; C = 6% HOAc in H$_2$O; D = pyridine-water (40:60).

Paper Chromatography. Ellagic acid gives discrete, nontailing spots on paper with a satisfactory R_f in relatively few solvents. Our results are shown in Table II. Ellagic acid and the substance recrystallized from the wine precipitate were not separable or distinguishable in any of the four developing solvents. The crude wine precipitate contained smaller amounts of two additional substances detectable by phenolic-type reactions, particularly on chromatograms developed by solvent A. With this solvent discrete spots were formed without tailing or residue at the origin. The fastest moving spot was very faint and the intermediate spot was considerably weaker than the spot corresponding to ellagic acid. These additional substances were not identified.

Derivative Comparison. The tetra-carbethoxyellagate and the analogous derivative of the isolated substance gave a relatively sharp capillary melting point of 246–47° C. observed without magnification [literature 244°, 247° C. (2, 8, 13)]. Microscopic observation with a hot-stage, polarizing apparatus revealed that some melt formed at 240° C. and the last crystal disappeared at 249° C. Cooling the melt to 245° C. produced partial crystallization and upon reheating the last crystal again disappeared at 249° C. Identical melting behavior occurred with synthetic ellagic acid, the isolated substance, and an equal mixture of the two ground together.

Conclusions

The data presented are considered to prove that ellagic acid is the predominant and significant substance in the light-colored crystalline precipitate which forms slowly in some lots of loganberry wine.

Since the paper chromatographic, ultraviolet spectral, and other studies upon the crude precipitate prepared and

observed under very mild conditions show the characteristics expected from free ellagic acid, it is considered proved that the precipitate is predominantly free ellagic acid rather than an ellagitannin, free hexahydroxydiphenic acid, or other possible precursors of ellagic acid.

Ellagic acid has a very low solubility in aqueous solutions and is readily removed from solution or suspension by most fining, filtration, or adsorptive treatments. It therefore appears that the ellagic acid precipitate results from the slow generation of ellagic acid. In preliminary experiments with canned loganberries, free ellagic acid was not found. It appears most probable that the source of the ellagic acid is an ellagitannin present in the loganberries which is relatively soluble in wine and is produced or extracted in variable amounts to account for the different degree of instability of different lots of loganberry wine. The slow rate of formation of the crystalline precipitate presumably reflects the slow hydrolysis of the more soluble ellagitannin and perhaps delayed lactonization of the resultant hexahydroxydiphenic acid. It is possible, of course, that enzymes present in the berry or even "tannase" produced in an occasional moldy berry are involved and synthesis from simpler substances such as gallates could occur.

These considerations are strengthened by the fact that improved, but not always completely stable, clarity of the unstable loganberry wine has been achieved by heating or treatment with gelatin or activated carbon. It is probably significant that although we were unable to find a report of the presence of ellagates in loganberries, ellagic acid has been reported from raspberries (17). The loganberry is reported to be a cross between European red raspberries and California blackberries. A precipitate very similar to that described here has been noted in a commercial red raspberry wine (1). We have been able to locate no previous report of wine turbidity or sediment produced by ellagic acid, which is interesting in view of reports of its presence in grapes and grape wines (5, 6).

Acknowledgment

Literature Cited

(1) Coven, M., unpublished data.
(2) Feist, K., Bestehorn, H., *Arch. Pharm.* **263,** 16 (1925).
(3) Harborne, J. B., "Biochemistry of Phenolic Compounds," p. 56, Academic Press, New York, 1964.
(4) Hathway, D. E., *J. Chem. Soc.* **1957,** 519.
(5) Hennig, K., Burkhardt, R., *Naturwissenschaften* **44,** 328 (1957).
(6) Hennig, K., Burkhardt, R., *Weinberg Keller* **5,** 542 (1958).
(7) Herzig, J., Polak, J., von Bronneck, M., *Monatsh. Chem.* **29,** 263, 277 (1908).
(8) Hillis, W. E., "Wood Extractives," pp. 230–1, 242–5, Academic Press, New York, 1962.
(9) Jurd, L., *J. Am. Chem. Soc.* **78,** 3445 (1956).
(10) *Ibid.,* **81,** 4610 (1959).
(11) Kung-Krause, H., *Arch. Pharm.* **259,** 193 (1921).
(12) Pearl, I. A., McCoy, P. F., *Anal. Chem.* **32,** 1407 (1960).
(13) Reichel, L., Schwab, A., *Ann. Chem. Justus Liebigs* **550,** 152 (1942).
(14) Scott, A. I., "Interpretation of the Ultraviolet Spectra of Natural Products," pp. 148–9, Macmillan, New York, 1964.

A TEST OF FRACTIONAL ADDITION OF WINE SPIRITS TO RED AND WHITE PORT WINES[1]

V. L. SINGLETON and J. F. GUYMON[2]

A number of reports, particularly from France (2,3,5,7,8) and Russia (9-11), state that adding wine spirits in some manner other than the usual single massive addition gives improved quality in the production of fortified sweet wines. The quality improvement is generally described as better "assimilation" of the added alcohol, so that it "stands out" less and the wine appears less alcoholic and therefore more aged. Gerasimov (9) stated that fractionally fortified wines containing 35% alcohol were estimated to have only 16-18% alcohol by expert tasters. Such a quality improvement is naturally of interest as a potential means of improving the early marketability of dessert wines—in effect a quick-aging process.

It is stated or implied in reports on various methods of fractional wine spirits addition that there exists an interaction between the technique, the alcohol, and the wine which results in a direct improvement in the wine's quality merely by the manner of the blending in of the added wine spirits. In several cases the authors of those reports advanced no explanation of the nature of this presumed direct effect. Gryaznov (11) believed that aging was required to break up the association between the molecules of the added spirits, and that therefore the improvement resulting from the addition of the spirit as vapor was because the molecules entered the wine already "disassociated" from each other. Although this does not seem to us to be a very plausible hypothesis, Deibner and Benard (5,6) and Gryaznov have

described special techniques and equipment for transferring the alcohol as vapor and condensing it in the wine. The resultant wine is rated as improved even more than wine produced by adding the wine spirits in two or more portions, which, in turn, is reportedly better than wine from a single fortification (5-8).

Indirect effects may also be operating in fractional fortification. Gerasimov (10) noted that adding some of the fortifying alcohol before or during fermentation is important not only for its contribution to improved "assimilation" of the alcohol, but also because the growth of organisms other than wine yeasts is suppressed (as in the Semichon process), resulting in a cleaner fermentation. Flanzy (7) suggested that the improvement from fractional fortification was a result of slower fermentation produced by the early addition of alcohol. Fraisse (8) suggested that the improvement resulted from a larger proportion of secondary products such as glycerol produced by the slower fermentation at a higher alcohol content. If part of the alcohol was added before screening, in the case of port or muscatel fermentation in the presence of the skins, the added alcohol would promote the extraction of color and flavor from the skins, as shown by Berg and Akiyoshi (4), and thus affect quality. These possible indirect effects do not appear to explain entirely the improved "assimilation" of alcohol that is claimed for fractionally fortified wines, although they probably contribute.

The claims for improvement of sweet wines by adding wine spirits in fractions are persistent, the first reports having been made as early as 1924. Commercial use of the technique has been recommended by both the French and Russian workers, but it apparently has not been widely adopted or tested elsewhere. Perhaps much of the reluctance to try this technique has

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stemmed from the fact that the reasons advanced for the special direct effects attributed to fractional fortification did not appear sufficiently logical. Considering the tax and regulatory problems with multiple spirits additions, improvement possible by other means (slow fermentation by temperature control, etc.) would appear to be preferable in California. In addition, sensory evaluation of the fractionally fortified wines in the cited tests was not thorough enough to be entirely convincing.

It seemed to us that a more satisfactory explanation than has heretofore been advanced for the direct effect of fractional fortification lay in the reduction of congeners, particularly "heads", by the fermenting yeast in the portions of alcohol added before the level became sufficiently high to stop fermentation. This effect, of course, could not occur to an appreciable degree after a single addition of the same total amount of alcohol. The reduction of heads added to solutions undergoing active fermentation by wine yeasts has already been clearly elucidated by Guymon and co-workers (12-15).

Several observations appear to favor this hypothesis. The aldehydes and associated compounds present in heads have been believed to be the source of much of the "hotness" that causes added brandies to "stand out" and make wines containing them seem too "alcoholic". Wine spirits of various types have been reported to give different responses in fractional fortification (3). The data reported can be interpreted in the light that the most neutral high proof was dependable in giving good dessert wines, but gave relatively little improvement upon fractional fortification. Less neutral brandies gave wines of various qualities, but they were often considerably improved by fractional fortification. This would be expected from our hypothesis. The reports that distillation of the brandy into the wine was even better than fractional liquid addition may be explained on the basis that the more volatile heads, particularly in a pot-still type of apparatus such as Deibner and Benard (5) used, would be the portion entering the wine first, and therefore acted upon more com-

pletely by any fermentation that occurs before too much alcohol has been distilled.

We therefore set about estimating the magnitude of the effect upon sweet wines of adding the alcohol in three increments and testing our hypothesis that a probable basis for the reported quality effect of fractional fortification lay in reduction of brandy constituents by the continuing fermentation.

MATERIALS AND METHODS

The ports were prepared from Carignane grapes picked in the University vineyard at Davis, California, on Oct. 3, 1961. On the following day they were destemmed and crushed into a large vat to which was added 75 ppm SO_2 and 0.5% by volume of a strongly fermenting wine yeast culture (Montrachet strain). This was thoroughly stirred into the must. It was then continuously stirred, and separated into 4 lots by dipping 1 gallon into each separate container in sequence until about 60 lb were in each lot. Fermentations were conducted in 13-gallon polyethylene drums in a controlled-temperature room at 21°C. All dissolved solids determinations were made with a refractometer after removal of the alcohol by distillation, and correcting to 20°C. The fermenting mass was well stirred before each sampling and after each addition of alcohol. The wine was pressed from the pomace at the appropriate time in a single portion in a basket press with a 17.5-in.-diameter plunger at 40 psi for about 1 min. The pomace was discarded and the wine volume measured. The fermentation results are shown in table 1. Note that lots A and B were prepared by a single fortification—A with a clean high-proof brandy, B with 5% heads added to the high proof. Lots C and D received three fortifications—C with heads first, and then clean high proof, and D with high-proof brandy containing 5% added heads. On the seventh day all four wines were racked and transferred to glass containers in the 10°C cellar, where they were held for chemical and sensory analysis.

Four similar lots of white port were prepared from Grillo grapes from the Davis vineyard. The grapes were picked on Oc-

TABLE I

The Course of Fermentation and Sequence of Wine Spirits Additions for the Ruby Ports[a]

Hours	Operation	Lot A	Lot B	Lot C	Lot D
0	Extract sample	20.5	20.6	20.5	20.6
10.8	Extract sample	20.8	21.0	21.0	20.8
17.5	Extract sample	19.6	20.0	19.5	19.5
17.5	Spirits addition	....	....	189 ml H	189 ml HP
28.8	Extract sample	17.0	16.6	17.7	17.3
29.0	Spirits addition	....	....	1890 ml P	1890 ml HP
29.5	Wine pressed	....	....	19.4 l	19.4 l
41.5	Extract sample	14.4	14.2	....	....
45.0	Extract sample	12.7	12.6	....	....
45.0	Wine pressed	18.0 l	18.2 l	....	....
45.3	Spirits addition	3400 ml P	3400 ml HP	....	....
52.0	Extract sample	....	....	15.8	15.7
73.5	Extract sample	....	....	13.7	14.4
100.5	Extract sample	....	....	13.1	14.1
100.5	Spirits addition	....	....	1450 ml P	....
116.0	Extract sample	....	....	....	13.2
116.0	Spirits addition	....	....	....	1450 ml HP

[a] Extract was measured by refractometer after distillation of alcohol. P = high proof brandy, H = concentrated heads, HP = 5% H in P. The final result is lot A = single spirits addition with high proof, lot B = single addition high proof plus 5% concentrated heads, C = triple addition — concentrated heads followed by high proof, and D = triple addition of high proof containing 5% concentrated heads.

tober 9, 1961, and crushed and pressed on the following day. The fluid must was divided into four 6.5 gal lots in 12-gal Pyrex bottles. They were each inoculated with 200 ml of Montrachet strain yeast culture, 50 ppm SO_2 added, and then allowed to ferment in a room maintained at 21°C. Lot A was fortified at 13° Brix with clean high proof. Lot B was similarly fortified with high-proof brandy plus 5% concentrated heads. Lot C was fortified in 3 portions. About 5% of the total brandy requirement was added at about 2% alcohol by fermentation, and the remainder in two portions, at about 17° Brix and at the final residual sugar. Concentrated heads made up the first portion, and clean high proof the other two. Lot D was the same as lot C except that high proof containing 5% added heads was used in all 3 portions. The high-proof brandy and concentrated heads used were the same for all wines in these experiments.

The final fortification was made at the end of two days for lots A and B, and at 15 days for lots C and D. Lots A and B were racked at 3 days; lots C and D were racked at 8 days. Further racking, as necessary, was accomplished as the wines were held for tasting and for analysis in glass containers in the 10°C cellar.

The brandy used in fortification was a highly rectified neutral high-proof spirit from a continuous still (190° proof, 11.2 mg ester/100 ml, 1.08 mg aldehyde/100 ml. and 9.8 mg total fusel oil/100 ml). The concentrated heads (800 ml, 16 mg aldehydes/ml) were prepared by redistilling a brandy heads cut from a continuous still (4500 ml, 190° proof) in a 5-liter laboratory pot still with two 30-in. concentrating sections packed with Berl saddles.

Analyses were according to standard methods for wines (1) except that total aldehydes were determined by the procedure of Jaulmes and Dieuzede (16), and esters by a ferric hydroxamate method (17). Spectra were obtained with a Spectronic 505 recording spectrophotometer with 1 cm cells, samples diluted as necessary with water. Sensory analyses were made by a twelve-member panel familiar with wines and with sensory testing. Samples were presented in pairs of all possible combinations (6 pairs/judge/session), and each wine was scored according to our standard scoring system: 20-17 superior, 16-13 standard, 12-9 commercial but defective, 8-5 unsalable, 4-1 spoiled. Each pair was judged as to which was preferred, or had preferred color, higher fruity aroma, more hotness, more bitterness, more sweetness, and appeared to have had the better wine spirits added. The panel's judgments were replicated four times on successive days on all samples to give a total of 48 judgments on each pair and 144 scores on each wine.

RESULTS AND DISCUSSION

The chemical analyses of the wines and musts are shown in table 2. The wines were made so that there would be as little variation as possible in composition except for heads reduction. The ports were all very close together in composition except for red color (absorbance at 520 mμ) and volatile aldehyde. The white ports showed differences in volatile aldehyde and were not so closely matched in extract, owing to difficulties in getting the fermentation to proceed as far in lots C and D as in A and B.

The differences in volatile aldehyde content were as expected with both types of wine. In the samples receiving a single fortification, samples A, with clean brandy, had less than half as much aldehyde as samples B, which received brandy plus heads. The fractionally fortified samples C and D had less aldehyde than samples B, owing to reduction by the yeast. Samples D had more than A because all fractions of their brandy contained heads, and therefore the later portions were not completely reduced. Sample C in the port series contained the same aldehyde as sample A and in the white port series less was found in C than in A. These facts suggest that the aldehyde level was low enough in the "clean" brandy that the aldehyde found in the wine primarily reflects the natural equilibrium, i.e., the minimum aldehyde content resulting from our yeast fermentation. The reduction of added aldehydes is computed on this basis, as shown in table 3, which summarizes the

TABLE 2

Composition of the Original Musts and the Resulting Ruby and White Port Wines Produced
by Different Methods of Spirits Addition[a]

	Extract (g/100 g)	Alcohol (% V/V)	Acid total (g tartaric/100 ml)	pH	Tannin (mg gallic/l)	Volatiles			Relative Absorbance (A(1 cm) x diln.)		
						Aldehydes (mg HAc/l)	Acid (g HOAc/100 ml)	Esters (mg EtOAc/l)	520 mμ	440 mμ	280 mμ
Ruby ports											
Must	21.5	......	0.68	3.55	......	......	......	....	......	......	......
A	10.3	19.7	0.43	3.67	610	43	0.005	44	1.81	1.31	24.0
B	10.9	19.3	0.47	3.66	630	156	0.008	34	2.28	1.58	20.5
C	10.8	18.9	0.43	3.70	610	46	0.008	34	1.58	1.20	20.5
D	10.2	19.1	0.45	3.71	585	58	0.010	48	1.58	1.23	24.0
White ports											
Must	22.5	......	0.63	3.51	......	......	......	....	......	......	......
A	10.7	22.4	0.50	3.41	182	114	0.010	56	0.04	0.10	5.50
B	10.9	22.0	0.52	3.39	176	253	0.007	85	0.04	0.10	6.10
C	12.0	22.0	0.49	3.54	166	62	0.010	63	0.02	0.07	5.50
D	12.7	21.6	0.49	3.55	168	156	0.012	54	0.03	0.08	5.60

[a] A = one addition, high proof spirit.

B = one addition high proof spirit plus 5% concentrated heads.

C = three additions, concentrated heads once then high proof spirit twice.

D = three additions, high proof spirit plus 5% concentrated heads.

aldehyde balance.

These data, together with the analyses (Table 2), show that samples A and C may be compared fairly as examples of single and multiple fortification because the extra aldehyde in sample C has been completely reduced. Samples B and D may be compared as examples of single and multiple fortifications with brandy containing appreciable heads.

Table 4 summarizes the sensory tests. The pairs are evaluated by the chi-square test, and the quality scores by analysis of variance and the Duncan multiple-range test. Significant differences were found between treatments in the analysis of variance test, based upon the error variance. However, there was a significant interaction between treatment and individual tasters such that if this was used as the error term, treatment differences could have resulted by chance. This means that variation between individual taster's preferences among the samples is too great to allow statistical justification of drawing general conclusions from these tests (such as which wine would be preferred by the population at large). This is a common finding in small-panel sensory testing, but so far as this panel in this test is concerned, the results are significant as shown.

In the white port series, the over-all quality scores are in the sequence ACDB and the residual content of volatile aldehydes in the sequence CADB with the highest aldehyde content receiving the lowest score. This result is reinforced by the other sensory judgments. For example, sample A (one addition, clean brandy) is judged to have had better spirits added than B (one addition, heads in brandy), and C (3 additions, heads then clean brandy) better than D (3 additions, heads in brandy). Sample B was noticeably hotter and more bitter than C. The few apparently extraneous or opposing factors, such as that D was sweeter than B or A, and that C had preferred color to A, apparently did not override the judges' reaction to the presence of aldehydes. With the white port

TABLE 3

The Effect of Fractional Wine Spirits Addition on the Amount of
Aldehyde Remaining in the Resulting White and Ruby Port Wines[a]

	Aldehyde found (mg HAc)	Aldehyde added (mg HAc)	Aldehyde found (% of expected)[b]	Aldehyde reduced (% of added)
Ruby ports				
A	920	37	------	--------
B	3370	2817	90.1	12.1
C	959	2740	26.0	99.7
D	1209	2924	31.1	91.2
White ports				
A	3409	57	------	--------
B	7565	4267	99.3	1.3
C	1854	3702	26.3	140.5
D	4664	4396	60.2	70.1

[a] A = one addition, high proof spirit.
 B = one addition, high proof spirit plus 5% concentrated heads.
 C = three additions, concentrated heads once then high proof spirit twice.
 D = three additions, high proof spirit plus 5% concentrated heads.

[b] Expected aldehyde equals the amount of aldehyde added in wine spirits to each lot plus the calculated amount produced by fermentation based on samples A corrected for the amount added to samples A in wine spirit.

series, then, the conclusion is that fractional addition of wine spirits had no evident effect other than contributing to quality by enabling heads reduction and that high heads content was discriminated against by the judges. If the brandy is carefully distilled and has low heads content, no practical advantage to fractional fortification is seen in white port production.

The red port series is more difficult to interpret, because the type and manner of addition of the alcohol in the different samples also produced effects upon color and perhaps other factors. Sample D (3 additions, heads in brandy) was not rated better than B (one addition, heads in brandy), but C (3 additions, heads then clean brandy) was rated better than A (one addition, clean brandy). The most clearly different sample was B, which was rated better than A even though it had the most heads (volatile aldehyde). However, C had less volatile aldehyde than any other sample except A, and was rated as second in quality with A lowest in quality rating. Note that B was significantly better in fruity aroma than D, and probably better than A. Sample B's high over-all rating

TABLE 4

Sensory Analysis by an Experienced Panel of Wines Produced by Different Methods of Wine Spirits Addition[a]

% of judgments above chance as higher in quality named, 48 judgments.

	Ruby ports[b]						White ports[b]					
	A/B	A/C	A/D	B/C	B/D	C/D	A/B	A/C	A/D	B/C	B/D	C/D
Preferred color	—42	19	33	46	44	6	— 8	—16	—10	14	—10	2
Fruity aroma	—15	—12	4	12	19	5	—16	17	33	2	— 2	8
Best spirits	6	—20	— 8	—21	4	6	14	6	4	— 4	— 8	23
Hotness	— 3	16	12	10	12	—7	—12	3	— 7	16	0	—13
Bitterness	1	14	10	26	7	—4	—13	13	15	17	—15	—14
Sweetness	— 3	— 9	2	—16	—15	—1	— 7	— 7	—14	— 9	—16	6
Preference	—25	—12	— 8	15	17	4	6	5	8	— 2	— 2	12

Significance levels of the difference by the two-tailed χ^2 test:
> 24 = p 0.001, 20-23 = p 0.01, 16-19 = p 0.05, 14-15 = p 0.1.

Mean quality scores, 1 low to 20 high scale, 132-144 judgments

Ruby ports	B	C	D	A
	14.44	14.11	13.85	13.74

White ports	A	C	D	B
	14.02	13.76	13.64	13.58

Samples not underlined by the same line differ significantly at p 0.05 by the Duncan multiple-range test.

[a] Wine A had one addition, high proof spirit.
Wine B had one addition, high proof spirit plus 5% concentrated heads.
Wine C had three additions, concentrated heads once then high proof spirit twice.
Wine D had three additions, high proof spirit plus 5% concentrated heads.

[b] The letters A/B, etc., refer to the wine lots paired for comparison. The value —42 for A/B indicates that ruby port B was preferred over ruby port A in color in 92% of the judgments minus 50%, the chance level. Similarly, the value 17 shows that white port B was judged as more bitter than white port C in 17 + 50 = 67% of the trials.

was in spite of the fact that C was judged to have had better spirits added, B was more bitter than C, and appeared less sweet. Considering all factors, the conclusion drawn from the red port data is that the effect of fractional fortification appears to be smaller than other factors. Reduction of heads appears to be considered desirable by the panel under some circumstances, but under other circumstances heads either contribute favorably or are overshadowed by other factors. The suggestion that heads may contribute favorably in ruby port to color retention and under some circumstances to desirable aroma is contrary to our expectations, and should be reinvestigated.

SUMMARY

The hypothesis that the major effect of fractional addition of wine spirits, compared to single addition, is the result of heads reduction appears to have been verified by this research. Heads, as measured by volatile aldehyde, appear definitely undesirable at high levels in white port, and the quality of the white port as measured by this panel of judges was a function of the degree of heads reduction produced. The relationship between heads content and quality is less clear in ruby port, because the effect of heads on sensory properties was smaller than other factors in these experiments.

ACKNOWLEDGMENTS

LITERATURE CITED

1. Amerine, M. A. Laboratory procedures for enologists. University of California, Davis. 130 pp. (1960).

2. André P., P. Benard, and L. Deibner. Influence du mode d'alcoolisation et de la nature des alcools sur les propriétés organoleptique des vins doux naturels. Compt. Rend. Acad. Agr. France 43:161-2 (1957).

3. Benard, P., P. André, and L. Deibner. Influence du mode d'alcoolisation et de la nature des alcools sur la qualité des vins doux naturels. Ann. Technol. Agr. 7:111-25 (1958).

4. Berg, H. W. and M. Akiyoshi. Further studies of the factors affecting the extraction of color and tannin from red grapes. Food Res. 23:511-17 (1958).

5. Deibner, L. and P. Benard. Nouveau procédé d'alcoolisation des vins spéciaux par diffusion graduelle des vapeurs alcooliques. Ind. Agr. Aliment. (Paris) 69:881-6 (1952).

6. Deibner, L. and P. Benard. Recherches sur la maturation des vins doux naturels. 1. Influence du traitement thermique prolongé a l'abri de l'air. Ann. Technol. Agr. 5:357-76 (1956).

7. Flanzy, M. Recherches sur la vinification des vins doux naturels. Ann. Technol. Agr. 8:285-320 (1959).

8. Fraisse, R. Les vins doux naturels à appelation controlée: leur élaboration par une alcoolisation globale ou par des affusions successives d'alcool. Rev. Vit. 95:2, 43 (1948).

9. Gerasimov, M. A. Tekhnologia Vinodeliya. Pischepromizdat, Moscow, 1952. p. 180-1.

10. Gerasimov, M. A. Izbrannye Raboty po Vinodeliyu 1925-1955. Pishchepromizdat, Moscow, 1955. p. 377-8.

11. Gryaznov, V. P. Diffuzionnyĭ sposob spirtovaniya vinomaterialov. Vinodel. i Vinograd. 12: No. 5, 14-18 (1952).

12. Guymon, J. F. and J. A. Nakagiri. Utilization of heads by addition to alcoholic fermentations. Am. J. Enol. 6:12-25 (1955).

13. Guymon, J. F. and J. A. Nakagiri. Effect of acetaldehyde, acetal, and ethyl acetate upon alcoholic fermentation. Am. J. Enol. 8:1-10 (1957).

14. Guymon, J. F. and Allen Pool. Some results of processing heads by fermentation. Am. J. Enol. 8:68-73 (1957).

15. Guymon, J. F. and M. S. Jaber. Reduction of aldehydes during alcoholic fermentation. Application to processing of heads. J. Agr. Food Chem. 7:576-8 (1959).

16. Jaulmes, P. and J. Dieuzede. Le dosage de l'acetaldehyde dans les spiriteux. Ann. Fals. Fraudes 47:9-14 (1954).

17. Libraty, V. Ester determinations and their application to wines. M. S. thesis, University of California, Davis, 76 pp. (1961).

ANTHOCYANIN COLOR LEVEL IN PORT-TYPE WINES AS AFFECTED BY THE USE OF WINE SPIRITS CONTAINING ALDEHYDES

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The hue and intensity of the color of a red wine are certainly important factors in the wine's quality. The short period available for anthocyanin extraction during port production and the relatively low anthocyanin content of many of the grapes used for port make the obtaining of adequate color a particularly acute problem for wines of the ruby port type in California.

The chemistry and technology of wine pigments are complex because grapes contain several different anthocyanin derivatives and leucoanthocyanidins, the color exhibited by each pigment is dependent upon the nature of the solution, and reaction products are produced from these pigments in wines (1, 2, 7, 9, 10). Because of this complexity the reactions of anthocyanins in wines are not yet always predictable, and more complete understanding is needed.

Singleton and Guymon (11) reported that red color (absorbance at 520 mμ and sensory-panel color rating) was considerably greater for a lot of port fortified with aldehydic brandy than for another portion of the same base wine made with the same brandy without added aldehyde or "heads." This result was unexpected, because several studies had shown that acetaldehyde in red wine reacts with anthocyanins and other components with the formation of precipitates, causing a decrease in red color and phenolic content of the wine (3, 6, 8, 9, 12).

It appeared desirable to determine whether the presence of aldehydes in the added spirit does consistently increase the red pigment in new red dessert wines. This report describes five replicate tests on this question and some analyses for estimation of the nature of the apparent color effect.

MATERIALS AND METHODS

Five varieties of grapes from the University vineyards at Davis, California, were picked on October 7-15, 1963, having the respective dissolved solids contents (degrees Brix): Souzão, 21.8; Trousseau, 23.8; Mission, 22.7; Carignane, 18.1; and Grenache, 21.8. The grapes were processed by destemming, crushing, adding 75 ppm SO_2, inoculating with wine yeast, and fermenting. The cap was punched down frequently, and the wine was drained and lightly pressed from the pomace after the hydrometer reading had fallen to between 13 and 15° Brix. The resultant fermenting wine from each variety of grapes was divided into two equal portions. One half received wine spirits sufficient to stop the fermentation and raise the alcohol content to about 20%. The other lot was identical except that it received the same spirit, containing 5% by volume of added concentrated aldehydic heads. The aldehydic concentrate was prepared as before (11) by fractional redistillation of a commercial type of continuous brandy still "heads" cut.

The wines (about 5 gallons each lot) were stored in full, cork-stoppered glass containers without further processing other than rackings after about 1 day, 2 weeks, and 6 weeks. The Mission lots received an additional 50 ppm SO_2 on December 11. Analyses were made on samples drawn after about 3 months. Spectra were determined on filtered and water-diluted samples with a Bausch and Lomb Spec-

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TABLE I

Composition, Absorbance and Quality of Port-Type Wines Prepared with and without Aldehyde in the Added Spirits

	Grape Variety									
	Souzão		Trousseau		Mission		Carignane		Grenache	
	Sa	SAb	S	SA	S	SA	S	SA	S	SA
Composition										
Vol. aldehyde, HAc mg/l	62	159	89	199	81	192	43	177	87	206
Vol. esters, EtOAc mg/l	46	76	47	75	47	73	43	68	37	71
Vol. acid, HOAc g/100 ml	0.007	0.007	0.011	0.014	0.012	0.010	0.006	0.008	0.009	0.008
Alcohol, % v/v	19.9	20.0	19.9	20.5	20.8	21.0	20.0	19.5	19.1	19.6
Total acid, Tartaric g/100 ml	0.64	0.64	0.49	0.51	0.37	0.36	0.58	0.58	0.46	0.42
pH	3.86	3.82	4.14	4.17	4.26	4.21	3.66	3.66	4.23	4.22
Extract, g/100 ml	11.5	11.6	12.6	12.0	11.0	11.0	11.4	11.4	11.8	11.8
Tannin, gallic acid mg/l	1189	1208	580	490	510	480	390	220	455	436
Absorbance										
520 mμ, 1 cm x diln.	6.56	9.25	2.87	3.35	0.65	0.63	0.91	1.25	0.66	0.77
440 mμ, 1 cm x diln.	4.33	6.42	2.17	2.46	0.58	0.57	0.69	0.86	0.72	0.83
280 mμ, 1 cm x diln.	43.0	50.0	30.0	28.2	17.3	16.0	11.8	11.7	16.2	15.8
Ratio $\dfrac{520 \text{ m}\mu}{440 \text{ m}\mu}$	1.52	1.44	1.32	1.36	1.12	1.10	1.32	1.45	0.90	0.92
Quality										
Rating of wine (20 max.)	14.3	15.0	14.6	14.4	12.2	12.6	13.2	13.6	12.8	13.8

a S = Wine spirits.

b SA = Aldehydes (heads) added, 5 Vol. % of a concentrated heads to the same wine spirit.

tronic 505 recording spectrophotometer. Alcohol and volatile aldehydes were determined according to the method of Guymon and Crowell (4, 5). Pigment fractions were studied by the pH and polyamide adsorptive methods of Berg and Akiyoshi (2). The other analytical methods were as reported before (11). Sensory quality ratings on a 20-maximum scale (11) by 9-11 expert tasters were obtained with these samples mixed randomly in coded sets with other port-type wines.

RESULTS

The compositions of the wines produced are shown in table 1. The variation between wines was rather great. This was a desirable result, reflecting a spread of characteristics in the varieties chosen. Souzão and Trousseau usually make high-quality ports, whereas the other three make standard ports. Grenache and Mission are notably low in anthocyanin content. These typical differences are borne out in these wines, as shown by the quality scores and absorbances at 520 mμ in table 1. The alcohol contents and extract values for all the wines are typical and similar. Total acidity, pH, and volatile acidity were very similar for each varietal pair, and apparently were not affected by the nature of the spirit added.

The wine spirit employed analyzed 191° Proof, 14 mg acetaldehyde equivalents per liter, and 177 mg ethyl acetate equivalents per liter. The addition of 5% (by volume) concentrated heads raised the

TABLE 2

Relative Composition, Quality and Color Characteristics of Port-Type Wines Produced from Five Grape Varieties by the Use of Aldehydic Spirits

	Variety[a]					
	Souzão	Trousseau	Mission	Carignane	Grenache	Average
Composition						
Vol. aldehyde	256	222	238	413	236	273
Vol. ester	167	160	153	159	191	166
Tannin	102	84	94	56	96	86
Quality						
Rating	105	99	103	103	108	104
Color Characteristics						
Absorbance at 520 mμ	141	117	97	138	117	122
Absorbance at 440 mμ	148	113	99	125	115	120
Absorbance at 280 mμ	116	94	93	99	98	100
Absorbance ratio 520 mμ/440 mμ	95	103	98	110	102	102
Eluted red pigment[b]	81	93	68	77	77	82
Tightly absorbed red pigment[c]	99	128	67	120	117	106
% tightly adsorbed[d]	113	128	94	154	140	126
Acid responsive red pigment[e]	74	24	57	42	57	49

[a] Relative values are the ratio, measured value for wine receiving aldehydic spirit divided by measured value for wine receiving plain spirit, times 100.

[b] Wine passed on to a polyamide (Nylon) powder column (ref. 2), the red pigment eluted with methanol-acetic acid-water 70:25:5, and measured by spectral absorbance at 540 mμ in butanol-conc. HCl 80:20.

[c] Red pigment not eluted from polyamide measured by dissolving the column in butanol-conc. HCl 80:20.

[d] The absorbance at 540 mμ of the tightly absorbed pigments divided by that of the elutable plus the tightly adsorbed.

[e] The relative absorbance increase at 525 mμ when the pH was lowered from 3.30 to 2.30.

volatile aldehyde figure to 804 mg/l and the volatile ester to 398 mg/l. Use of these two spirits produced the expected differences in the wines (Table I), i.e., the aldehydic spirit produced wines higher in volatile aldehydes and esters than did the plain spirit.

Except for the Mission pair, all of the wines showed considerably more absorbance at 520 mμ when the aldehydic spirit was used. Thus, in 5 of 6 cases this effect has been found and the single exception was a very low-colored, relatively brown wine. It thus can be concluded that fortification with spirits containing heads nearly always produces a more intensely colored port. These color differences are large enough to be easily seen by the eye and are probably the reason that the panelists tended to rate the "heady" wine as of better quality. However, the score differences were not consistent enough to be statistically significant. This is believed to be another (11) manifestation of different reactions of the panelists to improved color but "heady" odors in the wine.

Table I also shows that the Folin-Denis "tannin" values tended to be lower with more aldehyde in the fortifying spirit and that this was generally correlated with absorbance at 280 mμ, as expected. A wine with a higher absorbance at 520 mμ compared to absorbance at 440 mμ is a relatively red-colored wine, and as the 520/440 absorbance ratio falls the wine is relatively more brown or tawny. Table I shows that, although the Grenache and Mission pairs of wines were rather brown and the others were not, the aldehydic spirit did not produce more browning than the plain spirit.

Table 2 illustrates some of these results more forcefully by showing comparisons within the pairs of wines, and thus correcting for the fact that the pairs of wines are quite different from each other. The anthocyanin color (520 mμ) is seen to be, on the average, 22% more intense when the aldehydic brandy was used. Maximum absorbance in the visible region characteristic for anthocyanins was determined by the methods of Berg and Akiyoshi (2) following pH adjustment and ad-

sorption on polyamide columns.

The bottom of table 2 shows the relative amount of red pigment which was readily eluted from the polyamide, expressed as the ratio of the absorbance obtained in the standardized manner from the wine with extra aldehyde divided by that from the wine without added aldehyde. The relative amount of readily elutable pigment is seen to be greater for the wine without the added aldehyde. The relative amount of pigment adsorbed on polyamide, determined similarly, was variable, but the proportion adsorbed relative to the total recovered was considerably (26% on the average) greater for the wines which had received extra aldehyde in the spirits added. The figures given were obtained from the absorbance in the anthocyanin (visible) region of the cold solutions of the polyamide eluant and the dissolved polyamide column. The same acidic solutions, upon heating, gave absorbance increases which can be attributed to leucoanthocyanins. These data are not shown, but the results are consistent with those obtained with the unheated solutions, and no relationship is indicated between aldehydic brandy and leucoanthocyanins.

The increase in absorbance in the anthocyanin-red region which results from adjustment of the pH from 3.30 to 2.30 was determined. The increase was greater for the wines which received low-aldehyde brandy than for the higher-aldehyde brandy samples (Table 2). The pigment which fails to give appreciable color increase in response to this pH change has been postulated (2) to be an associated form of anthocyan which is relatively unaffected by the pH-related equilibria of normal anthocyanidins. Table 2 shows that the red color of wines without the added aldehydes averaged about twice as responsive to pH, indicating that the high aldehyde wines had a higher proportion of modified anthocyanins.

Table 3 shows the effect of longer time upon the 1961 wines (11). The absorbance at 520 mμ for the two wines became similar with time, and the wines became more brown. The aldehyde decreased, but dif-

ferences between the two lots persisted. The tannin decreased more in the higher-aldehyde wine, but there was relatively less browning. Therefore, although the intensity of the red pigmentation measured at 520 mμ was similar after 27 months, the higher-aldehyde wine was still exhibiting relatively more or purer red color in proportion to the total "tannin" (which includes the anthocyanins).

DISCUSSION

Although these results have some practical implications in the marketing of young ruby ports with more intense red color, their major value appears to be in clarifying the complex changes of anthocyanins in wines. It is now clear that halting the fermentation of a port-type wine with alcohol containing some heads usually produces a more intensely red wine after 2-3 months than is produced if a low-heads, high-proof spirit is used. The

major components other than ethanol in concentrated heads are highly volatile carbonyl compounds and esters. The concentrated heads used in the previous study (11) did not contain appreciable esters, and the same effect was observed. The major carbonyl components are acetaldehyde and acetal, which almost entirely reverts to acetaldehyde in wine (5). It will require comparable experiments with pure aldehydes to prove conclusively that the enhancement of red color is caused by the aldehyde in the spirit, but this appears to be true.

The wines which exhibited more red pigmentation because of aldehydic spirits addition were characterized by: 1) a higher proportion of pigment adsorbed tightly on polyamide (Nylon) powder; 2) a resistance to color increase upon lowering of the pH; and 3) a lowering of the polyphenol content as measured either by the Folin-Denis reagent or by absorbance at 280 mμ. The Folin-Denis reaction appears

TABLE 3

Changes in Composition and Absorbance During Storage of Two Port-Type Wines
Prepared with and without Aldehydes in the Added Spirit[a]

| | Storage period (months) | | | | Change (%) | |
| | 2 | | 27 | | | |
	S[b]	SA[c]	S	SA	S	SA
Composition						
Vol. aldehyde, HAc mg/l	43	156	30	113	—30	—28
Vol. esters, EtOAc mg/l	44	34	47	44	+ 7	+29
Vol. acid, HOAc g/100 ml	0.015	0.018	0.006	0.005	+20	—40
Total acid, Tart. g/100 ml	0.43	0.47	0.40	0.40	— 9	— 9
pH	3.67	3.66	3.72	3.79	+ 1.4	+ 3.6
Alcohol, % v/v	19.7	19.3	20.3	19.6	+ 3.0	+ 1.6
Extract, g/100 ml	10.3	10.9	10.7	11.1	+ 3.9	+ 1.8
Tannin, gallic acid mg/l	610	630	559	427	— 8.4	—33.3
Absorbance						
520 mμ, l cm x diln.	1.81	2.28	1.82	1.84	+ 0.6	—19.3
440 mμ, l cm x diln.	1.31	1.58	1.48	1.44	+13.0	— 8.9
280 mμ, l cm x diln.	24.0	20.5	18.6	19.2	—22.5	— 6.3
Ratio 520 mμ/440 mμ	1.38	1.44	1.23	1.28	—10.9	—11.1
Ratio 280 mμ/520 mμ	13.3	9.0	10.2	10.4	—23.3	+13.5

[a] Produced from Carignane grapes in October 1961 and stored in corked glass containers.
[b] S = Wine Spirit.
[c] SA = Aldehyde (heads) added, 5 Vol. % of a concentrated heads to the same wine spirit.

to be affected somewhat more than the 280 mμ absorbance. These facts suggest that the aldehydes (heads) have caused a reaction which modifed a portion of the anthocyanins so that they became resistant to further change and yet absorbed light so as to remain red. The masking of some of the phenolic groups is suggested, plus a change which prevents conversion of a sizeable portion of the anthocyans to colorless forms such as otherwise occurs at the pH of wine, as illustrated by Jurd (7).

A hypothesis which would explain these observations is that reactive carbonyl compounds, such as acetaldehyde, react with the anthocyanins (and presumably other flavonoids and tannins present) via an acid-catalyzed Baeyer (phenol-formaldehyde-Novolak) reaction. This postulated reaction could produce methylol intermediates of the form R-CH(CH$_3$)-OH, dimers of the form R-CH(CH$_3$)-R, and perhaps larger polymers by further substitution. The R groups would be phenolic compounds with linkage formed at an originally unsubstituted position *ortho* or *para* to the phenolic hydroxyl. With the anthocyanins found in wine, reaction with acetaldehyde to link at positions 6 and 8 in the A or phloroglucinol ring appear most probable.

This substitution would not affect the conjugation, and therefore should not affect the anthocyanin's color. Particularly in the dimer or larger state it would be expected, however, that the adsorption on polyamide would be tighter. Masking of phenol reactivity to the Folin-Denis reagent and resistance to pH effects would appear reasonable. Insolubilization or rendering inactive of other "tannins" and polyphenolics as well as anthocyanins would explain the greater total loss in these measures.

The necessary conditions for this reaction appear compatible with the nature of wine as a reaction medium, particularly considering that an apparently slow reaction rate and relatively small concentration changes are involved. Another fact which makes this hypothesis attractive is that it is very difficult and usually impos-

sible to get a good chromatogram on paper of the "anthocyanins" of red wine more than a few months old. However, grape skin extracts and very young wines chromatograph readily to show typical patterns of 8 or so discrete red pigment spots. The tendency of the pigments in older wines to stay at or only smear from the origin would be the expected result with the heterogeneous, molecularly large reaction products visualized.

Other hypotheses could be advanced for the observed aldehyde-pigment effect, such as oxidation-reduction effects via the alcohol dehydrogenase system as the fermentation is terminated by fortification. Further work will be needed to prove the true mechanism, but these experiments appear to have provided a new tool for unraveling the complex chemistry of wine pigments.

SUMMARY

The presence of heads in wine spirits used in the production of red sweet (port-type) wines produced an enhancement of the red pigmentation, measured 2-3 months after production. Other chemical and sensory effects are also reported. These effects all correlate with the volatile aldehyde content of the added heads. On longer storage, differences between samples prepared with and without aldehydic spirits diminished but did not disappear entirely.

The results suggest that the anthocyanin pigments are modified to form more adsorbable, less reactive, presumably larger, but still red products. A possible mechanism to explain these effects is discussed, namely the Baeyer reaction between phenols and aldehydes in acidic solution.

ACKNOWLEDGMENTS

REFERENCES CITED

1. Albach, R. F., R. E. Kepner, and A. D. Webb. Comparison of anthocyan pigments of red vinifera grapes. II. Am. J. Enol. Viticult. **10**: 164-72 (1959).

2. Berg, H. W. and M. Akiyoshi. Color behavior during fermentation and aging of wines. Am. J. Enol. Viticult. **13**:126-32 (1962).

3. Cantarelli, C. Invecchiamento naturale lento ed invecchiamento accelerato del vino in rapporto ai caratteri organolettici ed al valore biologico de prodotto. Accad. ital. vite e vino, Siena, Atti **10**:192-227 (1958) (Pub. 1959).

4. Guymon, J. F. and E. A. Crowell. The chemical determination of alcohol in wines and stillage by dichromate. J. Assoc. Offic. Agr. Chemists **42**:393-98 (1959).

5. Guymon, J. F. and E. A. Crowell. Determination of aldehydes in wines and spirits by the direct bisulfite method. J. Assoc. Offic. Agr. Chemists. **46**:276-84 (1963).

6. Joslyn, M. A. and C. L. Comar. Role of acetaldehyde in red wines. Ind. Eng. Chem. **33**:919-28 (1941).

7. Jurd, L. Anthocyanins and related compounds. I. Structural transformations of flavylium salts in acidic solutions. J. Org. Chem. **28**:987-91 (1963).

8. Mareca Cortés, I. and M. de Campos Salcedo. Sur la combinaison de l'ethanal et des polyphénols dans les vins rouges. Ind. Agr. Aliment. (Paris) **74**:103-6 (1957).

9. Mareca Cortés, I. and E. del Amo Gili. Evolution de la matière colorante des vins de Rioja au cours du vieillissement. Ind. Agr. Aliment. (Paris) **76**:601-6 (1959).

10. Ribéreau-Gayon, P. Recherches sur les anthocyannes de végétaux, application au genre **Vitis.** Librairie Générale de l'enseignement, Paris. 111 p. (1959).

11. Singleton, V. L. and J. F. Guymon. A test of fractional addition of wine spirits to red and white port wines. Am. J. Enol. Viticult. **14**:129-36 (1963).

12. Trillat, A. Sur les diverses destinations de l'aldéhyde acétique dans le vin rouge. Bull. soc. chim. **5**:546-9, 550-5, 555-8 (1909).

TABLE I

Composition of Wines Aged at 128°F under Oxygen or Nitrogen

| Composition | Days aged | Table wine | | | | Flor sherry | | Dessert Wine | | | |
| | | Dry white | | Dry red | | | | White sweet | | Red sweet | |
		O_2	N_2	O_2	N_2	O_2	N_2	O_2	N_2	O_2	N_2
Absorbance[a] at 420 mμ	0	139	112	712	639	308	319	286	287	3175	3085
	10	190	129	868	658	460	353	430	318	3190	2790
	20	232	134	992	676	509	399	520	346	3205	2745
Voltaile aldehydes (mg HAc/l)	0	55	52	40	45	615	603	121	106	48	48
	20	65	50	72	40	624	610	137	114	72	53
Total esters (mg EtOAc/l)	0	654	612	878	1022	785	875	925	979	805	856
	20	945	833	933	1370	1240	1183	1032	1074	1082	1108
Tannin (mg gallic acid/l)	0	144	158	670	675	204	.210	232	238	675	700
	10	146	156	655	655	204	210	227	232	670	705
	20	136	158	680	655	219	210	238	250	715	705

[a] Absorbance values shown are the readings for l-cm light path, water reference, times 1000 times dilution (the red sweet was diluted 1/5 with water).

flor sherry, which might be expected to behave differently). Grape aroma rating was not high in any of these wines, and upon heating in the presence of oxygen it fell in 10 days to zero or nearly so. Heating of inert-gas-treated wine tended to decrease this rating, but to a lesser degree and more slowly.

Hotness rating (the peppery, sharp, high "alcohol" mouth sensation characteristic of unaged brandy) was low in the table wines and appeared to decrease upon heating in

inert atmospheres. Sherry and dessert wines, as expected, were rated higher than the table wines in hotness, but each of them appeared to react differently to the heat-gas treatments. Perhaps because these dessert wines were made with wine spirits already low in hotness (7), we were unable to demonstrate clearly the improvement in smoothness that others noted when fortified sweet wines were heated in inert atmospheres. Astringency ratings showed no clear relationship to the treatment, but

TABLE 2

Effect on Mean Sensory Ratings of Aging Wines at 128°F under Oxygen or Inert Gases[a]

Factor rated	Days heated	Table wine				Flor sherry		Dessert Wine			
		Dry white		Dry red				White sweet		Red sweet	
		O₂	Inert	O₂	Inert	O₂	Inert	O₂	Inert	O₂	Inert
Bottle bouquet (8 max.)	0	0.0	0.3	0.0	0.0	0.0	0.5	0.5	1.3	0.0	0.3
	10	0.0	1.5	0.0	1.5	0.0	0.5	0.5	1.7	0.0	0.8
	20	0.0	3.3	0.0	2.3	0.0	0.7	1.0	1.5	0.0	0.5
Oxidized flavor (8 max.)	0	1.5	0.8	0.0	1.3	4.0	4.5	1.0	1.0	0.0	0.2
	10	2.5	0.5	1.0	0.2	5.0	2.5	2.5	1.7	1.0	0.7
	20	6.0	1.5	1.0	0.3	3.0	4.8	3.0	2.0	1.5	1.0
Color change related to age (8 max.)	0	1.5	0.8	0.5	0.2	3.0	4.0	4.0	3.3	2.0	2.2
	10	4.0	1.5	6.0	1.5	8.0	3.0	8.0	4.7	3.0	2.2
	20	8.0	1.7	8.0	3.8	8.0	4.7	8.0	6.0	4.0	1.8
Grape aroma (8 max.)	0	0.5	0.7	1.0	1.2	0.0	0.0	0.0	0.5	1.0	1.0
	10	0.0	0.7	0.0	1.0	0.0	0.0	0.0	0.5	0.5	1.0
	20	0.0	0.0	0.0	0.7	0.0	0.0	0.0	0.2	0.5	0.8
Hotness 8 max.	0	1.5	0.7	0.0	0.2	2.5	1.5	1.5	2.0	2.0	2.2
	10	0.0	0.5	1.0	0.3	0.5	1.5	0.5	2.5	2.0	2.2
	20	0.0	0.0	1.0	0.0	0.5	1.7	1.0	1.3	4.0	3.0
Astringency (8 max.)	0	0.5	0.5	0.5	0.5	0.5	0.5	1.0	0.5	1.0	0.7
	10	0.5	0.8	1.0	0.3	0.5	0.5	0.5	0.5	1.0	0.7
	20	0.5	0.3	1.0	0.2	0.5	0.3	1.0	0.5	0.5	1.0
Bitterness (8 max.)	0	1.5	0.7	0.0	0.2	2.0	0.5	1.0	0.2	2.0	1.5
	10	0.5	0.3	1.0	0.3	2.0	1.5	1.0	1.2	2.5	1.7
	20	0.5	0.5	1.0	0.0	1.5	1.5	1.5	1.7	3.0	1.2
Complexity or richness of flavor (8 max.)	0	1.5	1.0	1.0	0.8	1.5	2.0	1.5	1.3	1.5	1.0
	10	3.0	1.3	2.0	1.7	3.0	3.0	1.5	1.3	1.0	1.0
	20	1.0	3.7	1.5	1.3	2.5	2.8	3.0	2.3	2.0	1.3
Quality score (20 max.)	0	14.5	15.2	13.5	12.3	14.0	14.5	15.5	14.5	14.5	13.8
	10	12.5	15.3	10.5	15.0	16.0	15.5	14.0	14.2	13.0	13.7
	20	11.5	16.3	9.5	13.3	14.0	14.5	13.5	14.7	13.0	13.7

[a] Inert gases consisted of nitrogen, hydrogen, and carbon dioxide.

none of the wines were very astringent. Bitterness relationships were also not clear, but the effect was not great. There was a suggestion in the data for red wines and sweet wines that heating, especially with oxygen, gave a small increase in apparent bitterness.

The striking feature of these tests, however, was the apparently important increase in bottle bouquet rating when the wine was heated in the absence of oxygen, especially in the table wines (particularly the dry white one). Heating for at least some period increased the complexity rating of the wines, again most notably for the dry white table wine heated 20 days in inert gas atmospheres. Over-all quality scores for the wines were consistently decreased (except for flor sherry) by heating with oxygen. Heating in the absence of oxygen was judged to improve the dry white table wine considerably, and the highest score in the other cases was for one of the heated samples when inert gas was used.

Reviewing these results by wine type, the dessert wines did not appear to be greatly altered from a sensory viewpoint by heating in the absence of oxygen, although some of the effects (such as increased complexity) appeared favorable. The data appear to suggest, as recommended by Soviet workers (12), that warming in combination with and without oxygen is appropriate for dessert wines. The effects on the submerged-culture flor sherry can be best judged in connection with the comments made on the tasting sheets. This wine was very rich in the flor aroma but, relative to other sherries, was low in color and low in aged-oxidized flavors other than the flor odor. The longer period of heating in the absence of oxygen produced a wine with greatly decreased flor character and little baked sherry quality either. In the presence of oxygen the flor odor was also almost completely lost or modified, and although good baked sherry character was produced there seemed to be no great advantage to starting with a flor shermat. Therefore, at least under the limited range of conditions tested, the baking of a simple submerged-culture flor sherry does not appear as attractive as blending or other possible

methods of production of wines more similar to aged Spanish *fino* sherry.

The dry table wines, especially the white wine, reacted most favorably to heating in the absence of oxygen, and most unfavorably to heating in the presence of oxygen. The production of bottle bouquet, an increased complexity, and an improved over-all quality score from heating 20 days at 128°F in an inert atmosphere certainly warranted further investigation.

An experiment was conducted with a Thompson Seedless dry white table wine containing 0, 20, and 200 ppm of added sulfur dioxide. The wine was bottled after sweeping with nitrogen as before, and heated at 128°F for 0, 8, 16, and 32 days. Table 3 shows ratings by a panel of 10 judges on the same scale used in the first experiment. It can be seen that this wine did not respond as did the previous sample. The quality score decreased and bottle bouquet did not develop. In the opinion of the judges who served in both experiments, the quality of the odor was not nearly as similar to that of fine wines after long bottle aging. It was thought that this lack of favorable effect stemmed from the grape variety and the relatively low level of quality of the original wine. This experiment did demonstrate that 32 days of heating at 128°F did not darken this wine

TABLE 3

Effect of Sulfur Dioxide and Aging under Nitrogen at 128°F on the Mean Sensory Ratings of Thompson Seedless Dry White Table Wine

Factor rated	SO₂ added (ppm)	Days of heating			
		0	8	16	32
Bottle bouquet (8 max.)	0	2.4	3.4	2.9	3.3
	20	1.8	1.7	2.7	3.1
	200	3.0	....	....	4.3
SO₂ level (8 max.)	0	1.2	1.2	1.3	1.2
	20	2.5	1.2	1.1	2.4
	200	1.7	....	....	1.2
Over-all quality (20 max.)	0	12.7	11.8	10.5	10.2
	20	12.1	12.2	11.4	10.1
	200	13.0	....	....	10.2

Mean Sensory Scores of Wi

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Wine number[b]	Bottle bouquet[c] (10 max.)	Complexity and richness[c] (10 max.)	Grape aroma[c] (10 max.)	Oxidized flavor (10 max.)
1	y 5.7 c 4.3 x 4.2	y 5.0 c 4.4 x 3.8	c 5.8 y 4.1 x 4.0	y 3.9 x 3.6 c 3
2	y 5.6 x 5.2 c 4.2	y 4.7 x 4.3 c 4.0	c 4.8 y 4.6 x 4.5	y 3.7 x 3.3 c 2
3	y 3.8 x 3.2 c 2.4	x 3.9 y 3.4 c 2.9	c 3.6 x 2.9 y 2.2	y 3.4 x 2.4 c 1
4	y 4.1 x 3.5 c 3.0	x 3.4 y 3.2 c 3.1	x 4.0 c 3.9 y 3.6	x 2.4 y 2.4 c 2
5	x 4.3 y 4.2 c 3.6	y 4.0 x 3.6 c 3.5	c 4.4 x 3.9 y 3.5	x 4.4 y 3.6 c 3
6	y 3.6 x 3.1 c 2.8	x 3.4 c 3.4 y 3.2	c 4.1 x 3.3 y 2.2	y 3.2 x 2.0 c 1
7	y 4.1 x 4.0 c 2.6	x 4.0 y 3.8 c 3.2	c 4.6 x 2.9 y 2.0	y 3.2 x 2.8 c 1.
8	y 3.3 x 3.2 c 2.6	y 3.7 x 3.2 c 3.1	c 4.2 x 2.4 y 2.4	y 2.6 x 2.4 c 1.
9	x 4.4 y 3.9 c 3.4	x 4.1 c 4.0 y 3.9	c 3.6 x 3.2 y 2.5	x 4.7 x 4.4 c 3.
10	y 4.0 x 4.0 c 3.3	y 3.8 x 3.6 c 3.6	c 5.1 x 3.2 y 2.7	y 4.6 x 4.0 c 1.
11	y 3.4 x 3.4 c 2.8	x 3.8 y 3.8 c 3.7	c 5.2 x 2.9 y 2.6	y 3.8 x 3.2 c 2.
12	y 4.6 x 3.9 c 2.9	y 4.1 x 3.7 c 3.5	c 4.3 x 3.9 y 3.0	y 4.2 x 3.4 c 2.
13	x 3.4 y 3.2 c 2.5	x 3.0 y 2.9 c 2.8	c 2.8 x 2.4 y 2.2	x 3.3 y 2.6 c 2.
14	y 3.6 c 3.4 x 3.2	c 4.5 x 4.1 y 4.0	c 6.4 x 4.3 y 4.0	y 4.0 x 3.3 c 2.
15	y 4.1 x 3.8 c 3.0	y 4.2 x 3.8 c 3.4	c 4.2 x 3.4 y 2.8	y 3.6 x 2.8 c 1.
16	y 4.4 x 3.8 c 3.0	x 3.7 x 3.4 c 3.3	c 4.2 x 3.1 y 2.6	y 4.0 x 2.7 c 1.
17	y 4.5 x 4.1 c 2.8	x 4.0 y 3.8 c 3.6	c 4.4 x 3.0 y 3.0	y 3.6 x 2.9 c 1.
18	y 4.2 x 3.6 c 2.4	y 4.6 c 3.6 x 3.4	c 3.6 x 2.9 y 2.0	y 3.4 x 2.4 c 2.
19	y 4.0 x 3.8 c 3.2	x 4.3 y 3.8 c 3.8	c 4.8 x 3.6 y 2.9	y 3.7 x 2.8 c 2.
20	y 4.2 x 3.5 c 2.6	y 4.2 x 3.9 c 3.8	c 3.9 x 3.0 y 3.0	y 3.9 x 2.7 c 2.
21	y 3.3 x 3.1 c 2.1	x 3.4 y 3.1 c 2.8	c 3.5 x 2.3 y 1.8	y 4.3 x 4.3 c 2.
22	y 4.6 x 3.8 c 2.6	y 4.4 x 4.0 c 3.0	c 4.0 x 4.0 y 3.3	y 3.3 x 2.3 c 1.
23	y 5.0 x 4.6 c 2.7	y 4.5 x 4.5 c 3.4	c 4.4 x 3.6 y 2.9	y 4.2 x 4.0 c 2.
1-23	y 4.17 x 3.78 c 2.98	y 3.90 x 3.80 c 3.35	c 4.35 x 3.34 y 2.89	y 3.66 x 3.14 c 2.
24	y 3.7 x 2.9 c 2.4	y 3.6 x 3.5 c 3.2	c 3.2 x 3.2 y 2.6	c 2.5 x 2.3 y 2.
25	y 3.1 x 3.0 c 2.2	x 3.8 c 3.7 y 3.6	c 2.8 x 1.9 y 1.7	y 3.2 x 2.8 c 2.
26	y 3.8 x 3.6 c 2.5	y 3.5 x 3.3 c 3.2	c 3.1 x 2.4 y 2.2	y 3.2 x 3.0 c 2.
27	y 4.0 x 3.5 c 3.2	x 3.8 c 3.8 y 3.5	c 3.4 x 3.0 y 2.9	y 3.0 x 2.6 c 2.
28	y 3.8 x 3.2 c 3.0	y 3.7 c 3.7 x 3.5	c 3.3 x 2.8 y 2.6	y 2.8 x 2.4 c 1.
24-28	y 3.69 x 3.24 c 2.65	y 3.57 x 3.56 c 3.52	c 3.15 x 2.65 y 2.41	y 2.91 x 2.60 c 2.

[a] Scores are arranged in decreasing order of means. Scores not underlined by the same line differ significantly at Duncan multiple-range test.
[b] Wines 1—23 are dry white table wines, 24—28 dry red table wines.
[c] Treatments are: c= control, x = 15 days at 128°F, and y = 30 days at 128°F.

in the Absence of Oxygen[a]

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Color (Age-like Change) (10 max.)			Bitterness (10 max.)			Score (20 max.)		
y 4.4	x 4.2	c 3.4	y 3.9	x 3.8	c 3.7	c 13.0	y 12.3	x 11.9
y 5.4	x 4.5	c 3.8	x 4.0	y 3.6	c 3.6	c 13.5	x 13.3	y 12.5
y 3.6	x 3.2	c 2.8	y 2.6	x 2.1	c 1.6	x 12.9	c 12.8	y 11.7
y 4.0	c 3.8	x 3.3	x 2.4	y 2.2	c 2.1	x 11.7	y 11.6	c 11.6
x 4.5	c 4.4	y 4.4	y 4.0	x 4.0	c 4.0	c 11.7	y 11.2	x 11.2
y 3.2	x 2.8	c 2.2	y 3.0	x 2.2	c 1.8	c 13.4	x 12.9	y 11.4
y 3.2	x 3.0	c 2.0	x 2.8	y 2.6	c 2.4	c 13.2	x 12.5	y 11.7
y 3.4	x 3.4	c 2.5	c 2.4	y 2.4	x 2.1	c 12.4	x 11.9	y 11.5
x 5.0	y 4.8	c 4.2	y 3.4	x 3.2	c 2.4	c 12.7	x 12.7	y 11.5
y 3.8	x 3.7	c 3.6	y 3.5	x 3.4	c 2.7	c 13.3	x 10.7	y 10.0
y 3.8	x 3.3	c 2.2	y 2.5	x 2.4	c 1.8	c 14.1	x 11.8	y 10.8
y 5.6	x 4.3	c 3.6	x 3.7	y 3.6	c 3.0	c 13.1	x 12.7	y 11.7
x 4.4	y 4.2	c 2.7	x 2.6	y 2.5	c 2.4	y 10.6	c 10.5	x 10.4
y 3.8	c 3.4	x 3.3	y 3.5	x 2.7	c 2.5	c 14.6	x 12.7	y 11.5
y 3.8	x 3.8	c 3.0	y 3.2	c 2.6	x 2.4	c 12.5	x 12.2	y 11.8
y 5.3	x 4.2	c 2.9	y 3.2	x 2.6	c 2.2	c 13.2	x 12.5	y 11.2
y 3.8	x 3.4	c 2.7	y 3.6	x 3.2	c 2.6	c 14.0	x 12.6	y 11.3
y 4.4	x 3.6	c 2.8	y 3.0	x 2.8	c 2.5	c 12.0	x 11.8	y 11.4
x 4.0	y 3.8	c 3.1	x 3.2	y 3.0	c 2.7	c 13.4	x 13.2	y 11.4
y 3.4	x 3.2	c 2.8	x 3.2	y 2.9	c 2.5	c 13.2	x 12.4	y 11.8
y 5.2	x 5.0	c 3.0	y 2.7	x 3.6	c 3.1	c 9.8	x 9.1	y 8.5
y 3.0	x 2.9	c 2.6	c 2.9	x 2.8	y 2.6	x 12.9	y 12.7	c 12.6
y 5.4	x 5.2	c 4.2	y 3.6	x 3.3	c 2.8	c 12.7	x 12.4	y 11.4
y 4.18	x 3.84	c 3.14	y 3.16	x 2.97	c 2.63	c 12.75	x 12.10	y 11.37
y 3.8	x 3.8	c 3.2	y 2.5	c 2.2	x 2.1	x 12.8	y 12.4	c 12.2
y 4.6	x 3.8	c 2.8	x 2.7	y 2.5	c 2.3	c 12.1	x 11.9	y 11.2
y 4.0	x 3.7	c 3.0	y 3.2	x 2.6	c 2.1	c 11.6	x 10.4	y 10.1
y 4.3	x 3.8	c 3.2	y 2.8	x 2.8	c 2.6	c 13.6	x 12.7	y 12.6
y 3.8	x 3.6	c 3.0	y 3.7	x 3.6	c 3.2	c 12.6	y 11.9	x 11.7
y 4.09	x 3.74	c 3.06	y 2.94	x 2.76	c 2.48	c 12.41	x 11.90	y 11.62

% confidence level by the

to unusability, and that sulfur dioxide level was apparently not causatively related to bottle bouquet production.

The next experiment consisted of purging and bottling under nitrogen a series of 23 dry white and 5 relatively low-colored dry red table wines. Samples of each wine were heated, as before, for 0, 15, and 30 days. The samples were then tested for pH, redox potential, and sensory factors. The average pH of the wines, in order of increasing heat treatment, was 3.337, 3.306, and 3.326 for the white wines, and 3.614, 3.560, and 3.652 for the red wines. The difference between the means for the white wines heated for 15 and 30 days was statistically significant. This increase in pH with the longer heat treatment is probably the result of esterification of tartaric and other acids present with ethanol, as shown by the increase in total esters as in table 1. The other differences were not statistically significant.

Redox potentials, measured by a platinum electrode versus the saturated calomel electrode, averaged 112.8, 119.0, and 108.5 mv for the white wines, and 112.8, 112.2, and 97.0 mv for the red wines, in the order of 0, 15, and 30 days of heat treatment. These differences are about as expected since they show that wines stripped of oxygen with nitrogen averaged relatively reduced in potential and tended to become more reduced by heating. The potentials obtained with individual wines, however, varied from —81 to +234 mv and were by no means lowered consistently with heating, nor did redox potential or pH of individual samples appear to correlate with sensory ratings.

Difference testing was used to determine whether each of the heated samples differed significantly from the control samples of each wine before the samples were presented to the panel for rating the various individual qualities. In all 28 cases a significant difference was produced by warming 15 or 30 days under nitrogen. In nearly all cases, 10 correct matchings were obtained in 10 trials.

The mean sensory ratings of these samples by the panel are shown in table 4. Each mean rating for each wine represents 20 to 22 individual judgments for each

criterion by a total panel of 15 of the most experienced and expert wine judges available in the department. Ratings not underlined by the same line differed significantly to at least the 19:1 confidence level. Note that a significant difference by treatment was found in at least one criterion for all wines but 4, 5, and 27.

Wines numbered 1-4 were from the Chardonnay grape variety, 5-8 were Sauvignon blanc, 9-11 Gewürztraminer, 12-13 Pinot blanc, 14 Orange Muscat, 15 Muscat Canelli, 16 White Riesling, 17 Sylvaner, 18 Chenin blanc, 19 Peverella, 20 Pinot gris, 21 French Colombard, 22 Red Veltliner, 23 Delaware, 24 Gamay Beaujolais, 25 Meunier, 26 Grenache, 27 Cabernet Sauvignon, and 28 Petite Sirah. The astringency ratings are omitted from table 4 because no significant differences were found in any one wine or in the combined data for red or for white wines.

It can be seen that the combined results for the red wines parallel those for the white wines except in the magnitude of the ratings for some of the factors and in the lower degree of significance resulting from fewer samples. The bottle bouquet rating was increased by the heat treatment, as were the complexity rating, the rating for color changes related to aging (browning), the oxidized flavor rating, and the bitterness rating. Grape aroma rating was lowered, as was the average over-all quality score.

The lowering of the panel's average over-all quality rating by the warming is believed to reflect several factors: a) the loss in grape aroma was weighted heavily; b) the color changes and flavor changes were interpreted as oxidation, and therefore undesirable; and c) the panelists were primarily familiar with young fruity types of table wine as commercially produced, and were not, as a group, highly experienced with bottle bouquet. Although these panelists rated the heated samples as increased in oxidized flavor, this is not justified, because we know from the manner of handling and from the redox potentials that these wines were *not* oxidized during the heat treatment. The panelists are believed to have erred by confusing the flavor and odor change produced with true oxidation,

and also because of bias resulting from the slight darkening of the wines' color.

That the loss of grape aroma was heavily weighted by the panel as a whole can be seen by comparing ratings on individual wines. Wine 14, an Orange Muscat, received the highest rating of any wine for grape aroma in the control sample, and was the only wine significantly poorer after 15 as well as 30 days of heating. Also, muscat wines are seldom found to have bouquet of the type we are describing, even after long bottle aging. Thus, the rating for this wine, and also the comparable ratings for wines 10, 11, 17, and a few others ,strongly suggest the high value placed upon grape aroma by this panel.

Conversely, wines 4, 13, and 22 were rated low or did not change much in grape aroma rating, and these wines were rated better in over-all quality after heating. The increase in complexity and richness indicated by these data is certainly a positive quality factor produced by the heat treatment (14). The bottle bouquet increase is also of high potential value. Some individual panelists valued very highly the bottle bouquet produced by heating, whereas other noted it but did not consider it an important plus factor in their concept of table wine quality. The quality of the odor produced by heating the wines for 30 days in an inert atmosphere varied somewhat from wine to wine, and the over-all effect of heating on flavor usually included an increase in bitterness (Table 4).

These data suggest that heating table wines *in the complete absence of oxygen* for about 30 days at about 128°F has value in treating selected dry table wines. Candidate wines for the treatment should be chosen on the basis of actual trial with a sample, because not all wines respond equally favorably. Wines with a good intense grape-derived aroma, particularly certain varietals such as Muscats, will probably not benefit from such treatment. Standard wines relatively low in grape aroma appear most likely to be improved. It appears to us that long bottle aging at low temperatures may retain somewhat more grape aroma than is the case for a similar degree of bottle bouquet develop-

ment by heating, but it is generally agreed that grape aroma does diminish during bottle aging and that its loss must be compensated for by the gain in aged bouquet. Note (Table 4) that grape aroma is greatly decreased before bottle bouquet reaches a high level during the warming treatment. These observations and limited experimental trials suggest that it should be possible to develop bottle bouquet by heating in an inert atmosphere of a wine low in fruity aroma and then blend this wine with a fruity young wine to produce a wine better (higher in grape aroma yet high in bottle bouquet) than if the fruity wine had itself received long bottle aging. More study along this line is needed, but preliminary testing indicates that although the bottle bouquet aroma is very volatile or easily lost it can be retained after blending and rebottling under nitrogen.

Bottle "bouquet" is a term often used but poorly defined. As distinct from aromas derived from the grape, bouquet is reserved for the odors resulting from aging or processing. Amerine and Joslyn wrote: "Bouquet is the aged odor characteristic of fine wines that have been aged in the bottle for one or more years" (2). However, it appears that there is more than one bouquet or bottle bouquet, because the wine passes through more than one stage during long aging in bottles. Most white table wines appear to pass through a stage of relative flavorlessness shortly after bottling (bottle sickness), but then the wine evidently completes reaction

TABLE 5

Effect of Length of Storage of White Dry Table Wines in Bottles at 53°F on the Bottle Bouquet Rating

Years of storage	No. of wines	Min. & max. rating (1-10 scale)	Average rating (1-10 scale)
2	25	0-4	1.36
3	31	0-7	2.71
4	51	1-8	4.10
5	35	1-9	3.94
6	15	1-8	4.13

with the oxygen received during bottling, falls to a "rest" redox potential, and recovers its fruity aroma. If stored at normally low temperatures, change from here on is slow and undefined until the appearance of an odor begins, which we find very similar to that of our wines heat-treated in the complete absence of oxygen. With greatly prolonged bottle aging even this odor is lost, and either maderization or a "mushroomy" odor generally appears.

The odor, which is the bottle bouquet we are describing, is observable in wines available in retail shops, but as a rule only in costly wines which have been more than three years in bottle. It is probably significant that most of these are foreign wines, and as such may have been subjected to longer and warmer bottle storage during shipment than is common for American wines. Some of our experienced panel members described this odor as "old fixed SO_2," or "old burned match," whereas others simply called it bottle bouquet. All agreed that it was related to age—therefore by definition a bottle bouquet—and was similar in wines warmed in inert gas and wines "naturally" aged in bottles for several years. The experiment with added SO_2 did not produce added bottle bouquet during heating under nitrogen, and it has been observed that wines with high levels of SO_2 do not develop this bottle bouquet during bottle aging as long as SO_2 is evident, so that bouquet appears later in wines which have received excessive SO_2. It is believed that the nature of the odor, which is pleasantly pungent and empyreal, has caused some people, without justification, to relate it to sulfur dioxide.

One of us (VLS) rated on an arbitrary scale of 1-10 the intensity of this bottle bouquet as found in a series of wines bottled in air and binned at 53°F for up to 6 years (Table 5). Only white dry table wines were rated, and all labrusca or muscat types were excluded. Although it was again noted that wines with noticeable sulfur dioxide content did not show appreciable bottle bouquet, such wines were included. Note that it appears to take 3 years or more in bottle for many wines

to achieve much bottle bouquet. Some wines did not achieve appreciable bottle bouquet even in 6 years, but none of these wines were overaged, for they still had acceptable color and lacked maderization.

If one assumes the normal temperature coefficient for the reaction producing the bottle bouquet, the rate would double for each 10°C of temperature increase. About 2 to 3 months at 128°F should be equivalent to 3 to 4 years at 53°F. The fact that we got rather similar results with 1 month of warming at 128°F compared to 3 to 4 years at 53°F suggests a rather good correspondence between cellar aging and heating without oxygen, considering the approximations involved and the fact that the cellar samples were bottled with air in the headspace, some were in large bottles, and some had been rebottled during storage, which probably slowed the reaction.

This proposed warming treatment is more drastic than has been previously recommended for white table wine. Although the quality of the bottle bouquet odor developed has seemed to us satisfactorily similar to "natural" bottle bouquet of the type described, better quality might be produced by longer treatment at lower temperatures. Since the assumption of a normal temperature coefficient seems valid, 128°F (53.4°C) for 30 days, 110°F (43°C) for 60 days, and 92°F (33.3°C) for 4 months should be about equivalent. Further study will be needed to determine the best time-temperature relationship. Ribéreau-Gayon and Peynaud (11) stated that bottled white table wines, especially if well sulfited, stored (presumably with air in the headspace) at temperatures up to 28°C (82.4°F) develop bottle bouquet more rapidly but that higher temperatures give **brûle** (burnt) flavors. They indicate 25°C as the maximum for red table wines if this taste is to be avoided. At these maxima they found bottle bouquet formation in "some months" rather than years. Gerasimov (12) recommended heating table wines in the absence of oxygen and not above 30°C for periods up to 1 month. For white wines bottled under CO_2, Lipis et al. (9)

recommended pasteurization for 1-2 minutes at 68°C followed by cooling and storage for 5 days at 40°C.

The new, more drastic treatment suggested by our data exceeds the time-temperature combinations tested by those previous workers. Presumably such conditions may have escaped trial by earlier workers because air in the headspace of their bottles produced more oxidative damage when the reactions were accelerated by heat. Or, as our data show, a loss of grape aroma and a period of relative tastelessness which precede the development of bottle bouquet may have caused them to stop too soon.

SUMMARY AND CONCLUSIONS

Heat treatment of a submerged-cultured flor sherry, with or without oxygen, decreased the flor character. Some apparent gain occurred in other aspects of sherry character, but indications are that the desired complexity can be produced more easily by other methods, such as blending, than by heating under the conditions tested.

Table and dessert wines heated in the presence of oxygen-filled headspace (40 cc per about 750 ml of wine) changed in ways that were predominantly undesirable. Nitrogen, hydrogen, or carbon dioxide for displacing the dissolved and headspace oxygen seemed equivalent in effect. In contrast to oxygen, these gases appeared in these tests to behave as if inert and to have effect upon flavor changes during heating of the wine because oxygen had been displaced.

Warming dessert wines at 128°F for up to 20 days in the presence of these inert gases caused definite chemical and sensory quality changes, most of them favorable. These changes appeared small, however, compared to the favorable effects produced in two table wines, particularly the white wine. Further studies have shown that not every wine responds equally favorably, though many are capable of improvement.

In selected table wines, heating in the complete absence of oxygen for 15-30 days at about 128°F (53.4°C) produces definite improvement in bottle bouquet and increased complexity. A negative factor can be a loss in grape aroma, if any is present.

The nature of the bottle bouquet produced is discussed, along with its similarity to bouquet in bottled white table wines aged 3 to 4 years at wine-cellar temperatures. The conditions of the reaction of bottle bouquet formation appear to preclude enzymatic participation in the reaction because the temperature coefficient appears normal yet the products become noticeable only after a relatively long time at temperatures causing relatively rapid protein denaturation.

This new treatment appears worthy of further trial. It appears to be capable of producing improved table wines with aged-type flavor from selected, pretested standard wines otherwise lacking notably high quality or desirable aroma. Blending such aged-type wine with fruity aromatic young wines might enable production of better white and light red table wines than if the fruity wine were itself subjected to long bottle aging.

ACKNOWLEDGMENTS

LITERATURE CITED

1. Amerine, M. A. Laboratory procedures for enologists. 130 p. University of California, Davis. (1960).

2. Amerine, M. A. and M. A. Joslyn. Table wines, the technology of their production in California. University of California Press, Berkeley. 397 pp. (1951).

3. Costa, E. N. Investigation into measuring redox potential in wines. Am. J. Enol. Viticult. 10:56 (1959).

4. Deibner, L. and P. Bénard. Effets of traitements thermiques à l'abri de l'air sur les qualités des vins doux naturels. Ann. Technol. Agr. 6(4):421-7 (1957).

5. Deibner, L. and P. Bénard. Utilisation des autolysate de levures et des lies de vin au course du traitement thermique prolongé des vins doux naturels à l'abri de l'air. Ind. Aliment. Agr. (Paris) 80(5):511-18 (1963).

6. Gerasimov, M. A., Z. N. Kishkovskiĭ, A. P. Smirnova, and O. T. Babkina. Novye metody termicheskoĭ obrabotki ordinarnykh kreplenykh vin. Vinodelie i Vinograd. SSSR 21(5):5-12 (1961).

7. Guymon, J. F. Effect of brandy on the quality and changes in composition of California-type sherry during baking. Am. J. Enology 6(2):36-41 (1953).

8. Libraty, V. Ester determinations and their application to wines. M. S. thesis. University of California, Davis. 65 pp. 1961.

9. Lipis, B. V., V. N. Nikandrova, and L. A. Kalgushkina. Sadovstva, Vinograd, i Vinodel. (Moldavia) 1959(3):28-30; Chem. Abst. 57: 14292d (1962).

10. Ough, C. S. and M. A. Amerine. Flor sherry production by submerged culture. Food Technology 14(3):155-9 (1959).

11. Ribéreau-Gayon, J. and E. Peynaud. Traité d'oenologie, Vol. 2, Composition, transformations et traitements des vins. Librairie Polytechnique ch. Béranger, Paris. 1065 pp. 1961.

12. Singleton, V. L. Aging of wines and other spritous products; acceleration by physical treatments. Hilgardia 32:319-92 (1962).

13. Singleton, V. L. Changes in quality and composition produced in wine by cobalt-60 gamma irradiation. Food Technology 17(6):112-15 (1963).

14. Singleton, V. L. and C. S. Ough. Complexity of flavor and blending of wines. J. Food Sci. 27:189-96 (1962).

15. Singleton, V. L. and Diana E. Draper. Ultrasonic treatment with gas purging as a quick aging treatment for wine. Am. J. Enol. Viticult. 14(1):23-35 (1963).

Changes in Quality and Composition Produced in Wine by Cobalt-60 Gamma Irradiation

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SUMMARY

Samples of ruby port, white port, dry red table wine, and dry white table wine purged with nitrogen and air-saturated dry sherry material were irradiated with 100,000, 500,000, and 1,000,000 rads of Co^{60} gamma radiation. Analyses and sensory evaluation showed considerable changes from irradiation. Visible color and spectral absorption were diminished in intensity, redox potential was decreased, and volatile aldehydes increased, but other analyses were not appreciably changed. Odor and flavor were drastically modified by higher levels of irradiation, though 100,000 rads was not detrimental and had some desirable attributes.

INTRODUCTION

The results of treating wines with ionizing radiation have been summarized and discussed elsewhere (Singleton, 1962). In all cases, quality was modified by irradiation, and some researchers have claimed rapid aging and quality improvement. It is difficult to believe that irradiation would accelerate all, and only the same, reactions that would occur during traditional methods of aging of wines and therefore result in an identical product in a shorter time. However, recent work (Singleton and Ough, 1962) has emphasized the value to wine quality of increased complexity of flavor, and a limited addition of new flavors by irradiation might improve the quality of a wine to a degree similar to that caused by aging it.

The irradiation of foods usually results in new flavors and other noticeable modifications. In products such as meat and fresh fruit these changes have been judged as off-quality and detrimental, perhaps partly because of a firmly fixed idea in the consumer's mind of the limited differences in flavor to be tolerated. With wines, in contrast, a degree of variation and distinctiveness even within a single type of product is appreciated and sought by the more sophisticated consumer. Under these conditions a distinctive flavor that is not definitely unpleasant, produced by irradiation or otherwise, would seem potentially desirable to most people, either as a distinctive note in a traditional type of wine or as a new wine product.

The installation of a Co^{60} irradiation facility at the University of California at Davis made it possible to treat wines to test these ideas. The objective was to estimate the potential value of such irradiation by treating representatives of several of the major types of wine produced in California with relatively high doses of radiation so as to produce extreme effects. These effects were then evaluated by chemical and sensory analysis. Previous experiments with wines (Singleton, 1962) suggest that at least 11,000 rads are required for noticeable changes, and that appreciable changes are commonly produced in foods with 1,000,000 rads. Irradiation of 100,000, 500,000, and 1,000,000 rads were arbitrarily chosen for this study.

MATERIALS AND METHODS

Wines. The wines were produced in the University experimental winery from grapes from the University Vineyard. Lot 9044 was a white port from 50% Aligote 50% Pinot blanc grapes, vintage 1958. The four others, vintage 1961, were: 9984, ruby port, Carignane variety; 9931, dry red table wine, Cabernet Franc variety; 9779, dry white table wine, Thompson Seedless variety; and 9779, fortified to 20% alcohol, dry sherry material. Herein these five wines are respectively designated as white sweet, red sweet, red dry, white dry, and shermat.

Treatments. The wines were placed in 1/5-gal. colorless glass, metal-screw-capped wine bottles. The samples were treated with finely dispersed nitrogen bubbles to purge other dissolved gases (approx. 5 min with 25 ml N_2/min) and tightly capped with 40 cc N_2-filled headspace. An exception was the shermat, which was similarly treated except that air was used instead of nitrogen. Four bottles of each wine were prepared, one as a control and three to be irradiated.

The irradiation was conducted in the 32,500-curie Co^{60} irradiator described in detail by Romani et al. (1962). The 15 bottles of wine to be treated were placed in the central 6×14×20-in. treatment box, which was then lowered into the well between the two 19×23½-in. plaques containing the Co^{60}. The cooling system was operated so that the samples stayed in the range of 52–64°F throughout the treatment. At the end of the time calculated from previous dosimetry to yield 100,000 rads, the box was raised, one bottle of each wine removed, and the box again lowered for the time to reach a total of 500,000 rads. The second bottle of each wine was removed at this dose, and the third was removed after 1,000,000 rads total irradiation.

The samples were shown to be free of radioactive contamination by direct monitoring. They were returned to the wine cellar (53°F) with the control samples after a total elapsed time of about 4 hr, and held there until analyzed. After about 2 weeks the samples were each opened and transferred under nitrogen to two 1/10-gal. bottles, one for taste-testing and the other for chemical analysis.

Analyses. The oxidation-reduction potentials were determined essentially as described by Costa (1959), the volatile esters by a colorimetric ferric-hydroxamate procedure (Libraty, 1961), tannins by the Folin-Denis-Pro procedure (Amerine, 1960), volatile aldehydes as described by Jaulmes and and Dieuzede (1954), and the other analyses by standard methods for wine (Amerine, 1960). Absorption spectra were determined with a Spectronic 505 recording spectrophotometer using 1-cm cells. The white wines, compared to a water blank, were undiluted for visible-region spectra, but diluted 1/25 with water for the ultraviolet. The red wines similarly required 1/5 dilutions for visible and 1/100 for ultraviolet spectra.

Sensory tests. The samples were coded with random two-digit numbers and presented to 10–14 members of a panel highly familiar with wines and with sensory testing. The judges were seated in individual booths with bright illumination. Samples were about 25 ml in 6-oz clear, plain wine glasses. The judges were advised that some of the samples to be evaluated had received large doses of gamma radiation, but were not themselves radioactive. They were asked to fill in completely a three-part score sheet. Part one asked for ratings on an arbitrary scale

Table 1. Physical and chemical analyses of irradiated wines.

	pH	Redox potential (mV) (observed potential vs. satd. calomel electrode at 24°C)	Tannin (mg gallic acid/L)	Titratable acid (g tartaric acid/100 ml)	Extract (g dissolved solids/100 g)	Ethanol (% V/V)	Esters (mg EtOAc/L)	Aldehydes (mg AcH/L)	Acid (g AcOH/100 ml)	Absorbance times diln. at 520 mμ	Absorbance times diln. at 440 mμ	Absorbance times diln. at 320 mμ	Wavelength of max. absorbance (mμ)	Absorbance times diln. at max.
Red dry														
Control	3.57	+ 33	684	0.533	1.7	10.2	110	11.2	.013	2.80	1.94	16.6	275	27.4
100 krad	3.48	+ 29	692	0.561	2.0	10.6	114	8.9	.018	2.39	1.74	16.0	275	27.6
500 krad	3.47	−138	666	0.537	2.0	10.2	111	19.3	.013	1.58	1.28	8.7	275	23.7
1000 krad	3.68	−460	656	0.528	2.1	10.3	111	64.8	.012	1.12	1.06	6.1	275	22.7
Red sweet														
Control	3.76	+173	555	0.403	11.7	18.9	24	56.2	.004	2.50	2.14	14.9	270	25.3
100 krad	3.77	− 90	545	0.406	12.0	18.9	24	47.7	.003	2.00	1.75	14.6	270	25.0
500 krad	3.79	−182	535	0.399	11.6	18.7	25	82.9	.003	1.19	1.24	10.0	270	21.9
1000 krad	3.83	−464	565	0.392	12.1	18.1	28	103.9	.004	.60	.74	7.4	270	20.0
White sweet														
Control	3.83	+ 93	233	0.359	12.7	19.8	74	118.3	.007	.087	.216	11.4	285	12.6
100 krad	3.89	− 30	227	0.360	12.6	19.3	72	113.3	.008	.062	.169	9.3	281	11.6
500 krad	3.91	−438	219	0.356	12.5	19.3	73	147.6	.006	.050	.141	3.9	273	9.4
1000 krad	3.92	−246	231	0.355	12.7	19.7	73	138.2	.006	.046	.135	2.5	273	8.9
Shermat														
Control	3.59	+160	154	0.418	1.8	20.4	143	30.2	.013	.055	.110	3.5	265	8.9
100 krad	3.52	+ 35	147	0.428	1.8	20.1	128	42.5	.008	.052	.105	2.9	265	8.5
500 krad	3.57	−225	147	0.435	1.8	19.5	132	69.8	.008	.043	.087	1.6	265	7.4
1000 krad	3.54	−312	180	0.409	1.8	20.5	136	68.9	.010	.043	.084	1.3	265	7.0
White dry														
Control	3.60	− 98	162	0.497	2.1	11.0	127	65.4	.016	.051	.112	6.7	265	10.0
100 krad	3.54	−123	168	0.496	2.2	10.9	123	51.4	.017	.048	.105	6.1	265	9.6
500 krad	3.52	−298	168	0.493	2.2	10.9	127	77.8	.017	.048	.098	2.4	265	8.6
1000 krad	3.60	−507	173	0.493	2.4	10.8	124	89.3	.016	.048	.105	1.9	265	8.2

of 0–10 of: a) color changes related to aging, b) grape aroma, c) bottle bouquet, d) oxidized flavor, e) hotness, f) astringency, g) bitterness, and h) complexity or richness. These terms were chosen for evaluation after consultation with several expert wine judges as being readily understood and reasonably clear in the minds of most persons well acquainted with wine. The second part of the score sheet asked for an over-all quality score on a scale used for some time in this laboratory (Ough and Baker, 1961), in which 20–17 indicates wines with outstanding characteristics and no marked defects, 16–13 standard wines without either outstanding features or defects, 12–9 commercially acceptable but with noticeable defect, 8–5 below commercial acceptability, and 4–1 completely spoiled. The third portion of the score sheet asked for description and comments upon any unusual flavors noted.

The judges received all four samples (control, 3 treatments) of a single wine together, but in random order. Cross comparison and time were not limited in any way, and, if requested (rare), more of any sample was supplied. The judge was informed that the wines were to be judged as the appropriate type, i.e., dry sherry, dry red table wine, ruby port, white port, or dry white table wine.

RESULTS AND DISCUSSION

Radiation produced obvious changes at the higher levels: the glass of the bottles changed from colorless to greenish-brown, the wines were under a small positive pressure when opened, the color of both white and red wine was bleached, and odor and flavor were changed.

Analytical results are summarized in Table 1. Changes were not evident in extract, ethanol, volatile esters, or volatile acid. Although there is a suggestion of an increase in pH and a decrease in total acidity upon irradiation, the effect is uncertain from these data, and too small to be directly important to quality. The changes in spectral absorption are great; absorbance diminished at every wavelength, but not proportionately. All the untreated wines showed a maximum at 265–285 mμ and a shoulder at about 320 mμ, and the red wines showed the typical maximum near 520 mμ. The red wines were much decreased in absorbance at 520 mμ by irradiation. Since absorbance at lower wavelengths in the visible region was decreased nearly proportionately, the hue shifted only slightly toward orange, and the primary visible effect was nearly equivalent to dilution. This was also true with the white wines. The absorbance at about 320 mμ, however, was greatly decreased, and in several cases the shoulder disappeared, leaving only the residual absorption from the more intense but less affected peak at 265–285 mμ. The latter maximum shifted in some samples as a result of irradiation. Absorption at these wavelengths by wines is attributed largely to their content of flavonoid, polyphenolic substances. No appreciable change in the "tannin" (total polyphenolic) content accompanied the decreased spectral absorption. Paper chromatographic studies of these samples showed definite qualitative and quantitative changes in the polyphenolic substances.

The oxidation-reduction potential was greatly lowered by irradiation. This fact and the disproportionate decrease of the 320-mμ absorbance suggest that quinoid forms of the polyphenolics were decreased and that relatively reducing conditions were produced by irradiation. Volatile substances reacting as aldehydes were also produced in considerable amounts. These analytical results confirm that definite, selective, specific, and interesting compositional changes are produced in wine by gamma irradiation at feasible dose levels.

Sensory evaluations are summarized in Table 2. These results were analyzed for statistical significance by the Duncan multiple-range test (Alder and Roessler, 1961). All mean ratings underlined by the same line do not differ significantly at the 95% confidence level. No significant differences in bottle bouquet, hotness, bitterness, or astringency resulting from irradiation were found in individual wines or in the combined scores for all wines. The fortified wines were rated hotter than unfortified, and the red wines more astringent than the white, as would be expected. Since the wines used were

Table 2. Sensory analyses of irradiated wines: mean score by treatment (arranged in decreasing order of means, scores not underlined by the same line differ significantly at the 95% confidence level by the Duncan multiple-range test. C = control; x = 100,000 rads; y = 500,000 rads; z = 1,000,000 rads).

Over-all Quality Score (Max. = 20)

Red dry	C 13.5	x 12.9	y 10.9	z 9.2
Red sweet	x 12.8	C 12.7	y 10.0	z 7.9
White sweet	x 14.7	C 13.8	y 13.0	z 12.3
Shermat	x 12.2	C 12.1	y 10.3	z 8.2
White dry	C 13.3	x 12.8	y 10.8	z 8.8
Combined data	C 13.08	x 13.07	y 11.00	z 9.28

Complexity, richness (max. = 10)

Red dry	x 3.4	C 3.1	y 2.7	z 2.0
Red sweet	x 4.2	C 4.0	y 2.7	z 1.8
White sweet	x 5.3	C 4.9	y 3.8	z 3.6
Shermat	C 3.8	y 3.4	x 3.2	z 2.4
White dry	y 3.7	x 3.4	C 3.3	z 2.9
Combined data	x 4.00	C 3.87	y 3.27	z 2.57

Color changes related to aging (max. = 10)

Red dry	z 6.4	y 4.8	x 2.8	C 1.9
Red sweet	z 5.8	y 4.9	x 4.1	C 3.9
White sweet	C 5.8	x 4.8	y 2.8	z 2.4
Shermat	C 3.6	x 2.5	z 2.1	y 1.8
White dry	y 2.5	C 2.2	z 2.2	x 1.8
Combined red data	z 6.08	y 4.80	x 3.48	C 2.96
Combined white data	C 3.80	x 3.00	y 2.34	z 2.23

Oxidized flavor (max. = 10)

Red dry	z 4.1	y 3.1	x 1.8	C 0.7
Red sweet	z 3.7	y 3.0	x 2.6	C 2.2
White sweet	y 3.4	z 3.3	C 2.8	x 2.4
Shermat	z 3.8	y 3.2	x 2.5	C 2.1
White dry	z 3.8	y 2.8	x 1.8	C 1.6
Combined data	z 3.77	y 3.12	x 2.27	C 1.92

Bottle bouquet (max. = 10)

Red dry	C 3.0	x 2.5	z 2.3	y 2.2
Red sweet	C 2.6	x 2.5	y 2.5	z 2.5
White sweet	C 3.2	x 3.2	z 2.8	y 2.6
Shermat	x 2.5	C 2.1	z 2.1	y 1.5
White dry	C 2.8	y 2.5	x 2.2	z 1.7
Combined data	C 2.72	x 2.58	z 2.28	y 2.25

Hotness (max. = 10)

Red dry	x 1.1	y 1.0	z 1.0	C 0.8
Red sweet	C 3.8	x 3.5	z 3.5	y 3.1
White sweet	x 3.8	y 3.8	z 3.6	C 3.4
Shermat	z 3.9	x 3.8	y 3.2	C 3.1
White dry	y 1.1	z 1.1	C 1.0	x 1.0
Combined data	x 2.72	z 2.72	y 2.54	C 2.51

Bitterness (max. = 10)

Red dry	C 2.3	x 2.3	z 2.3	y 2.0
Red sweet	C 3.0	z 2.8	y 2.6	x 2.6
White sweet	z 2.2	C 1.9	x 1.9	y 1.8
Shermat	z 3.4	y 2.7	C 2.6	x 2.4
White dry	y 1.7	C 1.6	x 1.6	z 1.6
Combined data	z 2.47	C 2.32	x 2.19	y 2.19

Astringency (max. = 10)

Red dry	C 4.1	z 3.5	y 3.3	x 3.2
Red sweet	C 3.5	z 3.2	x 3.1	y 2.8
White sweet	x 2.3	y 2.2	C 2.0	z 1.9
Shermat	z 2.9	y 2.2	C 2.1	x 2.1
White dry	y 1.4	z 1.2	x 1.1	C 1.0
Combined data	C 2.55	z 2.53	y 2.42	x 2.37

Grape aroma (max. = 10)

Red dry	C 5.7	x 4.8	y 2.9	z 1.7
Red sweet	C 4.2	x 3.3	y 2.3	z 1.2
White sweet	x 5.2	C 4.7	y 3.3	z 3.2
Shermat	C 3.8	x 2.5	z 1.5	y 1.4
White dry	C 4.4	x 4.3	y 2.6	z 1.5
Combined data	C 4.54	x 4.02	y 2.51	z 1.82

not rated particularly high in any of these qualities, it may not be safe to conclude that irradiation would not reduce them significantly if they were more intense. It does appear valid to conclude, however, that irradiation of wine does not produce appreciable hotness, astringency, bitterness, or bottle bouquet.

Statistically significant differences did appear in all the other factors rated. Red color decreases as a red wine ages, whereas brown color increases as a white wine is aged. It is therefore consistent with the spectral data that the panelists rated the color of the red wines as more aged (less red) and that of the white wines less aged (less brown) after irradiation. Grape aroma rating was significantly decreased in all cases by the higher levels of irradiation. The dry red wine and the combined data for all wines showed a significant increase in rating for oxidized flavor. This is of interest because, although the redox potential decreased, indicating relatively reducing rather than oxidizing conditions, the volatile aldehydes increased. Increased aldehyde content is associated with sherry formation and oxidized wine tastes. Russian workers (Singleton, 1962) have reported favorable results in maderizing wine by irradiation, and these data appear to confirm their conclusion. It should be noted, however, that the sherry material was not rated as improved more, or by higher irradiation levels, than the other wine types.

The differences in complexity and richness ratings were large enough to show a significant decrease in the red sweet wine and in the data combined for all wine, as a result of the two highest levels of irradiation. The over-all quality rating indicated decreased quality by the two highest levels of irradiation.

Remembering that the wines were rated by experienced judges with reference to standard wine types, these data show that large changes were produced by 500,000 and 1,000,000 rads. The changes were great enough to show statistical significance in several ratings in spite of the great variation inherent in such sensory data and with only 10–14 replications (judges). The changes were great enough and unusual enough to cause the wines to be rated near the limit for commercial acceptability for their standard type in some cases. That is, the quality factors were varied to a degree sufficient to place the wine outside the limits of normal variability of good representatives of the type in commerce.

To estimate satisfactorily whether highly radiation-modified wines have a potential as new wine products is

not possible from the available data. Further testing does seem warranted. The radiation-induced flavor seems similar, regardless of the type of wine irradiated. Descriptive terms suggested by the panel for this flavor included cheesy, fishy, perfumy, leathery, sulfurish, fatty, metallic, aged-European-wine-like, whey-like, burned, mushroomy, and fruity. Comments were favorable on the flavor and odor induced by high levels of radiation by 5 of the 14 panelists. From the panel's comments and the fact that grape aroma, complexity, etc., decreased in rating with 1,000,000 compared to 500,000 rads, it is believed that even for possible new products doses should be less than 1,000,000, and probably less than 500,000, rads for most base wines.

The data in Table 2 show no significant differences (19 to 1 confidence level) between the untreated control and the sample receiving 100,000 rads. There appear to be some suggestive trends, however, which will require more replication to verify at this stringent confidence level. In 4 of the 5 cases the low-level-irradiated wine was rated more complex and richer than the control. In 3 of the 5 cases this sample was rated higher in over-all quality than the control. The 2 not so rated were the 2 dry wines, and it may be that their more delicate flavor was already over-treated at 100,000 rads. The possibility of favorably influencing the quality of standard types of wine by irradiation near or below 100,000 rads seems definitely worthy of further study.

REFERENCES

Alder, H. L., and E. B. Roessler. 1961. "Statistical Procedures." 3rd ed. 482 p. University of California, Davis.

Amerine, M. A. 1960. "Laboratory Procedures for Enologists." 130 p. University of California, Davis.

Costa, E. N. 1959. Investigation into measuring redox potentials in wines. *Am. J. Enol. Viticult.* **10**, 56.

Jaulmes, P., and J. Dieuzede. 1954. Le dosage de l'acetaldéhyde dans les spiritueux. *Ann. fals. et fraudes* **47**, 9.

Libraty, V. 1961. Ester determinations and their application to wines. M.S. thesis. University of California, Davis, 76 p.

Ough, C. S., and G. A. Baker. 1961. Small panel sensory evaluations of wines by scoring. *Hilgardia* **30**, 587.

Romani, R. J., E. C. Maxie, C. O. Hesse, and N. F. Sommer. 1962. Cobalt-60 gamma-ray irradiator. *Calif. Agr.* **16**(3), 2.

Singleton, V. L. 1962. Aging of wines and other spiritous products, acceleration by physical treatments. *Hilgardia* **32**(7), 319.

Singleton, V. L., and C. S. Ough. 1962. Complexity of flavor and blending of wines. *J. Food Sci.* **27**, 189.

THE TOTAL PHENOLIC CONTENT OF GRAPE BERRIES DURING THE MATURATION OF SEVERAL VARIETIES

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The development of various constituents of fruits during maturation and ripening has deservedly received considerable study from the viewpoints of both fundamental biochemistry and practical applications. Many studies have concentrated upon the sugars and acids, and relatively few on the phenolic, flavonoid, or "tannic" substances. Joslyn and Goldstein (13) recently reviewed the phenolics of fruit, particularly in relation to astringency. They noted the generally recognized importance of phenolic substances to grape and wine quality, but the more complete studies they reported on were made with apples, cherries, strawberries, peaches, pears, bananas, and persimmons.

Data on the phenolics of grapes and wine have been summarized in several publications (1, 2, 10, 11, 12, 15, 16, 18, 22). Much of this work, particularly in recent years, has centered on identification of individual components or groups of substances such as anthocyanins, and relatively little study has been devoted to total phenolic content, its development, distribution, or variability. The metabolic synthesis of flavonoids, phenolic cinnamic acid derivatives, and most of the other important phenolics of plants is interrelated, and knowledge of the total present in grapes is considered basic to determining the true importance of individual substances in grapes or grape-derived foods. Data from previous studies are incomplete or conflicting in several respects. For example, the total "tannin" has been reported to decrease in concentration during growth and maturation of the grape, but it is not clear whether or not there is a net synthesis, stasis, or decrease

in individual berries.

A program of research has been initiated to clarify such phenolic relationships. A study has been published (21) showing that the seeds can contribute major amounts of total phenolics to juice or wine left in contact with them. The present report continues the series with studies on several varieties with regard to total phenols during development, and later reports are planned on tissue distribution, weather relationships, and individual phenol components.

MATERIALS AND METHODS

The grapes were obtained from the Davis experimental vineyard. The varieties tested in 1962 included Calzin (A×R rootstock planted in 1949 and Calzin budded in 1956), Catawaba, Grenache, Petite Sirah, and Sauvignon blanc (all on their own roots and planted in 1949, except the last-named, in 1950). In 1963, Aligoté, Delaware, Emerald Riesling, French Colombard, Muscat of Alexandria, and Pinot blanc were studied. None of these were grafted and all were planted in 1949 except French Colombard (1955) and the Muscat (1950). Marked groups of 6-10 vines were sampled for each variety between 8 and 10:30 A.M. at weekly intervals from mid-July until the berries were ripe. In 1962, each harvest consisted of one cluster from each vine on each date. In 1963, 20-25 individual, normal, representative berries were snipped at random from all parts of each vine, giving a total of at least 200 berries at each harvest for each variety (19). The collected berries were brought directly to the laboratory and processed as quickly as possible. A representative portion of the berries was separated from all stem parts with minimal damage to the berry skin, counted (usually 500 in 1962, 200 in 1963), and weighed. Each sample was separated as rapidly as possible

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by hand (2 people) into skins, seeds, pulp, and juice. In 1962, the pulp and seeds were left together since very little phenolic substance occurred in the pulp.

The juice was obtained by squeezing by hand as forcefully as possible through 2 layers of cheesecloth. Each separated fraction was weighed, and the solid parts were covered with 95% ethanol and brought quickly to a boil (100°C bath), boiled 2 minutes to inactivate phenolases and other enzymes, and rapidly cooled to room temperature. Comparison of paper chromatograms of alcoholic extracts prepared rapidly without heat treatment indicated no differences produced by this treatment, and continued enzymic browning was prevented in treated samples held for short periods.

A small portion of the juice was refrigerated under nitrogen until analyzed. The solid tissue samples were held at room temperature until processing could be completed (the same afternoon). The extracts prepared from the solid tissue were stored in tightly capped prescription bottles under nitrogen, in the dark, in a freezer (about —20°C) until analyzed. This was ordinarily within 3 days, but storage for at least one year under these conditions produced no evident change in analyses.

The extracts were prepared by grinding the tissue for 1 min in a high-speed blender (Servall Omnimixer) with at least 2 ml of 95% ethanol per gram of solid, filtering, regrinding with chloroform (to remove lipids—discarded), regrinding with a similar portion of 95% ethanol, and finally regrinding with 50% ethanol. The intent, of course, was to produce quantitative recovery of the extractable phenolics in the combined aqueous ethanol extracts. It was desired to keep the extract as concentrated as possible to facilitate paper chromatography, which is to be the subject of later reports. This extraction procedure, reported previously (21), has been shown to be capable of recovering at least 90%, and usually 95% or more, of the total phenolic substance extractable by further grinding or long standing in fresh solvent.

The Folin-Denis-Pro procedure (17) was used to determine total phenolic content of the juice and extracts. With grapes having anthocyanin in the skin, the proportion of berries showing red color was determined. The degree of browning of the juice was determined by filtering a small portion and determining absorbance in 3/4-inch round cuvettes in a Spectronic-20 colorimeter at 440 mμ. The pH (glass electrode) and dissolved solids (Brix by refractometer) were determined on the juice.

RESULTS AND DISCUSSION

One of the ultimate purposes of this research program is to elucidate the functions of the phenolic substances in the quality of and reactions occurring in wines and other grape-derived food products. In order to develop an optimum harvest procedure and to understand the effects of processing and storage, it is necessary to know the qualitative and quantitative analysis of the phenolic substances in the ripening grape. A number of reports have given data on the polyphenol content of the fruit of various grape varieties, but were not entirely satisfactory for our purposes, for various reasons such as different but usually unspecified climate or weather, few or unfamiliar varieties, or limited sampling.

It is clear, from work such as reports on juice by Caldwell (6) and on wines by Amerine and Winkler (3), that there is considerable difference in tannin or total phenol content among different varieties of grapes. However, it is also well known, from observation as well as from research data, that there is considerable variation in "tannin" content among apparently similar products made from the same variety in different areas or years. Quantitative data upon the total phenol content of the typical berry of several varieties grown under identical conditions is needed, as has been emphasized by Caldwell (6), in order to relate the juice or wine data to the fruit itself.

In order to estimate the importance of harvest timing upon the phenol content of grapes it is necessary to know how the content changes during ripening. The relatively few reports which relate to this question are not in complete agreement,

but it appears most common that the phenol concentration in grapes decreases considerably as the grapes ripen (5, 7, 8, 9, 10, 14, 15, 16, 23). However, it is biochemically important to know whether or not there is a net synthesis or accumulation of phenolic substance within the entire berry as it enlarges, and no clear answer to this question was found in the literature.

Since the stems are generally excluded from consumption as completely as possible even in the form of wine extracts, they were not included in our study. Seeds may be consumed, and do contribute important amounts of phenolic substances to many wines (21). Therefore, data were collected on phenol content as measured by the Folin-Denis-Pro procedure for the entire grape berry, including the seeds but no part of the stem. The data for Sémillon are shown graphically in figure 1

as an example of typical results. The complete data for all varieties are shown in table 1. The relatively regular increase shown in figure 1 and in table 1 in pH, Brix, and berry weight is as expected during grape development and ripening.

The concentration of total phenolics per gram of berry weight was quite variable by variety, from a low near 2 mg/g, for Sauvignon blanc, and over 10 mg/g, for the highest samples of Pinot blanc. The total phenol content of anthocyanin-producing varieties was not clearly different from that of the white varieties from these data. In all cases (Figure 1, Table 1), there was less total phenol per gram in the later harvests than in the earlier harvests. However, this downward trend did not proceed in a simple, regular manner, and within the harvests near commercial ripeness there are several examples (Figure 1, Table 1) of appreciable increase in phenols
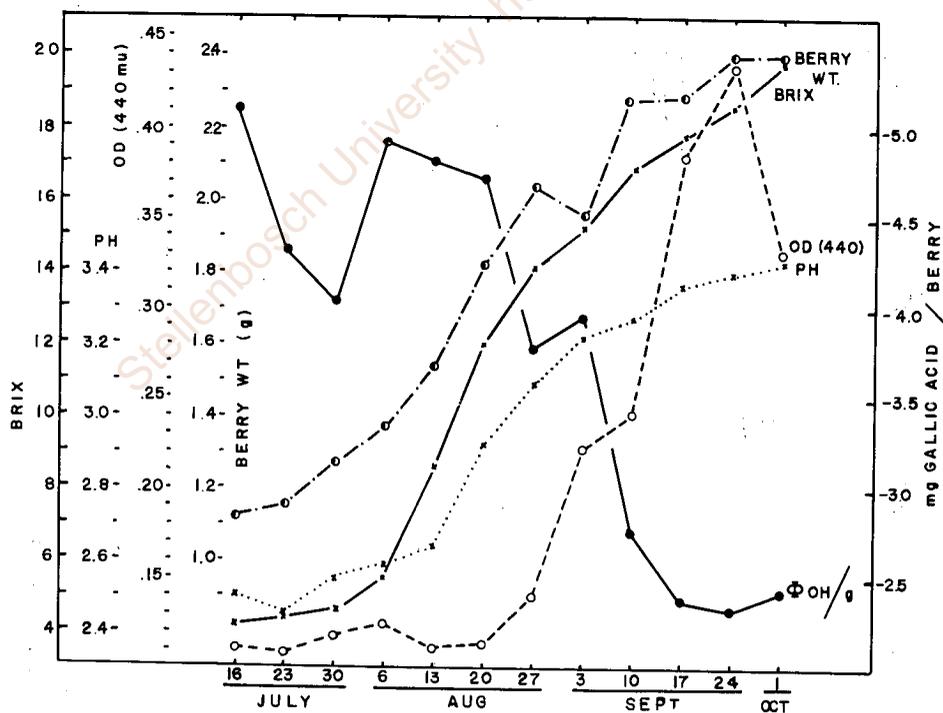
Figure 1. Typical data, change in composition and size of Sémillon berries harvested during the 1963 season.

TABLE I

Changes in Composition and Size of Grapes During Development and Ripening

Variety	Date	pH	Brix (Refrac- tometer)	Berry wt (g)	Total extractable phenols		Red berries (%)	Relative browning (A. 440 mμ)
					gallic acid (mg per g of berry)	gallic acid (mg per berry)		
Calzin (1962)	7/16	2.22	5.0	0.80	6.85	5.47	0.0	.59
	7/23	2.22	6.1	0.86	6.80	5.85	0.4	.48
	7/31	2.29	8.6	0.96	4.25	4.08	10.3	.53
	8/10	2.62	12.2	1.34	5.68	7.61	69.2	.65
	8/21	2.86	16.2	1.49	5.33	7.95	99.6	.82
	8/27	2.82	16.8	1.44	4.46	6.43	99.8	.53
	9/5	3.10	18.2	1.61	....	....	100.0	.52
	9/13	3.08	21.0	1.51	4.98	7.52	100.0	.66
	9/19	3.36	22.5	1.53	5.42	8.29	100.0	.51
Catawba (1962)	7/16	2.22	5.0	1.41	4.60	6.48	0.0	.59
	7/23	2.16	5.7	1.55	5.15	7.98	0.0	.32
	7/30	2.17	6.2	1.63	5.27	8.59	0.0	.53
	8/9	2.52	9.8	1.73	5.24	9.05	5.3	.87
	8/21	2.87	17.4	1.91	4.28	8.18	72.5	.64
	8/27	3.42	18.1	1.90	3.50	6.65	90.1	.60
	9/5	3.14	19.3	1.96	3.16	6.20	94.4	.48
	9/13	3.30	22.1	2.11	4.06	8.56	99.3	.57
Grenache (1962)	7/16	2.43	3.6	0.61	4.33	2.62	0.0	.83
	7/24	2.44	4.0	0.69	4.51	3.11	0.0	.37
	7/30	2.44	4.4	0.76	3.30	2.51	0.0	.64
	8/9	2.60	8.3	0.70	5.62	3.93	84.3	1.10
	8/21	2.86	15.1	0.91	3.46	3.13	77.6	.56
	8/27	2.95	17.2	1.07	3.07	3.29	83.6	.57
	9/5	2.94	16.3	1.21	2.39	2.89	83.7	.78
	9/13	3.08	20.9	1.19	2.85	3.39	94.1	.51
	9/19	3.38	22.9	1.22	2.92	3.56	98.5	.37
Petite Sirah (1962)	7/16	2.22	4.0	.73	5.30	3.88	0.0	.12
	7/23	2.16	5.3	.81	5.93	4.80	0.4	.15
	7/30	2.23	8.6	1.01	5.85	5.91	23.8	.29
	8/9	2.62	11.3	1.18	4.76	5.62	79.2	.38
	8/21	2.86	13.8	1.39	3.83	5.32	99.8	.67
	8/27	3.37	17.9	1.48	3.54	5.24	99.7	.37
	9/5	3.04	16.2	1.57	3.71	5.84	100.0	.62
	9/13	3.16	20.7	1.44	4.25	6.13	99.9	.38
	9/19	3.23	22.1	1.57	3.80	5.96	100.0	.22
Sauvignon blanc (1962)	7/16	2.26	6.1	1.06	4.45	4.73		.14
	7/23	2.37	10.5	1.31	2.82	3.69		.62
	7/30	2.55	13.7	1.62	2.52	4.08		.89
	8/10	2.87	17.2	1.95	2.29	4.46		.34
	8/21	3.17	19.6	2.15	2.07	4.44		.13
	8/27	3.23	21.8	2.21	2.09	4.61		.24
	8/30	3.33	22.6	2.23	2.25	5.02		.10
Delaware (1963)	7/16	2.36	5.3	.95	4.41	4.18		.30
	7/23	2.34	5.6	.98	3.90	3.81		.25
	7/30	2.46	6.8	1.02	4.32	4.39		.27
	8/6	2.54	10.5	1.09	4.48	4.90		.22
	8/13	2.69	14.2	1.24	4.92	6.08		.15
	8/20	2.92	18.2	1.24	4.87	6.05		.24
	8/27	3.04	20.6	1.28	4.36	5.57		.22

TABLE I (CONTINUED)

Changes in Composition and Size of Grapes During Development and Ripening

Variety	Date	pH	Brix (Refractometer)	Berry wt (g)	Total extractable phenols		Red berries (%)	Relative browning (A. 440 mμ)
					gallic acid (mg per g of berry)	gallic acid (mg per berry)		
Aligoté (1963)	7/16	2.37	3.8	.87	6.34	5.54		.18
	7/23	2.22	3.6	.92	6.18	5.65		.10
	7/30	2.33	4.2	.94	5.95	5.61		.15
	8/6	2.46	5.3	.97	8.14	7.86		.23
	8/13	2.47	8.9	1.08	6.89	7.47		.17
	8/20	2.88	13.3	1.22	6.78	8.26		.15
	8/27	2.77	15.1	1.29	6.54	8.43		.10
	9/3	3.04	17.6	1.35	5.22	7.04		.38
	9/10	3.03	18.2	1.48	4.67	6.92		.37
	9/17	2.94	19.6	1.49	3.66	5.44		.45
	9/24	3.13	20.7	1.58	3.24	5.12		.26
Emerald Riesling (1963)	7/15	2.51	3.7	.89	6.82	6.06		.21
	7/22	2.37	3.8	.99	5.14	5.06		.20
	7/29	2.53	3.9	1.05	5.25	5.50		.24
	8/5	2.56	3.9	1.10	5.78	6.36		.25
	8/12	2.50	4.2	1.12	8.21	9.17		.20
	8/19	2.53	4.5	1.16	7.76	8.99		.20
	8/26	2.78	6.5	1.20	7.87	9.47		.24
	9/2	2.72	10.4	1.27	4.92	6.24		.26
	9/9	2.57	14.6	1.47	5.48	8.06		.36
	9/16	2.72	16.4	1.64	5.04	8.27		.60
	9/23	2.92	17.7	1.71	4.10	6.99		.80
	9/30	3.14	20.6	1.70	5.47	9.27		.76
	10/7	3.17	21.8	1.73	5.32	9.20		.72
French Colombard (1963)	7/15	2.49	3.9	.88	5.55	4.86		.04
	7/22	2.43	4.0	.93	4.36	4.03		.06
	7/29	2.66	4.2	1.05	4.76	4.99		.06
	8/5	2.63	5.1	.99	4.54	4.51		.07
	8/12	2.79	7.8	1.08	5.16	5.55		.11
	8/19	2.86	10.2	1.36	4.32	5.86		.04
	8/26	3.16	12.2	1.43	3.98	5.70		.10
	9/2	2.99	13.8	1.58	3.14	4.98		.12
	9/9	3.03	15.7	1.55	2.33	3.61		.12
	9/16	2.91	16.5	1.67	2.49	4.16		.20
	9/23	3.00	16.9	1.73	2.25	3.89		.06
	9/30	3.15	18.0	1.87	2.74	5.13		.07
	10/7	3.21	18.7	1.79	2.53	4.53		.06
Muscat of Alexandria (1963)	7/15	2.48	4.5	1.73	4.90	8.46		.19
	7/22	2.34	4.6	2.08	3.39	7.06		.15
	7/29	2.24	4.7	1.96	3.54	6.94		.18
	8/5	2.46	5.3	2.30	4.49	10.34		.13
	8/12	2.47	6.4	2.27	4.68	10.60		.14
	8/19	2.58	8.0	2.38	5.16	12.31		.13
	8/26	2.91	11.0	3.01	4.43	13.31		.13
	9/2	2.83	12.2	2.96	4.03	11.92		.25
	9/9	2.73	14.3	3.40	3.08	10.46		.34
	9/16	3.15	15.8	4.02	2.61	10.48		.40
	9/23	3.22	16.6	3.74	2.28	8.52		.27
	9/30	3.32	18.0	4.33	2.60	11.28		.56
	10/7	3.40	18.8	4.12	2.84	11.69		.43

TABLE I (CONTINUED)

Changes in Composition and Size of Grapes During Development and Ripening

Variety	Date	pH	Brix (Refractometer)	Berry wt (g)	Total extractable phenols		Red berries (%)	Relative browning (A. 440 mμ)
					gallic acid (mg per g of berry)	gallic acid (mg per berry)		
Pinot blanc	7/16	2.48	4.7	.78	9.68	7.54		.15
(1963)	7/23	2.42	5.1	.81	7.22	5.83		.14
	7/30	2.52	5.8	.81	8.37	6.78		.15
	8/6	2.64	8.8	.90	10.85	9.78		.30
	8/13	2.77	12.5	1.07	10.11	10.78		.16
	8/20	3.01	15.2	1.12	9.63	10.80		.20
	8/27	3.10	17.6	1.24	8.34	10.38		.21
	9/3	3.04	18.4	1.26	6.81	8.58		.35
	9/10	3.18	20.1	1.30	6.06	8.09		.40
Sémillon	7/16	2.50	4.2	1.12	5.10	5.71		.11
(1963)	7/23	2.45	4.4	1.15	4.32	4.97		.11
	7/30	2.55	4.6	1.27	4.03	5.10		.12
	8/6	2.59	5.5	1.37	4.92	6.72		.12
	8/13	2.64	8.6	1.54	4.81	7.39		.11
	8/20	2.92	12.0	1.82	4.72	8.60		.11
	8/27	3.09	14.1	2.04	3.77	7.70		.14
	9/3	3.22	15.2	1.96	3.95	7.75		.22
	9/10	3.27	16.9	2.28	2.76	6.29		.24
	9/17	3.36	17.8	2.28	2.38	5.45		.38
	9/24	3.39	18.6	2.40	2.33	5.60		.43
	10/1	3.43	19.8	2.39	2.43	5.80		.33

with increasing ripeness. These data show that the total extractable phenols or "tannin" per ton of grapes does not always decrease in the latter stages of ripeness, and they suggest that changes in this group of components may deserve to be considered in deciding when to harvest grapes.

The period at which the berries begin to accumulate sugar rapidly (Brix increase) coincides reasonably well with the onset of anthocyanin production (Table I). This transition has been called "veraison" or the onset of ripening, and probably coincides with the transition from cell division to cell enlargement. It is a well known but often ignored fact (4, 20) that most fruits cease appreciable cell multiplication at a relatively specific and early stage, but continue to increase greatly in size by enlargement of the existing cells. The

data in figure 2 support this in that the "hesitation" in the increase in berry weight about 60 days before the fruit is ripe suggests the transition from cell division to enlargement and coincides with the beginning of rapid sugar accumulation. Figure 2 was prepared by interpolating from the actual data to obtain the date on which each variety reached 18.0° Brix. The analytical values obtained on that date (interpolated from bracketing data) were set as 100 for each variety, and relative values are calculated for all other dates to place all varieties on a comparative scale. The curves shown in figure 2 give the means of these values summarized for all 12 varieties and both years. The total extractable phenols *per typical grape berry* clearly increased considerably up until about 30 days before minimum ripeness, or a full month past the initial onset of

ripening. After this point the overall trend for all varieties appears to be a leveling off, or perhaps a slight decrease followed by a slight increase.

The data on total phenols per individual berry for the different varieties (Table 1) reflect the great differences in berry size between varieties. They show considerable fluctuation within each variety and not a simple regular trend. This fact is reflected in the scatter of the points shown in figure 2. The data clearly show, however, that there is a general and considerable increase in the grape berry of extractable phenolic compounds throughout much of the ripening period. Thus, the general decrease in total phenols per unit weight as the berry ripens is shown to be the result of a relatively greater increase in berry weight which dilutes and overshadows the nevertheless great accumulation and probable *in situ* synthesis of phenolic substances in the berry.

The fluctuation in total extractable
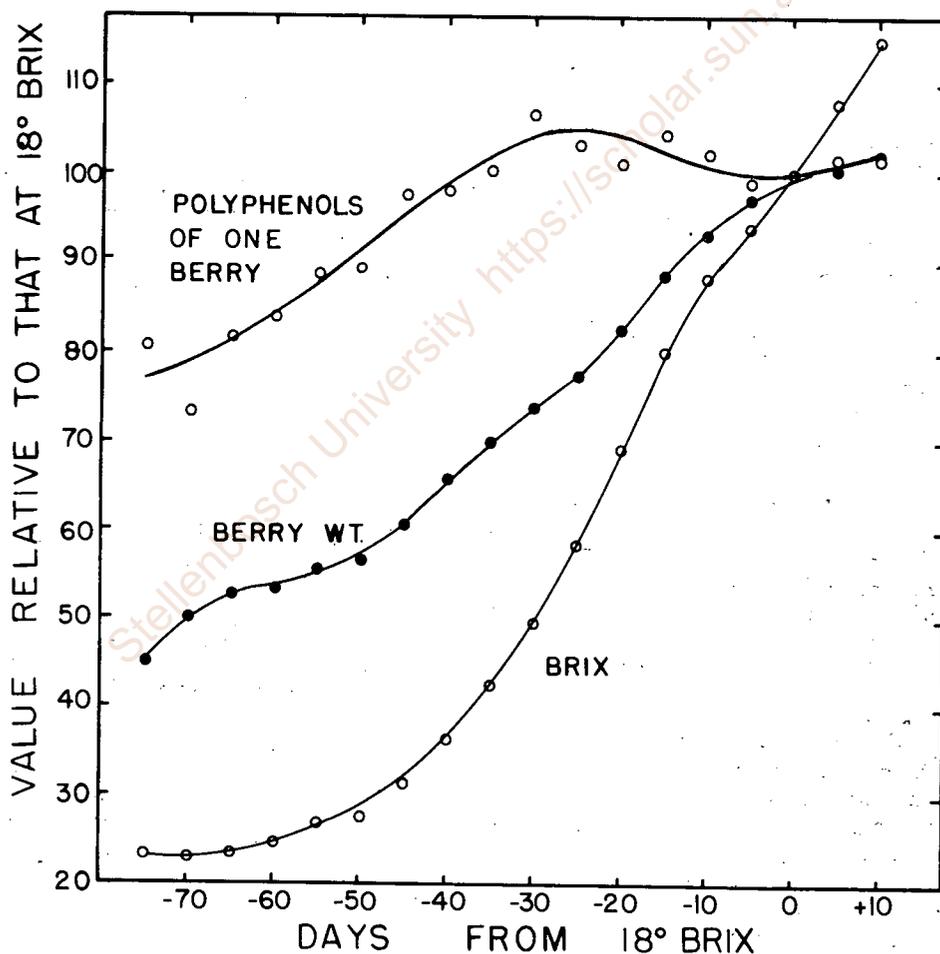
Figure 2. The relative changes during development and ripening with respect to the value at 18.0° Brix in total extractable phenols per berry, berry weight, and juice Brix, summarized for 12 grape varieties and two growing seasons.

phenol per berry by date is evidently real, and data suggesting important short-term weather relationships will be the subject of another report. These apparently rapidly alterations between high and low levels of total extractable phenols in a grape berry, as well as the general increase during much of the ripening period, suggest that the phenols as a group are much more active metabolically than previously believed. They certainly do not appear from these data to be the inert "storage" forms of "waste products" which was once assumed, particularly for the condensed tannins and related substances which are present to a considerable extent in grapes.

The data in table 1 (and figure 1) suggest that enzymic browning by oxidation of phenols in the juice is most rapid with the majority of the white grapes in the later stages of ripeness and at the time of onset of anthocyanin synthesis in the grape skin for red grapes. Such a conclusion from these data must remain tentative owing to some variability in the conditions pertaining to sample preparation (time to filter, etc.). It does appear however, that the known properties of the varieties, such as high susceptibility to browning of Emerald Riesling and low tendency to brown for French Colombard, are reflected correctly in the brownness of the juices.

SUMMARY AND CONCLUSIONS

A study was made during two seasons of the total extractable phenolic substances assayed by the Folin-Denis colorimetric procedure from representative entire berries of a total of 12 varieties of grapes (7 without anthocyanins, 5 with; 2 *Vitis labrusca*, 10 *Vitis vinifera*) from mid-July until they were commercially ripe.

A general trend downward in total phenols *per unit weight* of berry was found. However, the total phenol content *per berry* actually increased rather rapidly over a considerable portion of the development and ripening period. A considerably greater biochemical activity of the total phenolic substances carrying over into later periods of ripening than expected was observed not only as indicated by this net synthesis or accumulation in the berry,

but also by shorter-term increases and decreases of considerable magnitude. Additional criteria of practical use for harvesting grapes for improved quality, particularly regarding wine-tannin relationships as well as new understanding of the grape's physiology and biochemistry, are anticipated from further extension of these studies.

Great differences were found among the varieties tested in total extractable phenols per single berry or per unit weight of berry and in browning tendency of the juice. There appeared to be generally greater browning in the riper harvests for white grapes and during onset of anthocyanin synthesis in red grapes.

ACKNOWLEDGMENTS

LITERATURE CITED

1. Albach, R. F., R. E. Kepner, and A. D. Webb. Structures of acylated anthocyans pigments in **Vitis vinifera** variety Tinta Pinheira. Identification of the anthocyanidin, sugar, and acid moieties. J. Food Sci. **30**(1):69-76 (1965).

2. Amerine, M. A. Composition of wines. I. Organic constituents. Advances in Food Research **5**:353-510 (1954).

3. Amerine, M. A. and A. J. Winkler. Composition and quality of musts and wines of California grapes. Hilgardia **15**(6):493-673 (1944); Calif. Agr. Exptl. Sta. Bull. No. **794**:1-83 (1963).

4. Bain, J. M. and R. N. Robertson. Physiology of growth in apple fruits. I. Cell size, number, and fruit development. Austral. J. Sci. Res. **4B**:75-91 (1951).

5. Baragiola, W. I. and Ch. Godet. Chemisch-analytische Untersuchungen über das Reifen von Trauben und über die Entwichlung des daraus gewonnen Weines. Landw. Jahrb. **47**:249-302 (1914).

6. Caldwell, J. S. Some effects of seasonal conditions upon the chemical composition of American grape juices. J. Agr. Research **30**:1113-1176 (1925).

7. Cantarelli, C. and C. Peri. The leucoanthocyanins in white grapes: their distribution, amount, and fate during fermentation. Am. J. Enol. Viticult. 15(3):146-153 (1964).

8. Copeman, P. R. v. d. R. Some changes occurring during the ripening of grapes. Union S. Africa Dept. Agr. Divn. Chem. Ser. No. 75, Sci. Bull. 60:1-19 (1927).

9. Diemair, W., H. Janecke, and G. Krieger. Das Schicksal des Gerbstoffes der Weinrebe während des Wachstums (II). Z. Anal. Chem. 133:353-359 (1951).

10. Durmishidze, S. V. Tannin compounds and anthocyanins of grape vine and wines (Trans.). Academy of Science, Moscow. 323 p. (1955).

11. Hennig, K. and R. Burkhardt. Vorkommen und Nachweis von Quercitrin und Myricitrin in Trauben und Wein. Weinberg u. Keller 7(1): 1-3 (1960).

12. Joslyn, M. A. and M. A. Amerine. Dessert, appetizer and related flavored wines, the technology of their production. Division of Agricultural Sciences, University of California, Berkeley and Davis, 483 p. (1964).

13. Joslyn, M. A. and J. L. Goldstein. Astringency of fruits and fruit products in relation to phenolic content. Advances in Food Research 12:179-217 (1964).

14. Kelhofer, W. Untersuchung von Räuschlingtrauben in verschiedenen Reifestadien. Schweiz. z. Obst.- und Weinbau 17:65-68 (1908).

15. Laborde, J. Étude sur les matières tannoides du vin: matière colorante et oenotanin. Rev. Viticult. 30:169-173, 200-203, 533-537, 564-569 (1908); 31:7-10, 47-51, 178-184, 204-207 (1909); 32:453-457, 480-484, 561-567, 706-711, 733-741 (1909); 33:206-211, 238-242 (1910).

16. Negré, E. Les matières tannoides et la composition des vins. Bull. Off. Internatl. Vin 15(154):20-52 (1942); 15(155):25-56 (1943).

17. Pro, M. J. Report on the spectrophotometric determination of tannins in wines and spirits. J. Assoc. Offic. Agr. Chemists 38:757 (1955).

18. Ribéreau-Gayon, P. Les composés phenoliques du raisin et du vin. Ann. Physiol. Végétale 6:119-147, 211-242, 259-282 (1964).

19. Roessler, E. B. and M. A. Amerine. Further studies on field sampling of wine grapes. Am. J. Enol. Viticult. 14(3):144-147 (1963).

20. Singleton, V. L. Chemical and physical development of the pineapple fruit. I. Weight per fruitlet and other physical attributes. J. Food Sci. 30(1):98-104 (1965).

21. Singleton, V. L. and Diana E. Draper. The transfer of polyphenolic compounds from grape seeds into wines. Am. J. Enol. Viticult. 15(1):34-40 (1964).

22. Tanner, H. Phenolics Inhaltsstoffe des Obstes und der Trauben. Schweiz. z. Obst- und Weinbau 72(25):607-612 (1963).

23. Tsakov, D. D'bilnite i bagrilnite veshchestva v sortovete G'mza i Zarchin. Nauch. inst. vinarska i pivovarna prom. Nauchni Trudove (Sofia) 6:5-30 (1963).

DENSITY SEPARATIONS OF WINE GRAPE BERRIES AND RIPENESS DISTRIBUTION

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The separation of fruits and vegetables on a density or specific gravity basis into fractions useful for experimental study or commercial processing appears to deserve wider consideration. A mud-flotation process is used commercially to separate rocks and tramp iron from sugar cane before milling. There, the entire desired product is expected to float, and only the waste to sink. Analogous commercial processes, operating in the reverse direction, are those which clean peas and other products of leaves and low-density parts such as empty seed coats and hulls by density and froth-flotation methods. Fractional separations among the product units themselves and involving density have included such operations as selecting sweet, tender peas from over-mature peas (1), sorting potatoes for quality (2), and sorting of fruits for ripeness and quality (3, 4, 5).

The nature of the separation produced by the simple operation of collecting units which float as opposed to those which sink in a solution will depend greatly upon the nature of the product and, of course, the solution density and handling procedure involved. Abnormal conditions such as rot, corkiness, undeveloped seed kernals, etc., could strongly affect specific gravity of whole fruit. The density of a given healthy fruit is influenced by the specific gravity or Brix of the expressible juice. It may, however, be influenced by other factors, depending on the specific composition or structure of that kind of fruit. Particularly important in this regard may be the internal "air" content, either

in relatively large spaces, such as the seed cavities of apples or melons, or in finely dispersed spaces among the fruit tissue cells (4, 5). The presence of high- or low-density structures or parts may be important, depending upon the species of fruit, and practical problems may arise from air-bubble entrainment in skin folds, etc.

The only studies of which we are aware in which grapes have been segregated by specific gravity were those of Nelson et al. (3). They showed that 'Cardinal', 'Perlette', and 'Thompson Seedless' berries from a very variable population could be separated by flotation on sugar solutions into fractions with a relatively constant juice Brix. They further showed that such nondestructive segregation was very convenient in selecting whole berries with specific compositional differences for further testing—sensory analysis in their studies.

This paper reports further studies of separation of grapes. A major objective was to compare the juice Brix with the flotation solution Brix to estimate the importance of such factors as seed density or air content on fruit density in grapes. Another subject of the study was the population distribution of berry density and how it varied with area and date. The segregated samples were also used with considerable success to study some ripeness-composition relationships at a single harvest (not weather-confounded), and part of these results are reported.

MATERIALS AND METHODS

'Cabernet Sauvignon' and 'White Riesling' grapes were taken in 1965 from vines which had been marked in the Davis and Oakville university vineyards. The clusters on these vines were counted, and the samples were taken at periodic intervals (one or more weeks apart) by taking a constant portion of the average number

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of clusters per vine at random over the whole vine. For example, if each vine had 60 clusters and six harvests were planned, 10 clusters would be taken from each vine at each harvest. The berries were snipped with scissors at random from each cluster, usually 2 berries per cluster, with 777 to 2760 total berries per sampling.

These berries were selected randomly except that raisins, shot berries, or damaged berries were not included. They were snipped from the pedicel, flush with the skin of the berry so as to exclude the stem tissue but not damage the skin appreciably. The berries were harvested in the morning and brought to the laboratory for further processing during the afternoon. The segregation by density was completed the same day, but crushing and the determination of juice Brix, etc., was sometimes delayed until the following day. If so, the berries were held at 0°C and 91% relative humidity.

Sucrose solutions were prepared by weighing table sugar and distilled water to make solutions at intervals of 2.0 Brix from 12.0 to 32.0 Brix. The berries were dropped into beakers of each solution and separated into floaters and sinkers ("hovering" berries were rare, but were considered to be floaters). The berries which are categorized as the 16-18 density class, for example, would be those which sank in 16.0 Brix sucrose and all lower Brix solutions but floated on 18.0 Brix sucrose and all solutions of higher Brix. Both low-to-high and high-to-low dipping were tried, and made no apparent difference in results obtained, but dipping from high-to-low Brix solutions was easier from a manipulation viewpoint. In the early experiments the solutions were rechecked frequently with a refractometer and readjusted to within 0.2 Brix of the original value. In later experiments it was found more convenient to retest the solutions less frequently and re-dip the segregated berries in bracketing restandardized solutions after initial separation.

The temperature of berries and solutions was that of the air-conditioned laboratory, 24-25°C, but refractometric Brix values were corrected to 20°C. The juice was expressed by hand by firm squeezing through cheesecloth, and the pH and Brix were determined upon the composited juice. pH was determined with a Beckman GS meter and glass electrode standardized at pH 4.0. A sample of winery must from the whole harvested lot of grapes was also tested for Brix and pH to compare with the weighted average calculated from the data on the segregated fruit.

RESULTS AND DISCUSSION

The density of the fruit was determined for each segregated berry sample by measuring the volume of the grapes by displacement in water, weighing them, and calculating g berry per ml volume. This value was compared with that obtained by conversion of the sample's juice Brix into apparent density. It was found that the whole-fruit density for all samples averaged 97.6% of the respective juice Brix values, and that there was no apparent or consistent difference in this value by grape variety, date, or area. Therefore it is evident that the density of the juice is practically the same as the density of the whole fruit, and berry density variation should reflect Brix variation almost exclusively, at least above 12° Brix. The fact that the juice is slightly more dense than the whole fruit indicates that the solids (parts other than juice) of the berry are on the average less dense than the juice. This coincides with observations of separation of must in that most of the solids [except sometimes the seeds (3)] tend to float to the top of the juice with time, even without gas bubbles to buoy them up. These data also show that the "air" content in ripe grapes must be very low and not a major variable.

The average whole-fruit density for all samples as percentage of their juice density differed slightly by density interval. The fruit which sank in 14 Brix sucrose and floated on 16 Brix showed 96.6%; 16-18 Brix gave 96.9; 18-20, 97.3; 20-22, 98.0; 22-24, 97.8; 24-26, 98.8; 26-28, 97.3; and 28-30 Brix showed 96.9%. These data are interpreted as showing that the relative effect upon whole-fruit density by the lower-density non-juice components is

TABLE 1

Brix of Juice from Berry Samples Segregated by Flotation on Sucrose Solutions

Variety Location Date	Density class — Brix of sucrose solution where berries sank-floated										Weighted av. juice (Brix)	Must (Brix)
	12-14	14-16	16-18	18-20	20-22	22-24	24-26	26-28	28-30	30-32		
'White Riesling'												
Davis												
3 Sept.	13.0	16.0	15.9	18.8	20.4	21.8	22.9	**22.8**	......	......	20.1	......
10 Sept.	15.3	16.4	17.1	18.6	20.0	21.7	22.5	25.8	27.9	30.1	20.8	20.8
17 Sept.	......	16.2	18.2	20.3	22.1	22.7	24.6	26.0	29.5	32.5	22.8	21.4
24 Sept.	......	15.5	17.6	18.5	20.6	22.2	24.1	25.4	28.3	29.0	22.6	22.0
1 Oct.	......	......	17.6	19.4	21.5	23.1	24.3	25.4	28.4	30.7	23.4	22.2
Oakville												
16 Sept.	12.4	14.3	15.5	17.5	20.2	22.1	24.6	25.7	......	......	17.6	16.7
29 Sept.	12.8	14.7	16.4	18.9	21.0	21.8	25.3	26.1	......	32.1	18.6	18.1
12 Oct.	14.0	14.5	15.9	17.9	20.3	21.9	23.7	25.1	......	......	19.0	18.8
20 Oct.	14.0	14.7	16.1	18.1	20.6	22.3	23.5	24.9	......	......	19.6	18.9
27 Oct.	......	17.3	17.6	19.2	21.0	22.4	23.7	25.2	27.9	......	21.5	21.0
'Cabernet Sauvignon'												
Davis												
3 Sept.	11.3	14.0	16.5	17.6	19.3	20.7	23.0	25.0	......	......	19.1	......
10 Sept.	17.6	20.3	18.7	18.7	19.6	20.4	23.0	24.3	26.0	......	20.4	19.9
24 Sept.	......	......	15.0	18.7	20.9	22.1	22.0	25.3	28.4	31.4	23.6	22.7
1 Oct.	......	......	......	18.7	21.4	22.9	24.2	25.5	27.0	29.0	24.6	23.2
8 Oct.	......	......	......	20.0	20.8	22.0	24.5	25.4	26.6	28.9	24.7	24.2
Oakville												
29 Sept.	11.3	14.7	16.0	18.0	20.0	22.4	24.7	29.7	28.8	......	18.4	17.5
12 Oct.	......	16.3	16.1	17.6	19.6	22.0	23.3	25.4	28.5	30.8	19.4	18.7
20 Oct.	......	16.0	16.2	18.2	20.4	22.0	23.5	25.4	27.5	......	20.0	20.0
27 Oct.	......	14.7	17.6	18.4	20.0	21.4	23.0	25.0	28.5	......	21.2	22.1
4 Nov.	......	17.8	17.8	18.6	19.8	22.0	24.2	25.7	27.5	30.6	21.5	22.2
Average	13.5	15.8	16.8	18.6	20.5	22.0	23.8	25.5	27.9	30.5		

slightly greater in the less ripe (lower Brix) fruit. This is reasonable since the riper fruit is larger and has a higher proportion of juice up to a certain stage (max. berry weight coincided with about 22 Brix in these samples). The slight depression in the value in the very ripe (high Brix) samples is believed to reflect the onset of shriveling and water loss, which, again, would raise the relative contribution of the low-density non-juice portion.

No particularly great problem was encountered from entrained air bubbles. It was necessary to wipe off bubbles from a few grapes when they were first immersed in the flotation solutions, but as soon as the surface was wetted no further problem was encountered. A few late-harvested samples, particularly the last samples from Oakville, were shriveled enough to have fine wrinkles, which might have entrained microscopic air bubbles, but comparable values were obtained. Raisins, which were not included in this study, had been found to be nearly impossible to segregate in simple sucrose solutions, owing to air held in the surface grooves. A few trials indicated that if pedicels had been pulled from the berries rather than cut off, a bubble might have entered the space left by the "brush" and could have affected the results.

Table I gives the Brix of the juice samples by segregated groups. It is evident that the separation of grapes by density did give a separation by ripeness or juice
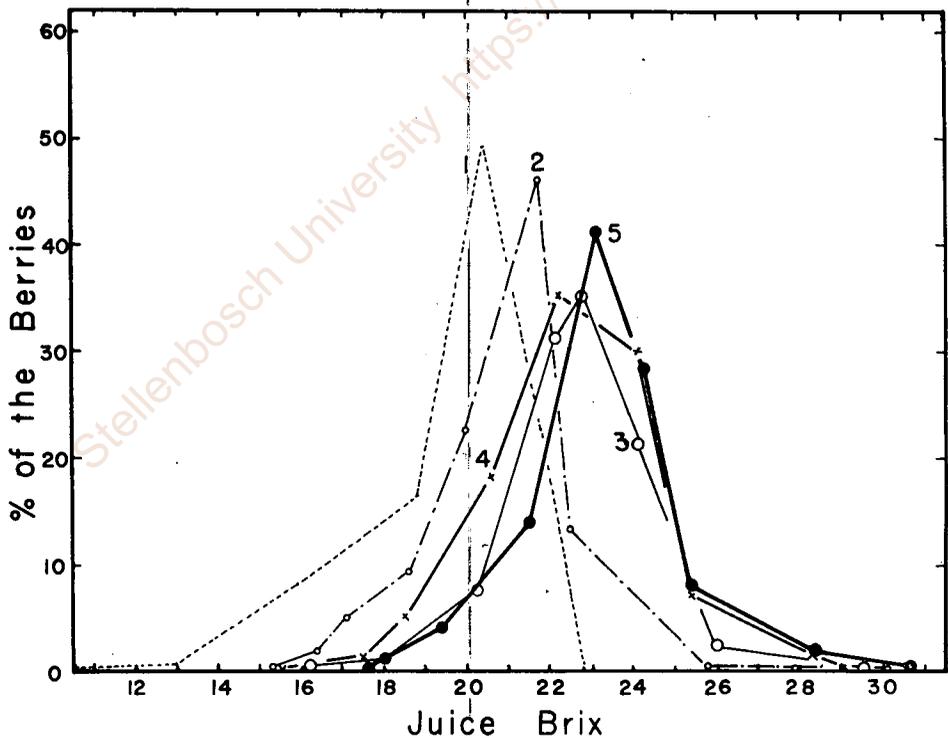

Figure I. 'White Riesling', Davis. Distribution of Brix of composited juice from density-segregated grapes versus the percentage of the total berry sample represented by each fraction. Harvests: 1) 3 Sept., 2) 10 Sept., 3) 17 Sept., 4) 24 Sept., and 5) 1 Oct., 1965.

Brix, as expected from the data of Nelson *et al.* (3). Although some variation is shown in the observed juice Brix for any one density class, much of this variation occurs in the extreme classes representing relatively few fruit, whereas in the intermediate classes (up to about 7500 berries) the juice Brix is rather constant regardless of variety, location, or date. This, again, indicates the fundamental correlation between juice Brix and grape density, because most other factors (turgor, berry size, acidity, etc.) varied considerably over this range of samples.

The flotation solutions differed from each other by 2.0 Brix units, and the average difference in juice Brix between the density classes, considering all samples, was 1.95 Brix units. This was not uniformly distributed, however, for the combined classes representing the most berries had differences as small as 1.5 Brix from each other, and the classes representing berries with either considerably less or more sugar content than the population mean were more than 2 units higher or lower than the neighboring classes. This is believed to result from the fact that fairly wide fractions with little or no overlap cut from a normally distributed population will not be normally distributed internally. The skewness within the fractions will distribute the means at less than average intervals near the peak, and at greater than average intervals in the sections with rapid slope. Statistical tests of the data for the fifth
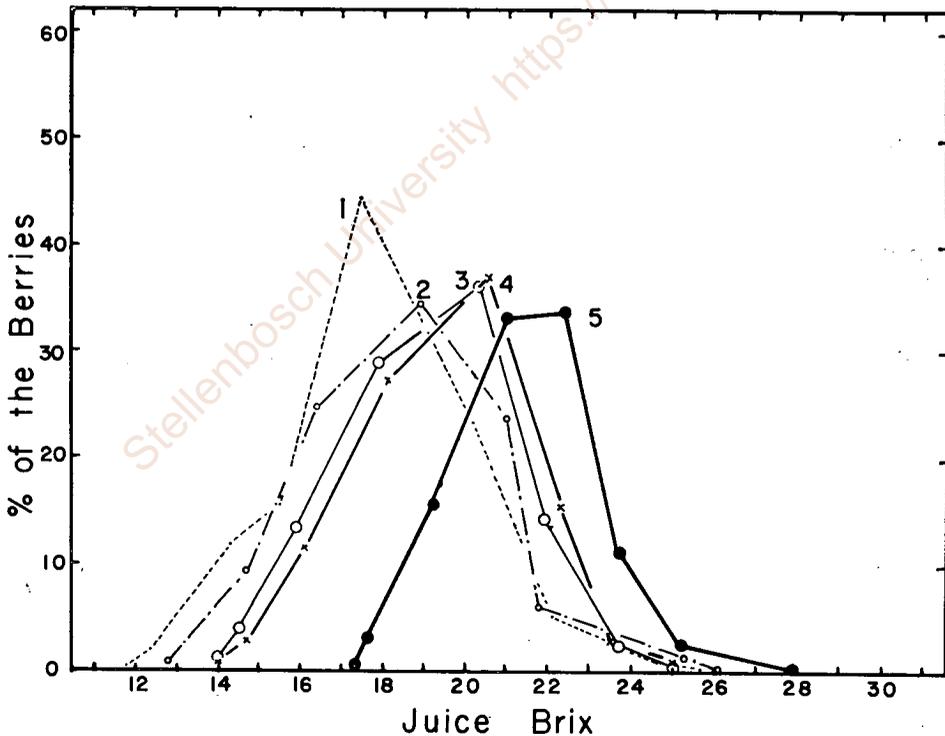
Figure 2. 'White Riesling', Oakville. Distribution of Brix of composited juice from density-segregated grapes versus the percentage of the total berry sample represented by each fraction. Harvests: 1) 16 Sept., 2) 29 Sept., 3) 12 Oct., 4) 20 Oct., and 5) 27 Oct. 1965.

maturity sampling of 'Cabernet Sauvignon' from both areas indicated that the distributions were very highly significantly different from normal with respect to both skewness and kurtosis. Considering the similarity of the curves (Figure 1-4), this is no doubt also true for the other harvests and both varieties. The kurtosis was positive, showing the flanks of the curves to be excessively steep. This is believed to reflect partly the experimental effect of the relatively wide (2.0 Brix) density classes, and partly the nature of the population, particularly on the high Brix side. This and the skewness suggest that the advance of Brix during ripening can be characterized as a rolling-wave type curve.

The data presented in table 1 show that a considerably finer separation could have been achieved by using flotation solutions at closer Brix intervals than 2.0. The juice Brix tended to match the Brix of the solution in which the berries first sank, e.g., a group of berries which sank in 22.0 Brix sucrose solution and floated on 24.0 contained juice at about 22.0 Brix. As would be expected from consideration of the normal distribution curve, the low-density samples yielded juice appreciably higher than the Brix of the most dense solution in which they sank. If intervals between flotation solutions had been narrower in Brix units, the juice Brix should be more nearly the same as the most dense solution in which they would sink. A few other varieties have also been tested but not
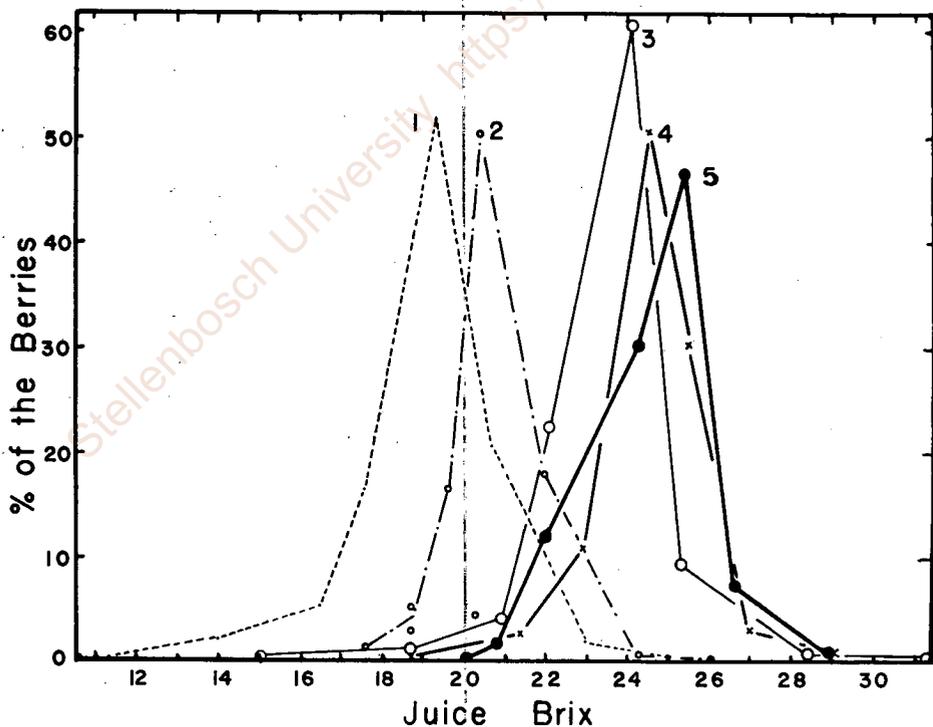
Figure 3. 'Cabernet Sauvignon', Davis. Distribution of Brix of composited juice from density-segregated grapes versus the percentage of the total berry sample represented by each fraction. Harvests: 1) 3 Sept., 2) 10 Sept., 3) 24 Sept., 4) 1 Oct., and 5) 8 Oct., 1965.

TABLE 2

pH of Juice from Berry Samples Segregated by Flotation on Sucrose Solutions

Variety Location Date	Density class Brix of sucrose solution where berries sank-floated								Weighted av. juice (pH)	Must (pH)
	14-16	16-18	18-20	20-22	22-24	24-26	26-28	28-30		
'White Riesling'										
Davis										
3 Sept.	......	3.04	3.40	3.19	3.25	......	......	......	3.23	......
10 Sept.	2.97	3.03	3.21	3.16	3.23	3.17	3.08	......	3.19	2.95
17 Sept.	3.03	3.09	3.07	3.08	3.08	3.08	3.17	......	3.08	3.02
24 Sept.	......	3.09	3.17	3.16	3.17	3.19	3.12	3.28	3.17	3.12
1 Oct.	......	3.16	3.16	3.30	3.31	3.31	3.30	3.24	3.30	3.26
Oakville										
16 Sept.	3.06	3.07	3.13	3.13	3.19	3.24	......	......	3.11	2.99
29 Sept.	3.07	3.12	3.17	3.20	3.09	3.11	......	......	3.15	2.99
12 Oct.	3.17	3.25	3.31	3.33	3.36	3.29	3.26	......	3.31	3.27
20 Oct.	3.26	3.33	3.42	3.44	3.44	3.33	3.37	......	3.41	3.29
27 Oct.	3.33	3.37	3.41	3.48	3.50	3.48	3.47	3.41	3.47	3.47
Cabernet Sauvignon'										
Davis										
3 Sept.	2.82	3.18	3.29	3.37	3.38	......	......	......	3.34	......
10 Sept.	2.97	3.03	3.21	3.16	3.23	3.17	3.08	......	3.19	3.11
24 Sept.	......	......	3.13	3.24	3.20	3.22	3.21	3.33	3.22	3.36
1 Oct.	......	......	3.17	3.31	3.36	3.42	3.44	3.43	3.42	3.50
8 Oct.	......	......	3.63	3.38	3.48	3.50	3.50	3.50	3.50	3.49
Oakville										
29 Sept.	2.96	3.06	3.11	3.14	3.25	3.19	3.26	......	3.12	3.13
12 Oct.	3.19	3.16	3.22	3.24	3.29	3.26	3.31	3.44	3.24	3.28
20 Oct.	3.09	3.27	3.32	3.36	3.42	3.47	3.44	......	3.36	3.30
27 Oct.	......	3.37	3.24	3.33	3.37	3.39	3.46	3.53	3.36	3.44
4 Nov.	3.37	3.43	3.40	3.42	3.51	3.52	3.54	3.59	3.48	3.41
Average	3.06	3.17	3.26	3.27	3.31	3.30	3.32	3.40		

reported here, and it appears to be a valid approximation that the juice Brix of maturing grapes is the same or slightly higher than the Brix of the solution in which they will sink if they will float on a solution one degree Brix higher.

Table 2 shows the pH of the juice of density-segregated grapes. Table 3 gives similar data on berry weight, and figures 1-4 give the distribution curves of juice Brix of segregated berry samples versus percentage of the total berries sampled by dates, varieties, and locations. All these data illustrate that information otherwise difficult or impossible to obtain can be gathered by first segregating the fruit by Brix. Table 2 shows that the pH generally rises, as expected for increasing ripeness

as measured by increasing juice Brix and fruit density. However, it also shows that the latest harvests gave higher pH even within a Brix-density class. Except for the Oakville 'Cabernet' samples, this was preceded by an initially moderate pH and then a drop to a minimum before the final rise. Since this minimum in pH occurred earlier in greener fruit, but in all cases between 10 and 29 September, it appears to have both weather and physiological components. These small differences are partially masked if only non-segregated berries are examined.

The berry weight (Table 3) is lower for 'Cabernet' than for 'Riesling', and lower in the later harvests for 'Riesling' and throughout for 'Cabernet' for Oakville than
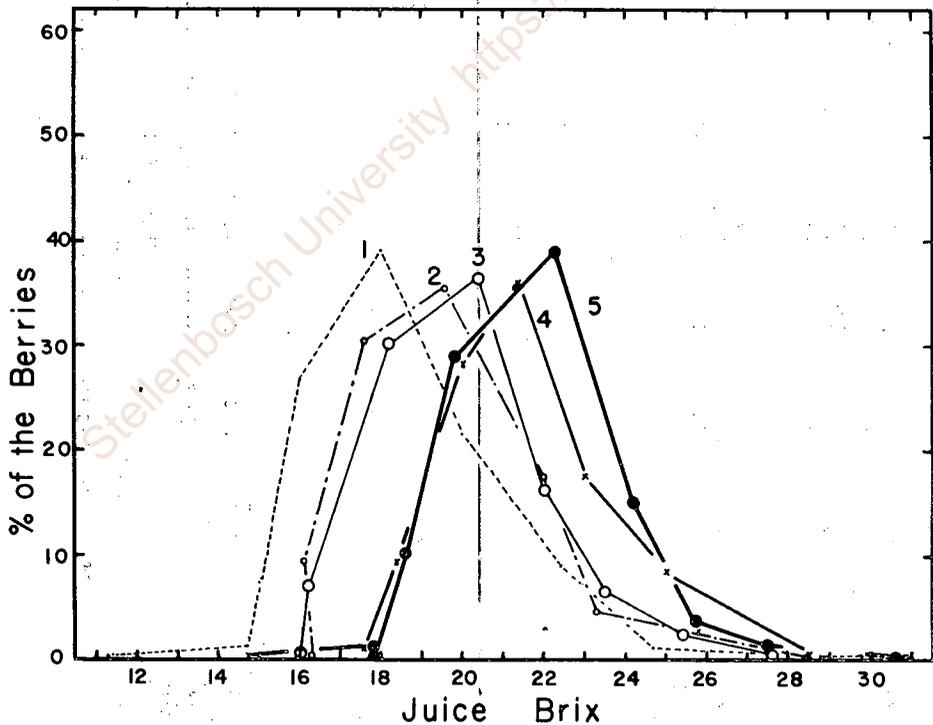
Figure 4. 'Cabernet Sauvignon', Oakville. Distribution of Brix of composited juice from density-segregated grapes versus the percentage of the total berry sample represented by each fraction. Harvests: 1) 29 Sept., 2) 12 Oct., 3) 20 Oct., 4) 27 Oct., and 5) 4 Nov., 1965.

TABLE 3

Average Weight (g) Per Berry from Samples Segregated by Flotation on Sucrose Solutions

Variety Location Date	Density class Brix of sucrose solution where berries sank-floated								Weighted av. berry wt. (g)
	14-16	16-18	18-20	20-22	22-24	24-26	26-28	28-30	
'White Riesling' Davis									
3 Sept.	.92	1.08	1.15	.96	1.30	1.16	....	....	1.08
10 Sept.	.94	.98	1.08	1.21	1.28	1.11	....	....	1.20
17 Sept.	....	....	1.12	1.24	1.30	1.26	....	....	1.28
24 Sept.	....	....	1.26	1.22	1.30	1.29	....	....	1.28
1 Oct.	....	....	1.20	1.30	1.32	1.27	1.09	....	1.28
Oakville									
16 Sept.	1.07	1.10	1.15	1.13	.95	1.06	.90	....	1.12
29 Sept.	1.15	1.23	1.35	1.29	1.15	....	....	....	1.28
12 Oct.	....	1.20	1.34	1.34	1.32	....	....	....	1.32
20 Oct.	....	1.14	1.23	1.23	1.17	....	....	....	1.21
27 Oct.	....	....	1.18	1.22	1.17	1.12	....	....	1.18
'Cabernet Sauvignon' Davis									
3 Sept.	.85	.84	.96	1.04	1.07	1.05	1.02	....	1.01
10 Sept.	.94	.98	1.08	1.21	1.28	1.11	....	....	1.20
24 Sept.	....	....	....	.94	1.12	1.07	.99	....	1.09
1 Oct.	....	....	....	....	1.02	1.11	1.01	....	1.04
8 Oct.	....	....	....	....	1.07	1.08	1.07	.98	1.08
Oakville									
29 Sept.	....	.92	.94	.98	.94	....	....	....	.94
12 Oct.	....	.85	.93	1.04	.95	....	....	....	.96
20 Oct.	....	.88	.94	.93	.94	.91	....	....	.93
27 Oct.	....	....	.89	.89	.92	.89	.88	....	.90
4 Nov.	....	....	.88	.83	.86	.86	.80	....	.85

for Davis. The highest density class has smaller berries than the intermediate density. This is expected from the fact that berries with fewer seeds are known to be smaller and tend to accumulate sugar earlier. The greenest berries were, of course, both smaller and lower in density and Brix. The density-segregated classes reveal, however, that at the same Brix level the berries increase in size up to a point and then decrease. This decrease is at least partly a matter of water loss and shriveling. This effect was particularly noticeable with the Oakville 'Cabernet', and nearly absent with the Davis 'Riesling' samples.

Figures 1-4 give the distribution in terms of the percentage of the berries at each harvest at the different juice Brix levels. Figures 1 and 3, Davis, show sharper peaks and a narrower distribution than Figures 2 and 4, Oakville, for both varieties. Testing the pooled distributions (all levels added together) for similarity by chi-square statistical techniques verified that both area and variety altered the shape of the curves to a significant degree. This shows that density segregation can be used to examine rather small differences within and between grape berry populations. Figure 1, particularly, and figure 2 to a lesser extent suggest that the progression of ripeness as measured by Brix increase in the normal grape population is characterized by a progressive increase in the most prevalent Brix level (by berry) up to a physiological limit (near 25-26 Brix in these examples). At this point the further increase in Brix on an individual berry basis would depend primarily on dehydration. On a population basis both dehydration and the catching up of the laggard berries would increase average Brix. Note the steep and constant front between Brix 24 and 26 in figure 1 for the last three harvests, and remember that table 3 shows less berry shriveling for these Davis 'White Rieslings' than for the others. Also, note the relatively great tailing, especially in the low-Brix direction in the first harvest, in both figure 1 and figure 3 compared to later harvests.

The Oakville samples (Figures 2, 4) apparently never reached their physiological limit. These harvests were characterized by an intermediate period during early October when the weather was cool and average Brix advanced very slowly. A period of warm weather then produced increased Brix, but this appeared to be partly true physiological accumulation of sugar and partly dehydration.

SUMMARY AND CONCLUSIONS

It was shown that juice Brix is the primary factor determining grape berry density and that the whole berry's density is about 98% of the density of its juice. This was true for several density classes of two seeded varieties harvested on several dates in two different locations. Preliminary tests indicate similar relationships with other varieties. Juice Brix from segregated berries based on these tests is expected to be near or slightly above the Brix of the most dense sucrose solution in which they will sink, if they float in a solution one Brix unit more dense.

The data presented show the validity and value of the relatively easy technique of density segregation in characterizing a grape berry population. Varietal wine prepared from two musts at essentially the same average Brix is often quite different. Part of this difference may lie in the ripeness distribution making up the average Brix, and density segregation can elucidate this point. Application of the methods outlined to the detection, study, and use in variety selection of a characteristic physiological limit to sugar accumulation warrants further study. The technique is capable of selecting fruit from a single harvest so that ripeness differences relatively free of weather differences can be studied. Berries of relatively rare composition can be selected from a large population. Further study may even disclose commercial utility in making quality separations among whole clusters or, eventually, mechanically harvested berries.

ACKNOWLEDGMENTS

nical assistance was provided by Kathryn Singleton, MaryAnn Graf, Judith Carroll, and J. A. Rossi, Jr.

LITERATURE CITED

1. Anonymous. Brine flotation test for separating peas according to maturity. U. S. Code of Federal Regulations. Title 7, Agriculture, Sections 52.2291-2 (1959).

2. Bettelheim, F. A. and C. Sterling. Factors associated with potato texture. I. Specific gravity and starch content. Food Research **20**: 71-80 (1955).

3. Nelson, K. E., G. A. Baker, A. J. Winkler, M. A. Amerine, H. B. Richardson, and Frances R. Jones. Chemical and sensory variability in table grapes. Hilgardia **34**(1):1-42 (1963).

4. Singleton, V. L., C. P. Sideris, and W. A. Gortner. Unpublished data (1957).

5. Singleton, V. L. Chemical and physical development of the pineapple fruit. I. Weight per fruitlet and other physical attributes. J. Food Science **30**(1): 98-104 (1965).

PAPER CHROMATOGRAPHY OF PHENOLIC COMPOUNDS FROM GRAPES, PARTICULARLY SEEDS, AND SOME VARIETY-RIPENESS RELATIONSHIPS[1]

V. L. SINGLETON, DIANA E. DRAPER, and JOSEPH A. ROSSI, JR.[2]

It was known, from our own experiments and from reports in the literature, that, as a group, the phenolic substances including the tannins are of critical importance as astringents and oxidation substrates in wine. For this reason we investigated, early in our wine quality-composition-aging research program, the phenolics in wines of various origins and ages. It was soon found, however, that paper chromatograms prepared from wines, particularly red wines, were very complex, highly variable from wine to wine, and difficult to relate to the origin and previous processing of the wines. It therefore was of interest to examine the phenolic composition of the tissues of the grape itself in order to simplify and clarify the wine studies.

At the time this work was started, many individual phenolic substances had been reported from grapes or wine. Particularly noteworthy were studies by Durmishidze (4) and Hennig and Burkhardt (6). While our work was in progress several additional pertinent studies and reviews have appeared (3, 7, 9, 16). This paper reports our findings and makes some comparisons with other studies. Emphasized are reproducible paper chromatographic "mapping" procedures such as have been so useful in studies on tea, etc. (2, 10, 15). Also emphasized are the phenolics in the seeds, which have been studied less but are very important, especially in red wines (13). Qualitative or quantitative differences between varieties and changes during berry development are discussed.

MATERIALS AND METHODS

The grapes examined were representative samples of at least 200 berries at each harvest for each variety. The technique of berry processing (weekly harvest, 12 varieties, 1962 and 1963) has been described previously (13). The separated skins, seeds, or pulp were ground with ethanol to prepare a concentrated extract representing essentially quantitative recovery of phenolic substances exhaustively extractable into water or ethanol as previously described (12, 13).

Because the high sugar content of the juice, particularly in the later stages of ripening, interfered with sample application and proper migration on the paper, alcoholic extracts of the juice were prepared after saturation with ammonium sulfate (11).

The extracts were stored under nitrogen in the dark, at about —20°C. Under these conditions no changes in the total phenol assay or in the paper chromatographic pattern were noted for at least one year, and all results reported here were obtained within a few months of collection of the samples. The extracts were analyzed for total phenol content, expressed as gallic acid, by Folin-Ciocalteu type of colorimetry by standard methods as modified in this laboratory (14).

For the standard two-dimensional paper chromatograms, Whatman No. 1 chromatographic sheets, 18 x 22 cm, were spotted (maximum final spot diameter 2 cm) with sufficient extract (approximately 0.02-

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2.0 ml) to contain 200 μg of gallic acid equivalent. The spotted paper was ordinarily equilibrated in a saturated atmosphere of the solvent for at least one-half hour, then developed descendingly with the organic layer from freshly prepared n-butanol:acetic acid:water mixture (BAW), 4:1:5 v/v. The chromatography was conducted at 24±2°C. The papers were air-dried and developed in the second dimension with 2% v/v acetic acid in water (HOAc). This sequence was used and is preferred for convenience and slightly more discrete spots; however, for extracts with very high polar solids (sugar) in relation to the phenols, developing ascendingly first with aqueous solvent can be helpful without appreciably altering the relative migration of the individual phenolic substances.

After complete development, the dry paper chromatograms were examined under 366-mμ ultraviolet light, and the fluorescent spots were marked. The paper was then examined under the same lamp while being contacted with ammonia gas arising from concentrated ammonium hydroxide solution, and the additional, or more intensely fluorescent, spots were marked. The paper was then allowed to lose ammonia and was sprayed with a freshly prepared 1:1 mixture of aqueous solutions of 1% potassium ferricyanide and 1% ferric chloride. This solution was allowed to react on the paper for 3 minutes, and then the chromatogram was placed in dilute HCl to stop the reduction and remove the excess reagent. The dipping in HCl was followed by thorough washing in water to remove the remaining acid and ferric ions and make the final dry chromatogram essentially permanent. Properly done, the chromatogram will have discrete spots of Prussian or Turnbull's blue corresponding to the location of the readily oxidizable substances (such as most phenolics) and an almost colorless background between spots. If the excess reagent is not removed, reducing sugars and other substances will react, eventually coloring the whole paper.

The procedure just described was found highly reproducible, sensitive, and satisfactory for the general "mapping" procedure to characterize the grape extracts. For identification of different classes of phenolics and flavonoids, additional chromatographic solvent systems and spot-locating reagents were used (5, 6, 8, 15); some of these are mentioned in the Results section. Most of these other reagents required 2-10 times as much phenolic substances on the chromatogram and did not permit detection as readily as did the ferricyanide reagent, particularly when considerable non-phenolic solid was present.

For estimating the relative quantities of the different substances separated on the chromatograms, several recognized procedures were tested. The weight of the area of tracing paper equivalent to each spot, planimetry, and direct photometry in comparison with standards gave similar results. However, direct visual estimation on an arbitrary scale also gave good agreement with these methods in estimating the relative quantities of each substance on a chromatogram. Since it was necessary to examine about 25 spots on each chromatogram, and 9-17 chromatograms for each of 12 varieties, this latter rapid (though more subjective) method was chosen. The arbitrary scale used was: 0 = absent (or fluorescent only), 1 = very faint blue, 2 = faint blue, 3 = light blue, 4 = medium blue, 5 = dark blue, and 6 = very dark blue (blue-black).

The seed extract was fractioned into three major fractions by column partition chromatography by modified procedures based upon those reported by Vuataz and co-workers (15). A fraction eluted by ether contained the major substances, designated E1, E2, and E3. The following fraction eluted by ethyl acetate contained the major substances A1-A6, and the substances staying at or near the origin on paper chromatograms (designated Oo, O1, O2, and C) were collected as an ethanol wash of the column. The E1-E3 fraction was subjected to preparative-strip one-dimensional chromatography on Whatman 3MM paper. The separated substances were eluted, determined to be unchanged by cochromatography with the original seed extract, and used in studies to identify the three substances.

RESULTS AND DISCUSSION

Phenols are not the only substances which will be oxidized by the ferric chloride-potassium ferricyanide reagent to result in blue spots on the paper chromatograms. In wines, ascorbic acid and sulfurous acid derivatives can be sources of such interference. Many substances which fluoresce are not phenolic. It must be borne in mind, therefore, that a few of the substances considered here as "phenolic" may ultimately prove otherwise. However, by combination of other considerations such as browning in the presence of phenolase, or dye production with diazonium salts, most of them are known to be phenols (flavonoids, tannins, etc.).

Many phenols do not fluoresce on paper chromatograms, and some, particularly monophenols of suppressed reactivity, may not produce blue spots under our reac-
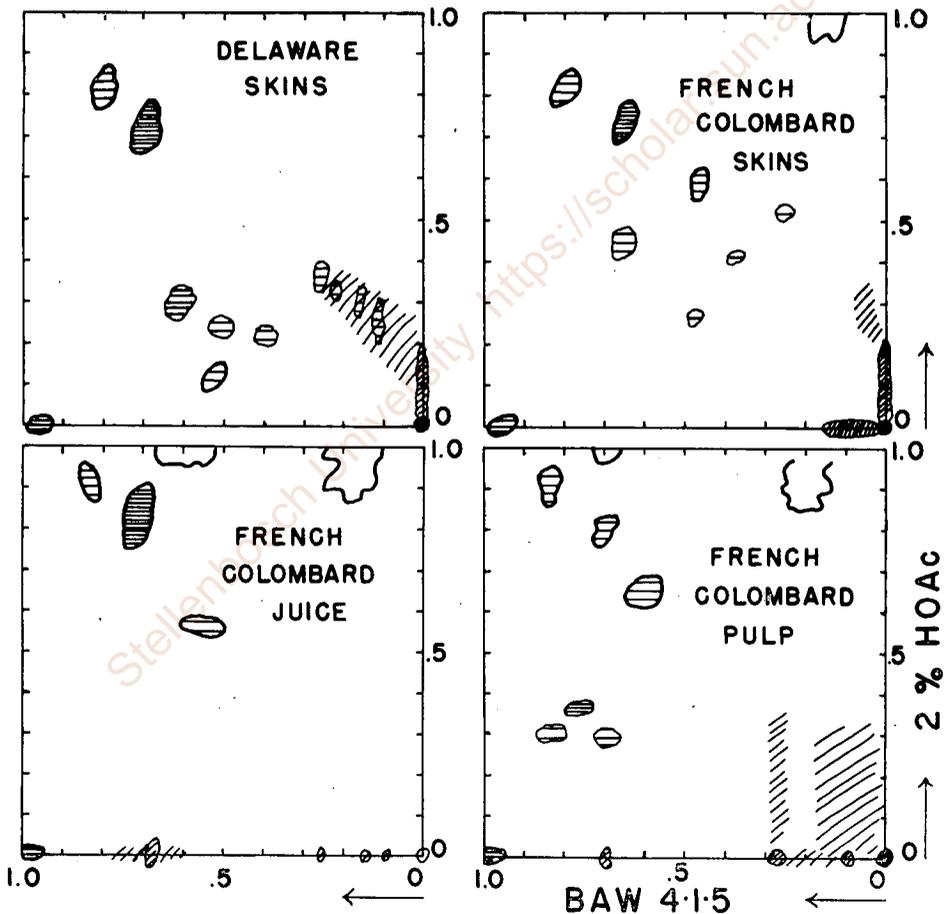
Figure 1. Two-dimensional paper chromatograms of the "phenols" of typical juice and pulp samples as represented by French Colombard grapes and of French Colombard compared to Delaware skins. A thick-line border indicates a flourescent spot and increased relative intensity (Turnbull's blue) is indicated by closer cross-hatching or darkening.

tion conditions. Therefore, no claim is made that all the phenolic substances of grapes or grape seeds have been indicated by our studies. In fact, we know that additional substances can be shown by heavier loading than the 200 μg of gallic acid equivalent per chromatogram used in this study. At least one spot and possibly others on our standard two-dimensional chromatogram can be two (or more) substances which migrate together.

It has become the fashion to name the substances responsible and consider them identified if migration in one or two solvents on paper and some color reactions are the same as those of a known phenol of plant origin. Certainly, most such assignments will prove correct, but, owing to the great number of possible compounds and the many similarities in properties and reactions, such identifications must be considered provisional pending more detailed studies. For this reason, statements of the identity of the phenolic substances are limited and qualified in this report. More detailed study of the identity and importance of the individual grape phenolics is in progress, and further reports are planned.

Figure I shows representative chromatographic "maps" of juice, skin, and pulp extracts. The French Colombard juice gave results very similar to those of most of the other grape varieties studied. One to three phenolic substances relatively mobile in both solvents predominated, although small amounts of other phenolic substances and considerable fluorescent but not readily oxidized (probably nonphenolic) substances were present. These predominant substances fluoresced bright blue-white and were enhanced by fuming with ammonia. They appear to be phenolic compounds of the chlorogenic acid class and it was interesting that double spotting, indicative of cis-trans isomerism of these cinnamic acid derivatives, was not detected in these juice samples but did appear in analogous chromatograms from wines.

Calzin juice was found to be unusual among the twelve grape varieties studied in detail in that it had a large proportion of phenolic material which did not move from the origin. This was mostly condensed tannin and it accounts for the fact that not only is Calzin wine very astringent, but shortening the fermentation on the skins has not reduced this astringency satisfactorily in experimental wines. Even a white wine made from Calzin evidently would be astringent. This property must be fairly rare among grape varieties, because among a total of 34 varieties only Calzin and Refosco had any noticeable condensed tannin (origin material) in the juice. Calzin is a cross between Zinfandel, which lacks the property, and Refosco. Additional varieties which lacked noticeable condensed tannin in the juice were Ruby Carernet, Aleatico, Touriga, Mataro, A r a m o n, Souzao, Trousseau, Carignane, Mission, Grignolino, Pinot Noir, Gamay Beaujolais, Cabernet Sauvignon, Black Malvoisie, Tinta Madeira, Barbera, Tannat, Pinot St. George, Charbono, and Napa Gamay.

The skin extracts were found to be quite different between varieties. Figure I shows French Colombard and Delaware as examples. Two chlorogenic-acid-like spots were similar to the juices and present in each variety, but additional substances were present that were qualitatively quite different between the two varieties. Owing to this diversity and a greater variation in the chromatograms, resulting from high but variable sugar content, summarizing the results with skins is difficult. Diversity between varieties in other phenolics in the skins is not particularly surprising in view of the known variation among the (phenolic) anthocyanins (16). In a similar manner. however, study of this diversity may prove practical in variety identification and useful in biochemical, genetic, and taxonomic studies with white grapes or as additional parameters in red varieties.

The pulp of the grapes had very little total phenolic substance compared to the seeds or skins, and that which was present seemed to contain no unique components (Figure I) but reflected the juice, the seeds (probably from the tiny aborted seeds inevitably missed), and sometimes the skins (also possibly from fragments overlooked during separation).

The seeds, which will be the subject of the remainder of the report, were rich in phenols and gave chromatograms which were quite similar for all varieties tested. Based upon Rf values, color reactions, relative positions, and relative amounts, the spots corresponding to the various substances were given code designations and the data are summarized in table I. The Rf values shown are the averages from all the two-dimensional chromatograms from all varieties which had the substance in question. For those spots regularly present in all varieties this would involve about 144 individual measurements. The standard deviation between the means for varieties and the grand mean was very similar

for all spots and averaged 0.04 for the BAW Rf's and 0.03 for the HOAc Rf's. This shows reproducibility as good as or better than is ordinarily expected for such data and means that if the same conditions are used by other workers (temperature solvents, paper, etc.) the average of a few replicates should agree within ±0.04 of the values given. As will be shown later (Table 2), the Rf's of more purified samples in one-dimensional runs also agreed well with these two-dimensional data.

Figure 2 was prepared by plotting the average Rf value by varieties on tracing paper, centering the origin, E2, and the rest of the spots for the best agreement, and circling the resultant group of points

TABLE I

Phenols (Oxidizable Compounds) Located on Chromatograms of Grape Seed Extracts

	No. of varieties exhibiting	Color test[a]	BAW-HOAc Rf Mean x 100	Relative amount[b]
Oo	12	BV	0-0	5.9
O1	12	BV	0-S	4.2
O2	12	BV	S-0	4.0
C	12	B	S-S	3.8
A1	12	B	29-51	2.2
A2	12	B	34-40	2.8
A3	12	B	38-47	1.9
A4	12	B	41-58	1.9
A5	12	BF	46-61	1.2
A6	10	B	54-68	0.8
A7	5	BF	63-69	2.0
A8	4	F	73-78	0.1
A9	3	F	83-79	0.1
B1	11	B	08-48	1.3
B2	12	B	39-81	1.1
B3	1	B	63-85	1.2
B4	2	B	27-72	0.3
D1	12	B	35-23	0.9
D2	12	B	44-30	0.9
E1	12	B	55-32	4.2
E2	12	B	65-40	4.2
E3	12	B	74-27	3.1
P1	4	BFV	99-0	0.9
X1	6	B	21-40	0.8
X2	1	B	54-54	0.2
X3	4	B	23-44	0.7
G1	1	B	38-07	0.5

[a] B = blue in the $FeCl_3$-$K_3Fe(CN)_6$ test; V = visible in normal lighting; F = fluorescent.

[b] On an arbitrary scale of spots (blue) intensity: 0 = absent or fluorescent only, 1 = very faint, 2 = faint, 3 = light, 4 = medium, 5 = dark, 6 = very dark.

for each spot. The spots which were fluorescent are outlined with a heavy line, and the relative level of the substance is indicated by the degree of darkening or cross hatching.

The O (origin) group (Figure 2) of spots included Oo, which moved in neither solvent; O1, which streaked in the HOAc direction; and O2, which streaked in the BAW direction. The C (curtain) area was more or less a uniform smear in both directions, sometimes divided by an empty streak in the HOAc direction to give C1 nearer the origin and the C2 area away from the origin. The other code series were assigned more or less in order from the origin in the BAW direction. The P spot was eluted by petroleum ether on partition column chromatography. It was visibly brownish green and included chlor-
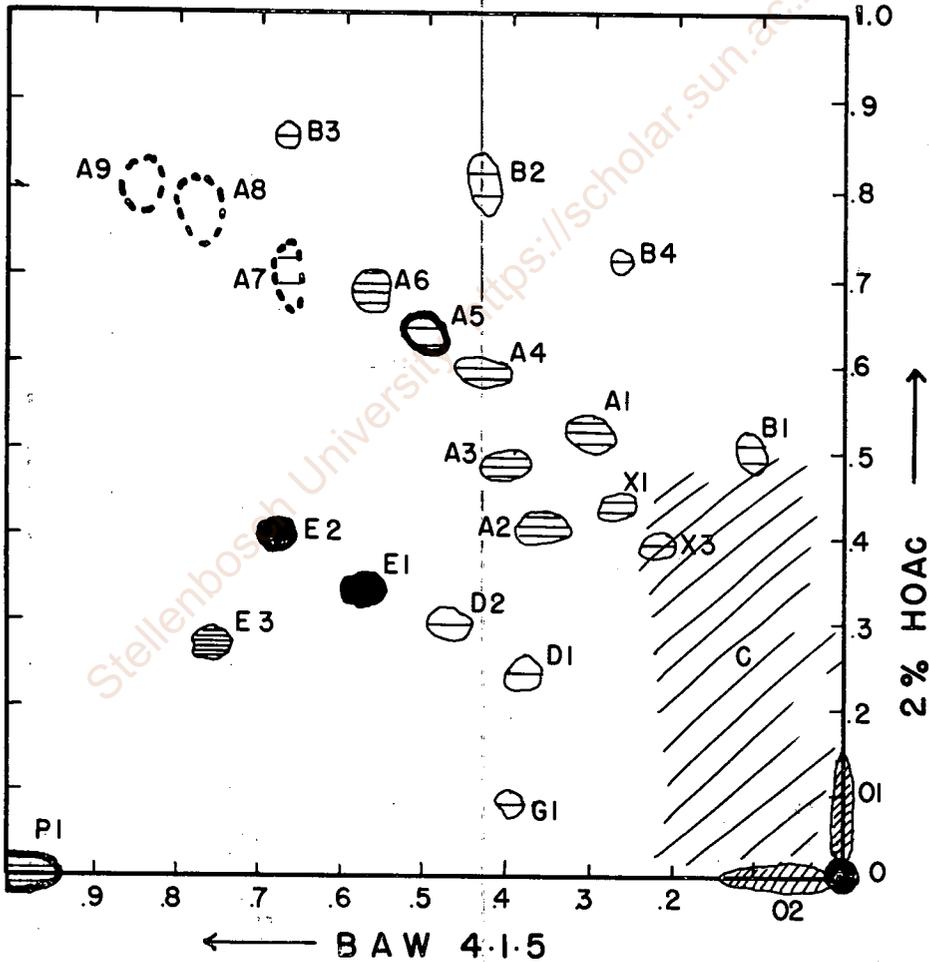
Figure 2. Grape seed phenolics, a combined paper chromatographic "map" for all varieties studied. A thick-line border indicates a fluorescent spot and increased relative intensity (Turnbull's blue) is indicated by closer cross-hatching or darkening. A dashed-line border indicates presence only if pulp accompanies the seeds.

ophyll degradation products. It was also present in the other berry parts (Figure 1) and was usually weak or absent unless green tissue was included with the seeds. Spots representing the substances desigated A8 and A9 (Figure 2, Table 1) were generally too weak to show the blue test, although they appeared to give blue at higher levels, and are shown as fluorescent only. They and A7 appeared on chromatograms only when pulp was included with the seeds (Calzin, Catawba, Grenache, Petite Sirah, and Sauvignon blanc) and not when it was excluded (the other 7 varieties). They probaly include the same substances noted as chlorogenic-acid-like in pulp and juice (Figure 1). Except with A7-A9 and sometimes P1, the inclusion of pulp appeared to make no difference in the chromatograms other than decreasing reproducibility slightly owing to the extra sugar and nonphenolic solid included.

The fraction eluted from partition columns of seed extracts with ethyl acetate

contained the A1-A6 series of substances and the fastest-moving part of the C material. Tested as a group, these substances gave positive tests for leucocyanidin. The fraction washed from partition columns containing the O material also gave strong tests for leucocyanidin and precipitated with gelatin, showing that this material is condensed tannin in nature.

The separated substances designated E1 and E2 were compared and cochromatographed (one-diminsional replicated 10 times) in 4 solvent systems with d-catechin and l-epicatechin samples obtained commercially. The data shown in table 2 indicate that E2 is catechin and E1 is epicatechin. Corroboratory data were obtained by comparative phenolase color reactions, colors produced by a series of diazonium salts (8), ultraviolet absorption-fluorescence behavior, etc. An authentic pure sample of epicatechin gallate was not available for direct comparison at the time this work was done, but the mobility data from the literature agreed well and

TABLE 2

Average Rf Values of the Major "Catechin Fraction" Components from Grape Seeds

Samples	BAW 4:1:5[a]		BAW 4:1:2.2		2% HOAc		30% HOAc
	Found	Lit.[b]	Found	Lit.[c]	Found	Lit.[c]	Found
E1	.58		.60		.31		.57
l-Epicatechin	.57	.65	.59	.58	.34	.37	.60
E1 + l-epicatechin	.58		.57		.34		.58
E2	.68		.69		.39		.64
d-Catechin	.68	.76	.70	.66	.39	.41	.66
E2 + d-catechin	.67		.68		.39		.64
E3	.78		.78		.26		.66
l-Epicatechin gallate		.86		.76		.28	
Ratios							
E1/E2	.85		.86		.80		.89
Epicatechin/catechin	.85	.86	.85	.88	.86	.90	.92
E3/E2	1.14		1.12		.66		
Epicatechin gallate/catechin		1.13		1.15		.68	1.02

[a] n-BuOH, 4 vol.; HOAc, 1 vol.; H_2O, 5 volumes.

[b] Bradfeld in Bate-Smith (2).

[c] Vuataz et al. (15).

E3 appeared to be epicatechin gallate. Pure E3 was hydrolyzed with acid and alkali under mild conditions in a nitrogen atmosphere, and the products found to include major amounts of gallic acid and E1 (or epicatechin) identified by several tests. For example, gallic acid and E3 gave blue spots with ferric ammonium sulfate sprays (indicating pyrogallol) under conditions that gave green spots with E1, E2, and other pyrocatechol derivatives. In the standard BAW-HOAc system, gallic acid and d-catechin coincided. However, E2 spots from grape seed extracts were found in each of several cases tested to be free of gallic acid by chromatography in solvent systems which will separate the two. Based on these data, color with diazonium salts (8), paper chromatographic comparison with tea extracts prepared in our laboratory, and literature identifications (2, 10, 15), E3 is believed to be and is provisionally identified as epicatechin gallate.

Although the general similarity in qualitative and quantitative composition of grape seeds was strikingly similar, considering the range of varieties tested, there were differences (Table 3). The relative lack of difference between the two varieties (Cataba and Delaware) with *Vitis labrusca* parentage compared to the *Vitis vinifera* varieties is particularly surprising and appears to indicate either dominant *V. vinifera* crossing or a fundamentally similar pattern of phenolic composition in *Vitis* seeds generally. A few substances appeared in one or only a few varieties. For example, G1 was found only in Muscat of Alexandria, B3 only in Grenache, and X2 only in Pinot blanc. In order to simplify the table, the pulp-related spots A7, A8, A9 and P1 are not shown in table 3, nor are the O or C series, which were present in all varieties but exhibited less evident variation. A few substances appeared in all but a few varieties; for example, A5 was missing from only Aligoté (Table 3).

A majority of the substances were detected in all the varieties, but not in constant relative amounts (Table 3). Note, for example, that Sauvignon blanc and Catawba were relatively higher in A1-A4 than were the other varieties. Muscat

of Alexandria appears notably low in E1, Petite Sirah high in E2, and Sauvignon blanc relatively high in all three "E" substances of the catechin series. Red-grape seeds did not appear to be consistently different from white-grape seeds in phenol composition.

The data on each variety indicated that a number of the phenolic substances changed in a regular way during ripening with respect to the fraction they contributed in a given amount of the total grape-seed phenol. It was desired to combine the data so as to generalize and verify it, and provide a better estimate by averaging the results for all varieties. The method chosen consisted of selecting the harvest at which the juice of the grapes of each variety tested nearest to 20° Brix, calling this the base value (zero weeks from 20° Brix), and averaging the relative phenol content estimates for all varieties by harvests (weeks) before or after this base date. The data obtained are shown in table 4. The pulp-related substances, A7-A9 and P1, appeared to decline as ripening progressed, but this may be related to a decreased percentage of pulp or pulp phenol in relation to the seeds. These data and the results for B3, which showed no clear trend, were left out of table 4 to save space.

Several very definite trends during development and ripening are shown in the combined data (Table 4) and in the individual varieties. In no case did individual varieties show trends contrary to each other or the combined data. One of the most clear-cut trends is the late appearance and then rapid increase of A6. Probably the most interesting is the decrease of E3 in the last weeks of ripening. This does not appear to result from simple hydrolysis of epicatechin gallate, because epicatechin did not increase in relative amount and free gallic acid did not appear in equivalent amounts. Hennig and Burkhardt (6) also found no free gallic acid until after the wine was made, but neither did they find epicatechin gallate in the seeds. This may have been because they examined seeds from grapes sufficiently ripe that the epicatechin gallate was nearly gone. We have found this compound to

TABLE 3

Mean Relative Amounts[a] of Individual Phenols in 200 μg of the Total Phenol

Assayed from Seeds of Different Grape Varieties

Phenol	Varieties[b]											
	P.S.	Cal.	Gre.	Cat.	Del.	M. A.	Sem.	E. R.	Ali.	P. b.	S. b.	F. C.
A1	2.9	2.8	2.6	3.0	2.0	2.1	1.8	1.9	1.3	1.4	3.7	1.0
A2	3.2	3.1	3.2	3.0	1.9	3.0	2.1	2.8	1.7	2.4	4.1	3.0
A3	2.3	1.6	1.8	3.4	1.2	0.3	2.0	2.1	1.6	1.2	4.0	1.5
A4	2.8	2.2	2.1	3.1	1.8	0.7	1.5	1.8	1.3	1.7	3.1	1.0
A5	2.0	0.9	1.9	1.4	0.9	0.5	0.4	1.2	0.8		1.6	1.2
A6	1.1	1.3	1.9	0.6	0.6		0.6	0.2	0.7	0.6		
B1	2.0	1.9	1.2	0.6	1.1	2.0	0.9	1.4	1.2	0.9		1.0
B2	3.0	1.4	1.3	0.4	1.6	0.9	0.4	0.5	0.5	0.1	1.6	0.4
B3			1.2									
B4						0.3	0.3					
D1	1.2	1.6	1.0	0.9	0.3	0.8	0.8	0.7	0.4	0.7	1.6	1.0
D2	1.4	1.4	1.1	0.9	0.3	1.0	0.7	0.9	0.4	0.3	2.1	0.5
E1	5.6	4.1	4.4	5.0	4.2	2.6	3.8	3.8	3.6	4.1	5.7	3.6
E2	4.7	3.9	4.4	4.4	4.2	4.1	3.9	4.3	3.3	3.4	5.6	4.1
E3	4.1	4.0	3.4	3.4	2.3	2.6	2.8	2.7	2.4	2.1	4.3	2.6
X1		1.0	0.8	1.0	0.4	1.3		0.9	0.2			
X2										0.2		
X3						0.5	0.8	0.7				0.1
G1						0.5						

[a] No entry = absent from all chromatograms of variety, O = missing or fluorescent only, 1 = very faint blue, 2 = faint blue, 3 = light blue, 4 = medium blue, 5 = dark blue, 6 = very dark blue (blue-black).

[b] Petite Sirah, Calzin, Grenache, Catawba, Delaware, Muscat of Alexandria, Sémillon, Emerald Riesling, Aligoté, Pinot blanc, Sauvignon blanc, French Colombard.

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Phenol	Varieties[b]											
	P.S.	Cal.	Gre.	Cat.	Del.	M. A.	Sem.	E. R.	Ali.	P. b.	S. b.	F. C.
A1	2.9	2.8	2.6	3.0	2.0	2.1	1.8	1.9	1.3	1.4	3.7	1.0
A2	3.2	3.1	3.2	3.0	1.9	3.0	2.1	2.8	1.7	2.4	4.1	3.0
A3	2.3	1.6	1.8	3.4	1.2	0.3	2.0	2.1	1.6	1.2	4.0	1.5
A4	2.8	2.2	2.1	3.1	1.8	0.7	1.5	1.8	1.3	1.7	3.1	1.0
A5	2.0	0.9	1.9	1.4	0.9	0.5	0.4	1.2	0.8		1.6	1.2
A6	1.1	1.3	1.9	0.6	0.6	0.6	0.2	0.7	0.7	0.6		
B1	2.0	1.9	1.2	0.6	1.1	2.0	0.9	1.4	1.2	0.9		1.0
B2	3.0	1.4	1.3	0.4	1.6	0.9	0.4	0.5	0.5	0.1	1.6	0.4
B3			1.2									
B4						0.3	0.3					
D1	1.2	1.6	1.0	0.9	0.3	0.8	0.8	0.7	0.4	0.7	1.6	1.0
D2	1.4	1.4	1.1	0.9	0.3	1.0	0.7	0.9	0.4	0.3	2.1	0.5
E1	5.6	4.1	4.4	5.0	4.2	2.6	3.8	3.8	3.6	4.1	5.7	3.6
E2	4.7	3.9	4.4	4.4	4.2	4.1	3.9	4.3	3.3	3.4	5.6	4.1
E3	4.1	4.0	3.4	3.4	2.3	2.6	2.8	2.7	2.4	2.1	4.3	2.6
X1		1.0	0.8	1.0	0.4	1.3		0.9	0.2			
X2										0.2		
X3						0.5	0.8	0.7				0.1
G1						0.5						

[a] No entry = absent from all chromatograms of variety, O = missing or fluorescent only, 1 = very faint blue, 2 = faint blue, 3 = light blue, 4 = medium blue, 5 = dark blue, 6 = very dark blue (blue-black).

[b] Petite Sirah, Calzin, Grenache, Catawba, Delaware, Muscat of Alexandria, Sémillon, Emerald Riesling, Aligoté, Pinot blanc, Sauvignon blanc, French Colombard.

TABLE 4

Mean Relative Amounts[a] of Individual Phenols (All Varieties Combined) at Weekly Intervals from the Harvest Giving 20° Brix Juice

Phenol	Weeks											
	−10	−9	−8	−7	−6	−5	−4	−3	−2	−1	0	+1
	Maximum number of varieties[b]											
	5	5	6	9	11	11	12	12	12	12	10	6
O0	6.0	6.0	6.0	6.0	5.9	5.9	5.8	6.0	5.8	5.8	5.8	5.8
O1	4.5	4.6	4.8	4.4	4.5	4.2	3.9	4.2	4.4	4.2	4.3	4.3
O2	4.8	5.0	4.8	4.1	4.0	4.2	4.3	3.9	4.1	4.1	4.0	2.8
C1	4.2	4.2	4.2	4.2	4.0	4.2	4.0	3.7	3.7	3.5	3.8	2.8
C2				2.0	2.0	2.2	3.2	3.4	3.8	3.2	3.6	3.8
A1	1.8	1.6	1.8	2.1	2.2	2.2	2.5	2.4	2.4	2.0	2.3	2.8
A2	2.8	2.4	2.8	2.9	2.9	3.2	2.9	2.8	2.5	2.1	2.1	2.7
A3	0.7	1.8	1.4	1.4	1.3	2.0	1.9	1.8	2.0	1.6	1.9	2.2
A4	0.2	0.6	0.8	1.2	1.2	1.9	2.1	2.3	2.4	2.5	2.6	3.2
A5	0.8	0.8	0.8	1.0	0.8	1.3	1.2	1.0	1.2	1.1	1.3	2.0
A6	0.0	0.0	0.0	0.0	0.0	0.1	0.2	0.9	1.5	1.7	2.1	2.2
B1	2.0	1.8	1.7	1.9	1.9	1.8	1.7	1.2	1.2	0.8	0.8	0.2
B2	0.6	1.0	1.2	1.4	0.9	1.1	1.2	0.8	0.8	0.7	0.9	1.2
B4	1.0	1.0	1.0	0.5	0.0	0.0						
D1	1.2	1.2	1.0	1.4	1.2	1.3	1.0	1.0	0.5	0.3	0.2	0.5
D2	1.0	1.0	0.8	1.2	1.2	0.9	1.4	1.4	0.7	0.6	0.4	1.0
E1	2.8	3.8	3.9	4.0	4.0	4.4	4.5	4.2	4.2	4.0	4.1	4.3
E2	3.8	4.5	4.6	4.4	4.1	4.3	4.7	4.2	4.0	3.8	3.7	4.0
E3	3.0	3.7	3.6	4.0	3.8	3.6	3.6	3.0	2.6	2.0	2.1	2.0
G1	1.0	1.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	
X1	1.0	1.0	1.0	1.2	1.0	1.0	0.7	0.4	0.6	0.0	0.4	0.0
X2			1.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
X3	1.0	1.0	1.0	0.7	0.7	1.0	0.3	0.6	0.3	0.0	0.3	

a No entry = no applicable harvest-variety data, O = missing or fluorescent only, 1 = very faint blue, 2 = faint blue, 3 = light blue, 4 = med. blue, 5 = dark blue, 6 = very dark blue (blue-black).

b The number of varieties having a sample at the indicated time; individual substances were not necessarily present in all.

be generally quite low or absent in grape seeds in wine pomace.

Other trends which appear to be definite in table 4 and the data for individual varieties include a general increase during ripening in the proportion of A4, A5, and A6, as already mentioned. A general decrease appears in A2, B1, B4, D1, G1, X1, X2, and X3. Several substances appear to increase in the early stages and then decrease slightly or become erratic in the later stages of ripening, for example, A1, B2, D2, E1, and E2. No regular change emerged for Oo, O1, or A3. The O2, C1, and C2 materials seem to change in a regular manner, the first two decreasing and the third increasing, but this may be related to the changes in sugars and other nonphenols, which can affect adsorption of these phenolics on cellulose. The major conclusion to be drawn is that not only is there considerable synthesis of total phenols in the grape berry during much of the ripening period (12), but there also are important metabolic changes in the relative proportion of the individual substances contributing to this total, with some appearing or increasing, and others decreasing.

Space limitations prevent an extensive review of the correlation between our results and reports of others. The most directly comparable work is that of Hennig and Burkhardt (6). In general, our results confirm their findings and extend the studies.

SUMMARY AND CONCLUSIONS

A simple standardized technique for two-dimensional paper chromatographic "mapping" and estimation of the relative amounts of the phenolic substances in grape extracts was presented. This technique was applied to the study of weekly harvests of 12 grape varieties. The berry pulp tended to reflect the juice and seeds in phenolic composition. The juice phenolics, as a rule, were predominantly blue-fluorescing substances resembling chlorogenic acid and its analogs. Calzin and one of its parents, Refosco, were unique among 34 varieties tested in having a considerable content of astringent tannin in the

juice. Skin extracts were quite variable in qualitative phenolic composition by variety, especially considering, but in addition to, the presence or absence of anthocyanins.

The phenolic composition of the seeds, however, followed a similar pattern in red or white grapes and in varieties with parentage from two species of *Vitis*. Among a total of about 25 differentiated substances readily oxidized by $FeCl_3$-$K_3Fe(CN)_6$ and considered as phenols, the majority were present in the seeds of all varieties at similar stages, and usually in roughly similar proportions. Varietal differences did occur with respect to the presence or absence of a few of the substances and the relative amounts of those present per unit of total phenol.

The nature of several of the substances was outlined, and, in particular, evidence was presented that one was catechin, one was epicatechin, and a third was epicatechin gallate. Although these flavanols have been reported in grapes (4, 6) previously, epicatechin gallate has not always been found in seeds (6).

A considerable metabolism of phenolic substances in seeds during ripening was found even up to the last stages, as shown by the appearance or increase of some and the decrease of others in relation to a unit of total phenol.

LITERATURE CITED

1. Amerine, M. A. Laboratory procedures for enologists. Department of Viticulture and Enology, University of California, Davis. 130 pp. (1960).

2. Bradfield, A. E. and E. C. Bate-Smith. Chromatographic behavior and chemical structure. II. The tea catechins. Biochim. Biophys. Acta 4: 441-444 (1950).

3. Cantarelli, C. and C. Peri. The leucoanthocyanins in white grapes: their distribution, amount, and fate during fermentation. Am. J. Enol. Viticult. 15(3):146-153 (1964).

4. Durmishidze, S. V. Tannin compounds and anthocyanins of grape vine and wines (Trans.). Academy of Science, Moscow. 323 p. (1955).

5. Geissman, T. A., ed. The chemistry of flavonoid compounds. Macmillan Co., New York. 666 pp. (1962).

6. Hennig, K. and R. Burkhardt. Detection of phenolic compounds and hydroxy acids in grapes, wines, and similar beverages. Am. J. Enol. Viticult. 11(2):64-79 (1960).

7. Joslyn, M. A. and J. L. Goldstein. Astringency of fruits and fruit products in relation to phenolic content. Adv. Food Research 12:179-217 (1964).

8. Pearl, I. A. and P. F. McCoy. Stable diazo salts for chromatographic spray reagents. Anal. Chem. 32(11):1407-1410 (1960).

9. Ribéreau-Gayon, P. Les composés phenoliques du raisin et du vin. Ann. Physiol. Végétale 6:119-147, 211-242, 259-282 (1964).

10. Roberts, E. A. H. Paper chromatography as an aid to the elucidation of the structure of polyphenols occurring in tea. In the chemistry of vegetable tannins, a symposium. Soc. Leather Trades' Chemists, Croyden, England. p. 87-97 (1956).

11. Singleton, V. L. An extraction technique for recovery of flavors, pigments, and other constituents from wines and other aqueous solutions. Am. J. Enol. Vitic. 12(1):1-8 (1961).

12. Singleton, V. L. The total phenolic content of grape berries during maturation of several varieties. Am. J. Enol. Viticult. 17(2):126-134 (1966).

13. Singleton, V. L. and Diana E. Draper. The transfer of polyphenolic compounds from grape seeds into wines. Am. Enol. Viticult. 15(1):34-40 (1964).

14. Singleton, V. L. and J. A. Rossi, Jr. Colorimetry of total phenolics with phosphomolybdic phosphotungstic acid reagents. Am J. Enol. Viticult. 16(3):144-158 (1965).

15. Vuataz, L., H. Brandenberger, and R. H. Egli. Plant phenols. I. Separation of the tea leaf polyphenols by cellulose column chromatography. J. Chromatog. 2:173-187 (1959).

16. Webb, A. D. Anthocyanins of grapes. In Phenolics in normal and diseased fruits and vegetables, a symposium. Plant Phenolics Group of North America, Montreal. p. 21-39 (1964, Pub. 1965).

CONTRIBUTIONS OF GRAPE PHENOLS TO OXYGEN ABSORPTION AND BROWNING OF WINES[1]

JOSEPH A. ROSSI, JR. and VERNON L. SINGLETON[2]

The reactions involved in the modifications of wines by oxygen are complex and far from completely understood in spite of considerable valuable research on many aspects of the subject. The major substrates readily susceptible to oxidation in most wines are the phenolic compounds. The effects of oxidation in wine can be beneficial in some cases and undesirable in others. For example, browning is one of the major results of oxidation, and it may be good in sherry and bad in table wine. Browning may be enzymatically catalyzed in the must, or nonenzymatically produced during processing and aging.

The brown-pigmented material in wines is evidently derived almost exclusively from phenolic compounds except in unusual cases, such as wines colored by the addition of caramel or wines whose production involves heating in the presence of appreciable sugar. Evidence for the phenol origin of brown pigments in wine includes the known participation of polyphenoloxidase, the fact that reduction of the phenol content by fining agents and adsorbents commonly delays or prevents browning (4, 8), and the knowledge that the conditions in wine are adverse to Maillard or other types of browning (6).

Individual phenolic substances, however, are not equally subject to oxidation, nor are their oxidation products equally brown. For example, chlorogenic acid is readily oxidized by polyphenoloxidase, but the products are not intensely colored (17). Nonenzymic oxidation proceeds in much the same manner as the enzymatic oxidation (10), and an increasing number of phenolic hydroxyl groups in the molecule, if they are not prevented from participating in a quinoid structure, increases susceptibility to oxidation (10, 20).

Studies on white wine, particularly those by Berg and co-workers (1, 2, 3, 8), have shown that the total phenol content does not correlate well with a wine's tendency to brown. These and other studies (4, 6) lead to the conclusion that the differing responses of wines to oxidation and browning are related to the different presence of or different relative amounts of specific fractions of the phenolic family of substances. However, the identity of the important fractions is not known. Therefore, we were interested in investigating the responses of wines and of purified fractions of grape phenols in regard to reaction with oxygen and in browning.

Since grape seeds contain a large and fairly representative complement of the phenolic compounds of the grape berry, with the major exceptions of the anthocyanins and simpler phenolic acids, they were used as a source of phenolics for this study. Research prior to this study had shown that the seeds contribute important amounts of phenols to wines if the juice or wine contacts the seeds for any appreciable time (19).

MATERIALS AND METHODS

The phenols from fresh grape seeds were extracted and separated into a catechin fraction, a leucoanthocyanin fraction, and a condensed tannin fraction as described elsewhere (17, 21). These fractions contained respectively 3, 5, and 2 different major phenolic substances (21), but did not overlap in phenol composition when chromatographed on paper. The condensed

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tannin fraction was freed of smaller molecules of contaminating nonphenolic substance by dialysis. All three fractions were converted to dry powders by low-temperature concentration and lyophilization from aqueous solution. These powders were relatively free of nonphenol contamination, as indicated by the fact that they gave only slightly lower color yield on a weight basis than did d-catechin with our standard total phenol assay, as expected (20).

A transistorized-regulated Bausch and Lomb Spectronic 20 containing a flow-through cuvette assembly was used to obtain absorbance values of the experimental solutions. Total phenolics were determined by the Folin-Ciocalteu assay (20). A vanillin-sulfuric acid assay (22) was used to determine the phenolic compounds with an unsubstituted phloroglucinol nucleus. This test, when used in conjunction with the Folin-Ciocalteu value, can be an indication of the amount of polymerization in the sample (22).

For the enzymatic studies, an active polyphenoloxidase preparation was isolated from grapes by a technique similar to that outlined by Siegleman for apples (18). Six hundred grams of Emerald Riesling berries were mixed in a Waring blender for 2 minutes with 600 ml of cold 0.1M phosphate buffer (pH 6) containing 0.05M ascorbic acid and chopped ice.. The mixture was filtered through four layers of cheesecloth and centrifuged at 13,000 xg for 15 minutes. The supernatant was discarded and the sedimented material re-suspended in 0.2M phosphate buffer (pH 6) and centrifuged again at 13,000xg. This washing was repeated two additional times and the solid then resuspended with a Ten Brock tissue grinder in 50 ml of the 0.2M buffer. Two-dimensional paper chromatograms of the total grape seed extract using n-BuOH:HOAc:H$_2$O, 4:1:5 (v/v), and 2% HOAc solvents (21) were developed and sprayed with the enzyme preparation. The sprayed papers were placed in a humid chamber at room temperature to enable the browning reaction to proceed without drying or denaturing the phenolase enzyme unduly.

For the nonenzymatic studies, a Thompson Seedless white table wine was decolorized with a small amount of charcoal and filtered. The total phenolic content of the wine after this treatment was 25 mg/l (gallic acid equivalents). The separated phenolic fractions, along with other selected phenolics, were added to this wine to study their effect on nonenzymatic browning. Samples in which the atmosphere was controlled were sparged for 10 minutes with nitrogen or oxygen through a sintered-glass distributor and tightly stoppered with rubber stoppers. Samples which were to undergo accelerated browning were placed in stoppered tubes in a 125°F oven.

The synthetic wine-model solution used in some of these tests consisted of 10% ethanol, 5 g/l tartaric acid, and 0.9 g/l sodium hydroxide to obtain a pH of 3.2. A Warburg constant volume respirometer and standard procedures (11) were used to measure the oxygen absorption by phenolic compounds.

RESULTS AND DISCUSSION

A sample of the total phenols extracted from grape seeds was added to grape juice, fermented, and the wine chromatographed after two months. No appreciable changes were noted after this treatment on the paper chromatograms and all the substances originally found in the seed extract were still detectable in the wine. Since it had already been shown that the phenols found in grape seeds were all represented in wine-like model-solution extracts of seeds (16, 19), this justifies further consideration of the reactions of phenols from the seeds in oxidation and browning of wines.

Oxygen consumption: The oxidation of phenols is related to the pH of the solution. The protonated phenols at acidic pH are more resistant to oxidation than their phenolate ions at alkaline pH. Many phenols are 50% ionized at about pH 10 (20), and at wine pH they would be poor in the phenolate form. In one of the few studies of quantitative oxygen consumption by wines (14) it had been shown that one liter of wine absorbed 30 to 40 ml of oxygen in 12 days at pH 3 and 20°C, but required only 15 minutes to absorb the same amount at pH 10.

Very little was taken up at strongly acid pH.

We conducted experiments on oxygen uptake by small samples (5 ml or less) of various wines in the Warburg apparatus. It was found that even with pure oxygen rather than air and at the maximum temperatures feasible with our equipment (60°C), the consumption of oxygen by wine at its normal pH was not as rapid as is desirable for convenient experimentation. Also, it did not reach even a greatly diminished rate, much less an end point, in experiments as long as a week. It was necessary to sterile-filter the wine, conduct the experiment as aseptically as possible, and examine the sample microscopically after the experiment to prevent erroneous results from microbial growth (both *Acetobacter* and yeasts were encountered) even at 60°C with 14% alcohol and pH 3. With these precautions, worthwhile results were obtained, but our hopes were not realized of developing a convenient analytical technique for determining the maximum oxygen consumption capacity of unmodified wines.

These tests confirmed the expected general relationship between the total phenol content and the absorption of oxygen in that high-phenol red wines consumed more oxygen under the same conditions in a given time than did moderate phenol rosés and rosés more than low phenol white wines. However, within a group of similar wines the correlation was not as clear, again illustrating the importance of differing phenol distribution within the total or perhaps other factors of wine composition. It was found that the consumption of oxygen was catalyzed by light and that CO_2 was released both in the light and in darkness. This shows that the oxidation goes beyond quinoid formation and results in ring scission, if (which remains to be proven) the phenols are the primary source of the CO_2. Figure 1 illustrates a typical experiment.

Since the absorption of oxygen by wine at its normal pH was slow and did not reach an end point in a reasonable length of time, the reaction with oxygen was studied under alkaline conditions. Figure 2 summarizes the reaction with oxygen by equal dry weights of the three purified phenol fractions and the catechin fraction at three pH values. It appears that oxygen reactivity in these preparations decreased in the order of catechin fraction, leucoanthocyanin fraction, and condensed tannin on a weight basis at pH 11; however, the main conclusion is that all consumed considerable oxygen. It was found that a rapid and relatively reproducible oxygen absorption by wines or by phenolic solutions resulted if the measurement followed 20 minutes of reaction in strongly alkaline solution. A bath temperature of 23.5°C was used, and the alkali (1.00 ml of 20% KOH) was added from the sidearm to 1.00 ml of wine or phenol solution in the body of a Warburg flask after flushing with oxygen, equilibrating the flasks, and setting the manometers. Appropriate thermobarometer corrections were made. Initial oxygen absorption was extremely rapid as indicated by the curve for the most alkaline solution in figure 2. After 20 minutes the reaction rate was very much slower.

Although some oxygen absorption often continued for a long time, this was thought not to relate to phenol oxidation, but rather to further reactions such as osone production from carbohydrates in the
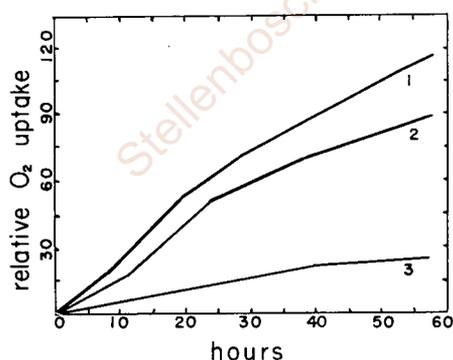
Figure 1. Oxygen absorption of a wine showing the effects of light and carbon dioxide liberation. 1) one ml of wine in the presence of light and with an alkaline wick to remove carbon dioxide. 2) same as 1, except with light absent. 3) same as 1, except with the alkaline wick absent.

strongly alkaline solution. The action of sugar solutions and sweet wines in separate tests agreed with this suggestion (Table 2). The twenty-minute reaction time was chosen to minimize such side reactions as much as possible and yet obtain as complete an endpoint in the reaction from the phenols as possible. Further effort to refine the method has been delayed until the value of this approach could be demonstrated.

Table 1 shows the oxygen taken up by various table wine samples under the alkaline condition described. Whether or not the alkaline uptake would be identical with the grand total of oxygen which could be taken up by a wine under normal acid conditions is not possible to determine from the available data. A typical dry red wine, however, appears to take up about 300 cc O_2/l under the alkaline

condition, and the naturally acidic wine shown in figure 1, for example, was consuming oxygen at nearly the initial rate as it approached 100 cc O_2/l total consumption. It therefore appears that the maximum oxygen consumption is at least of the same order of magnitude under both conditions. These maximum values may seem surprisingly high. It must be kept in mind that they represent an estimate of the total substance capable of reacting with oxygen under drastic conditions. The wine certainly would be completely maderized or sherry-like and spoiled, at least from a table-wine viewpoint, by such levels. These values are, however, about the same as indicated by previous table wine experiments (2, 14) and by oxygen levels used during conversion to baked sherry (5, 11).

Table 1 also shows that, per unit of
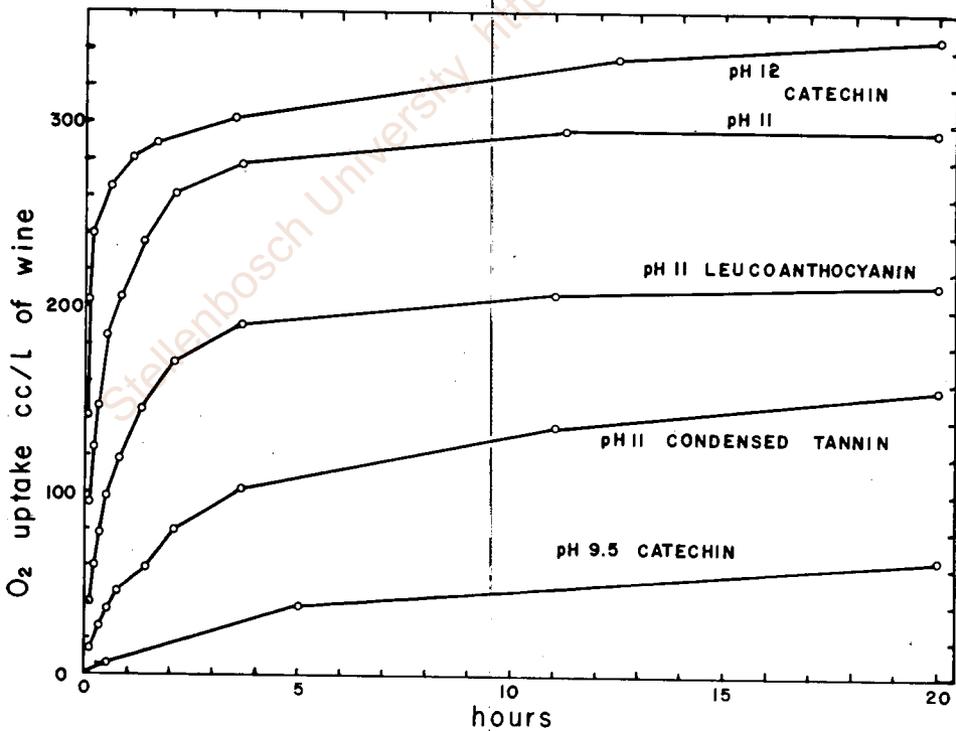
Figure 2. The absorption of oxygen by the three purified phenol fractions (added to wine-like model solution at 2000 mg/l) at pH 11 and by the catechin fraction at pH 9.5 and pH 12 in addition.

phenol by the Folin-Ciocalteu assay, the white wines consumed more oxygen than did the red wines. This may be related to the presence of malvidin in the red wines, which is phenolic but poorly oxidizable, owing to its methoxyl substitution. An alternative hypothesis that, owing to low total phenols, the relative effect of possible nonphenolic oxidation substrates such as SO_2 would be greater, would appear to be ruled out by comparison of samples 7 and 8.

Table 2 shows the oxygen uptake of solutions of the purified phenolic fractions in the white-wine range of total phenol concentrations. The apparent oxygen consumption capacity appeared to be (from greater to least) condensed tannin, leucoanthocyanin, and catechin. The data shown in figure 2 for pH 11 give the reverse order. This difference should not obscure the salient fact that all fractions absorbed important amounts of oxygen. Further work will be required to clarify the relative reactivity of these fractions. Different

preparations of the fractions were used, and differing phenol composition within the fractions could explain the results. Differing reactions in 10% KOH compared to pH 11 may be involved.

Enzymatic browning: On the paper chromatogram of the total phenolic extract from seeds, after being sprayed with the polyphenolase preparation, the three catechin compounds turned brown within seconds, whereas the other phenolic compounds were only faintly tan after 24 hours. The separate phenolic fractions were dissolved in the wine-like solution, and equal quantities of the particulate enzyme preparation were added to the solution. In this manner the amount of brown released into the solution could be measured (Table 3). It was found that very little brown pigment was released into the solution but that the flocculent precipitate which appeared upon addition of the particulate enzyme was dark brown in the catechin solutions and only tan in the leucoanthocyanin and condensed tannin

TABLE I

Oxygen Absorption Under Alkaline Conditions of Dry Table Wines
Compared with the Total Phenolic Assay

Description of the wine	A O₂ cc/l	B F-C mg/l	A/B[a]
1. Deep red, very astringent	597	2840	0.21
2. Deep red, very astringent	363	2270	0.16
3. Deep red, astringent	363	1840	0.20
4. Moderate red, astringent	361	2040	0.18
5. Deep red, slight astringency	335	1770	0.19
6. Dark red, medium astringency	218	1550	0.14
7. Dark rosé, no astringency	72	454	0.16
Average A/B of red table wine			0.18
8. Browned white	91	355	0.26
9. Browned white	93	255	0.36
10. Med. color white	70	262	0.27
11. Med. color white	54	241	0.22
12. Med. color white	72	234	0.31
13. Light color white	52	199	0.26
14. Light color white	49	163	0.30
15. Light color white	46	152	0.30
Average A/B of white table wines			0.30

[a] Values are an average of three or more assays.

solutions. The enzyme preparation also precipitated approximately four times as much of the catechin fraction (by assay difference) from solution when compared to the other phenolic fractions. These experiments indicated that the catechin compounds were the important enzymatic browning substrates and that the action of the enzyme resided mainly on the pulp surface yielding brown products which tended to be retained on the particulate matter.

Nonenzymatic browning: Twenty-five mg/l (dry weight) of the phenolic groups of compounds were added to aliquots of the decolorized white wine in order to study their individual effect on browning. The phenolic wine solutions were then divided into four sets. One set was reserved as the control, one set was sparged with oxygen, one with nitrogen, and the fourth set was subjected to accelerated browning at 125°F for four days. The absorbance of the solutions was determined at 440 mμ. Figure 3 gives the increase in absorbance of the control set over a 39-day period and indicates the catechin fraction to be the most significant contributor to nonenzymatic browning. The condensed tannin and leucoanthocyanin fractions had relatively little effect on the browning.

The sets containing the catechin fraction which were sparged with nitrogen and oxygen were significantly different from the control. The nitrogen-sparged set was
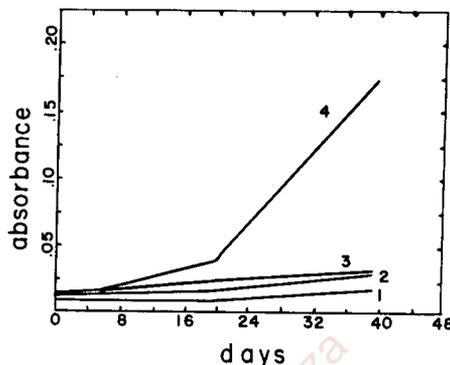
Figure 3. Nonenzymatic browning of the grape seed's phenolic fractions. 1) decolorized wine containing 25 mg/l of gallic acid equivalent. 2) decolorized wine with 25 mg/l of the leucoanthocyanin fraction added. 3) decolorized wine with 25 mg/l of the condensed tannin fraction added. 4) decolorized wine with 25 mg/l of the catechin fraction added. Samples 2-4 were added as gallic acid equivalents.

11% less brown, and the oxygen-sparged set 6% more brown, than the control set after the 39-day period. These results indicate that, although nitrogen and oxygen had the expected effects on browning, their effects were small when compared with the amount of the catechin com-

TABLE 2

Oxygen Absorption under Alkaline Conditions by Phenolic
Fractions from Grape Seeds

Sample	Dry weight[a] (A) mg/l	F-C[b] (B) mg/l	O₂ uptake (C) cc/l	Ratios	
				C/A	C/B
Total phenolic extract	387	245	40	0.10	0.16
Condensed tannin fraction	243	167	39	0.16	0.23
Leucoanthocyanin fraction	210	178	33	0.16	0.19
Catechin fraction	156	163	18	0.12	0.11
Glucose	50,000	....	68	0.0013	....

[a] Concentration by dry weight.

[b] Concentration by Folin-Ciocalteu (gallic acid equivalents).

pounds in solution. This suggests that the amount of oxidation required for a high level of browning is relatively low compared with maximum total uptake, as expected (2). The accelerated browning treatment on set four also increased absorbance more in the catechin samples than in the other samples. However, the absorbance increases in the solutions were not proportional to the room-temperature sets, owing to larger increases in absorbance of the condensed tannin and leucoanthocyanin fractions. The accelerated browning test, based on these results along with those of Caputi and Peterson (4), is not always a good indication of the browning tendency of a white wine.

Based on the often inconsistent comparisons between normal browning and the results of browning tests, as, for example, accelerated browning or absorption at 280 mμ (1), a more accurate method of determining the ability of a wine to brown would lie in a method which would measure the concentration of the catechins in the presence of the other grape phenolics.

One possibility in this direction is the use of the vanillin-sulfuric/Folin-Ciocalteu assay ratio. This ratio has been used to indicate polymerization in the flavonoid series based on the observation that some of the phloroglucinol moieties are masked and unable to react with vanillin and yet are able to react with the Folin reagent, thus lowering the ratio in polymers compared to monomers of the same type. Since caffeic acid and similar derivatives have no phloroglucinol moiety they do not react with vanillin and their van./F-C ratio would be zero.

The control set of samples used in the nonenzymatic browning tests was analyzed with these two methods, and the results are summarized in table 4. The catechin fraction had a higher flavonoid/total phenol ratio than any of the other fractions. The leucoanthocyanin fraction gave a lower ratio and the condensed tannin fraction a greatly lowered ratio, indicating some polymerization in the leucoanthocyanin fraction and the expected greater degree of polymerization in the

TABLE 3

Grape Polyphenoloxidase Effect on the Phenolic Fractions

Sample[a]	Increased absorbance[b]	Absorbance/ F-C $\times$ 10^{-4}[c]	Phenols precipitated (mg/l)[d]
Leucoanthocyanin group			
tube (1)	0.010	.10	392
tube (2)	0.033	.32	358
tube (3)	0.042	.41	358
Catechin group			
tube (1)	0.051	1.8	1318
tube (2)	0.046	1.4	1362
tube (3)	0.042	2.4	1435
Condensed tannin group			
tube (1)	....	......	275
tube (2)	....	......	318
tube (3)	....	......	318

[a] Tubes 1-3 are replications run at the same time.

[b] Final absorbance minus original absorbance read at 440 mμ through a 1-cm cuvette.

[c] Absorbance $\div$ mg/l $\times$ 10^{-4} gallic acid, assayed after filtering off the precipitate.

[d] Amount of phenol removed in the flocculent precipitate (mg/l before adding enzyme minus mg/l after enzyme precipitate removed).

tannin fraction. It appears that a high value of this ratio should prove useful for indicating a wine with a high tendency to brown. A vanillin assay procedure for catechin-derivative determination in wine has recently been proposed by German workers (12, 13). The ratio should be more informative than the vanillin value alone since it would tend to correct for the presence of flavanol polymers. Since catechins appear to be the predominant type of free flavonoid aglycone monomers in wine and the predominant browners, considerable importance can be attached to further study of them.

Compounds such as chlorogenic acid, which can have a synergistic effect in enzymatic browning (9), were added to aliquots of the wine-catechin samples but were found to have no effect. Also, combinations of the different phenolic fractions in the wine gave results comparable to those with their separate presence, indicating no important interaction between groups.

The color of white wines has been assumed to result, at least in part, from the flavonol glycosides, particularly quercitrin. However, Ribéreau-Gayon (15) recently reported that flavonols are not found to any extent in white wines and that, at the present state of knowledge, the compound responsible for the coloration of white wines is unknown. In this study, the pigment formed by oxidation

of the catechin compounds was undistinguishable, both visually and spectrally, from a "normal" white wine and, after a prolonged period, was characteristic of darker white wines, such as sherry.

The results obtained in these studies seem to help explain and to agree with those of other workers. For example, the correlation of browning tendency with the relative proportions of absorbance of white wines near 280 mμ versus that near 325 mμ (1) should relate to flavonoids (catechins particularly) versus chlorogenic acid or similar compounds. A previous study (7) has indicated a browning precursor in white wines whose properties can be interpreted as substantiating the conclusions drawn above. The occasionally opposite effects of usually favorable fining procedures in opposing browning (4) may relate to removing oxygen-consuming, but poorly browning, phenols and leaving a relatively increased proportion of catechins in the remaining mixture.

SUMMARY AND CONCLUSIONS

The reaction of oxygen with wine in its normal acidic condition was in proportion to the total phenol content if the wine samples differed considerably in phenol content, but the reaction was fairly slow and did not reach an end-point in a convenient length of time even at an elevated temperature. Maximum oxygen consump-

TABLE 4

Vanillin and Folin-Ciocalteu Assay of Decolorized Wine with Added Phenolic Groups

Sample	A F-C mg/l[a]	B Vanillin mg/l[b]	B/A
Decolorized wine	24.3	0.3	.01
Added cond. tannin group	55.7	2.0	.04
Added leucoanthocyanin group	52.6	4.2	.08
Added catechin + leuco. group	53.0	4.6	.09
Added catechin group	53.4	5.2	.10

[a] Total phenolics (gallic acid equivalent) determined with the Folin-Ciocalteu procedure using a one-cm light path.

[b] Vanillin value was determined by the procedure of Swain and Hillis (21) using d-catechin as a standard and a 12-mm light path.

tion by wines made very alkaline was rapid, and reproducible values were obtained with a Warburg technique. These values were of the order of 200-600 cc O_2/l for red table wines and 50-100 cc O_2/l for white table wines. The oxygen consumption per unit phenol of wines or different purified phenol fractions under alkaline conditions was not constant, indicating differences related to the relative content of specific phenolic substances. All phenolic fractions tested did, however, absorb considerable oxygen, and white wines took up relatively more oxygen per unit phenol than did red wine.

Studies of both enzymatic and non-enzymatic browning indicated that the catechin fraction was most important in browning. The catechin fraction's oxidation products appear to be capable of accounting for the color of most white wines, whether very light or a dark amber. An analytical technique using the vanillin versus Folin-Ciocalteu assays has also been proposed as a criterion of a wine's tendency to brown.

ACKNOWLEDGMENTS

LITERATURE CITED

1. Berg, H. W. Varietal susceptibility of white wines to browning. I. Ultra-violet absorption of wines. Food Research 18(4):399-406 (1953).

2. Berg, H. W. Varietal susceptibility of white wines to browning. II. Accelerated storage tests. Food Research 18(4):407-410 (1953).

3. Berg, H. W. and M. Akiyoshi. Some factors involved in browning of white wines. Am. J. Enol. Vitic. 7:1-7 (1956).

4. Caputi, A., Jr. and R. G. Peterson. The browning problem in wines. Am. J. Enol. Vitic. 16:9-13 (1965).

5. Crowther, R. F., et al. Variety tests of grapes for wine. Ann. Rept. Hort. Expt. Station and Products Lab. Vineland, Ontario, Canada (1953-54) (1957-58) (1961) (1963).

6. De Villiers, J. P. The control of browning of white table wines. Am. J. Enol. Vitic. 12:25-30 (1961).

7. Fuller, W. L. Browning of white wines. Master's Thesis, U. of Calif., Davis. p. 12-14 (1965).

8. Fuller, W. L. and H. W. Berg. Treatment of white wine with Nylon 66. Am. J. Enol. Vitic. 16:212-218 (1965).

9. Geissman, T. A., ed. The chemistry of flavonoid compounds. p. 529-531, Macmillan Co., New York (1962).

10. Harborne, J. B., ed. Biochemistry of phenolic compounds. p. 15-22, Academic Press, New York (1964).

11. Joslyn, M. A. The chemical changes occurring during the production of sherry flavor in wines. Wine Review 6(9):11-13, 24 (1938).

12. Rebelein, H. Beitrag zur Bestimmung des Catechingehaltes in Wein. Deut. Lebensm. Rundschau 61(6):182-183 (1965).

13. Rebelein, H. Beitrag zum Catechin und Methanolgehalt von Weinen. Deut. Lebensm. Rundschau 61(8):239-240 (1965).

14. Ribéreau-Gayon, J. Contribution à l'étude des oxidations et réductions dans les vins. Delmas, Bordeaux, 2d ed., 213 p. (1933).

15. Ribéreau-Gayon, P. Phenolic compounds of grapes and wine. Wines and Vines 46(5): 26-27 (1965).

16. Rossi, Joseph A., Jr. Studies on the nature of grape phenolics and their role in wines. Thesis, Master of Science in Food Science, University of Calif., Davis, 99 p. (1966).

17. Rossi, J. A., Jr. and V. L. Singleton. Flavor effects and adsorptive properties of purified fractions of grape-seed phenols. Am. J. Enol. Vitic. 17:240-246 (1966).

18. Siegelman, H. W. Detection and identification of polyphenoloxidase substrates in apple and pear skins. Arch. Biochem. Biophys. 56:97-102 (1955).

19. Singleton, V. L. and D. E. Draper. The transfer of polyphenolic compounds from grape seeds into wine. Am. J. Enol. Vitic. 15:34-40 (1964).

20. Singleton, V. L. and J. A. Rossi, Jr., Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic. 16:144-158 (1965).

21. Singleton, V. L., D. E. Draper, and Joseph A. Rossi, Jr. Paper chromatography of phenolic compounds from grapes, particularly seeds, and some variety-ripeness relationships. Am. J. Enol. Vitic. 17:206-217 (1966).

22. Swain, T. and W. S. Hillis. The phenolic constituents of Prunus domestica. I. The quantitative analysis of phenolic constituents. J. Sci. Food Agr. 10:63-68 (1959).

23. Umbreit, W. W., R. H. Burris, and J. F. Stauffer. Manometric techniques. Burgess Publ. Co., Minneapolis, 4th ed. 305 p. (1964).

FLAVOR EFFECTS AND ADSORPTIVE PROPERTIES OF PURIFIED FRACTIONS OF GRAPE-SEED PHENOLS[1]

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The phenolic compounds in wine constitute one of the most important groups of organic constituents affecting wine flavor, but they have received relatively little study from a flavor viewpoint. Without the astringency imparted by the tannins, most red wines would have an inadequate or unbalanced taste. The slight bitterness of some wines has also been assumed to be a property contributed by the phenolic content. A third phenolic effect on wine flavor is an interaction with the sensing of acidity and sweetness. The proper ratio of sugar, acid, and tannin has been shown to be much more important in wine quality than the sugar-acid ratio alone (5). The phenolic constituents decrease the apparent taste of acidity, while sugar decreases the effect of tannin on acidity, and tannin increases the detection threshold for sweetness (2, 3, 4).

Grape seeds are a major contributor to the phenolic content of red wines (8), and since they contain a fairly representative complement of the phenols of the grape berry, with the major exception of the anthocyanins, they have been used as a source of phenolic compounds for this study.

Whole grape seeds release variable amounts and types of phenolic compounds, depending mainly upon the duration, alcohol, and temperature of the fermentation. The extracted phenolics vary not only in type (e.g., catechins, leucoanthocyanins) but in size, ranging from small phenolic acids to flavonoid monomers with a molecular weight of about 300 (catechins) and on to polymeric condensed tannins with a high molecular weight. Owing to the presence of such a diversity of phenolic compounds, which in turn have different (but poorly known) flavor properties, generalizations as to their effects in wine are difficult to make, yet are often made. This research has attempted to evaulate the effects of different classes of phenolic compounds found in grape seeds as to their importance in the flavor of, and taste interactions in, a wine.

The different phenolic fractions have been found, as will be shown, to have different flavor effects and also, as is reported in a companion paper (7), different roles in oxidation and browning. It therefore becomes important to be able to control selectively the content of the different classes of phenolic substances in wine. With this end in view, an investigation was also made into the selective removal of these compounds from wine with appropriate fining agents.

MATERIALS AND METHODS

The total phenolic extract was prepared by extracting freshly separated and ground grape seeds with ethanol. The alcoholic extract was concentrated at low temperature under vacuum and separated into three distinct fractions with a liquid-liquid partition column chromatographic method similar to that of Vuataz and Brandenberger (11) and further modified in this laboratory. The three phenolic fractions separated from the total extract were obtained as an ether fraction, an ethyl acetate fraction, and an ethanol fraction, which were composed of different types of phenolic substances cleanly and completely separated from each other. Characterization of these fractions in detail will be the subject of other reports,

but, based upon solubility, paper chromatographic migration rate, color reactions, and literature comparisons, the three fractions are respectively described as the catechin fraction, the leucoanthocyanin fraction, and the condensed tannin fraction (10).

The total phenolic extract from seeds from mature grapes was usually composed of roughly equal portions of these three phenolic fractions. The catechin fraction contained three different compounds. The leucoanthocyanin group contains five major substances which appear to be very small polymers or monomers and give (as a group) a positive conversion to cyanidin when heated in acid (6, 10). The condensed tannin fraction also gives a high yield of cyanidin upon heating in acid, does not dialyze, and has an average molecular weight (by Mechrolab vapor pressure osmometer) of 5300 (6).

The total phenolic extract and the three fractions were converted to dry solids by low-temperature concentration to an aqueous phase, and lyophilization. The base solutions for comparison of these fractions were prepared either in distilled water, a "synthetic" wine model solution (10% ethanol, 0.5% tartaric acid, 0.15% potassium tartrate, pH 3.2), or in a Thompson Seedless dry white table wine (0.017 g gallic acid equivalent phenol per 100 ml). Two-dimensional paper chromatography with n-butanol-HOAc-H$_2$O, 4:1:5 and 2% HOAc was used to monitor the column chromatography qualitatively. For quantitation, the improved Folin-Ciocalteu phenolic assay (9) was used.

The sensory testing was by the paired-sample technique. Initial experiments to estimate the intensity of the flavor effect of the various fractions in water were conducted with a single taster who had been shown in studies not connected with this work to be neither insensitive nor hypersensitive to the main flavor factors in wine. He was asked to identify the sample to which a flavoring had been added. For each phenolic fraction several different concentrations were tested, and for concentrations near the threshold at least 15 separate judgments were made. Based upon these results, water solutions of the phenolic fractions under study were added to the base wine. These solutions were compared with wine receiving equivalent amounts (not over 20 ml/1) of water only. These samples were judged by a panel consisting of twelve experienced wine sensory panelists. Blind tastings were conducted at 3-5 P.M. for several days. Each judge was given five pairs per tasting, and between 35 and 45 individual judgments were made at each reported level. The judge was asked to indicate which member of each pair was more astringent, and which was more bitter, on the form illustrated in table 1.

Using this report form, the response to any effect on apparent acidity, along with the bitterness and the astringency imparted by the phenolics, could be evaluated separately. Opaque black serving glasses were used to prevent samples from being judged on the basis of the slight color imparted by the phenolic compounds.

For the studies on adsorption, solutions of the phenolic fractions were prepared in synthetic wine. Measured amounts of

TABLE I

Which Sample of the Pair Is:

Pair numbers	More acid	More bitter	More astringent
(2 digit			
random			
code			
numbers)			

standard stock solutions of gelatin, isinglass, or casein were added, and the effect was determined by analyzing the phenol content initially and after removal of the precipitated material. The maximum capacity and selective adsorptive properties of the synthetic resins, PVP[3] and nylon[4], were determined by a frontal analysis with columns packed with one part of the adsorbing resin and three parts by weight of acid-washed Celite 545 diatomaceous earth. The Celite increases the flow rate through the columns but does not affect the resins' abilty to adsorb the phenolic compounds, provided the optimum flow rate of the solution through the column is not exceeded.

RESULTS AND DISCUSSION

Sensory effects of the phenol fractions: Berg et al. (1) found that 0.02 g/100 ml of grape seed tannin could be detected by taste in water. Hinreiner et al. (4) found that grape seed tannin could be detected in red wines at 0.15 g/100 ml and in white wines at 0.10 g/100 ml. The carryover of astringency between samples was previously noted (4), and also caused some difficulties in establishing taste thresholds in this study. Table 2 shows estimations of the absolute thresholds in distilled water obtained in our tests.

Although these data are considered in-

[3] Polyclar AT (insoluble polyvinylpyrrolidone) from General Aniline and Film Corp., New York, N. Y.
[4] Polypenco Nylon (hexamethylenediamine adipate) from the Polymer Corp., Reading, Pa.

TABLE 2

Approximate Absolute Thresholds in Water for
the Phenolic Fractions

Sample	g/100 ml water
Total phenolic extract	.0025
Condensed tannin group	.00035
Leucoanthocyanin group	.002
Catechin group	.002

dicative only, and were used primarily to guide the following panel tests, two points are of interest. The total phenolic extract from grape seeds prepared by our method appears to be roughly ten times as potent from a flavor viewpoint as was the commercial grape seed tannin preparation in the previous reports. The judge's comments indicated that the condensed tannin fraction primarily contributed astringency, the catechin fraction bitterness, and the leucoanthocyanin fraction both bitterness and astringency.

Table 3 is a summary of over 700 individual tastings by the panelists to determine the difference thresholds of the total phenolic extract and its separated fractions in wine with an initial total phenol level of 0.017 g/100 ml.

Based on the panel's response, the significance levels of the tastings were calculated by a "one-tailed" Chi-square test, namely, the only responses accepted as correct were those which followed the panel's consensus judgment and chose the wine containing the added phenolic fraction to be more bitter, or more astringent, or less acid than the reference wine.

Table 3 shows that each of the fractions made important individual contributions to astrigency and/or bitterness, and interacted with the acid taste. Consideration of the relative proportions and flavor potency shows that the flavor effect of the crude total extract can be accounted for satisfactorily by the combination of the three separate groups of phenols. Astringency should not be confused with fundamental tastes such as bitterness, but involves the sense of touch, which is caused by an adsorption of phenolics on proteins at mucous membranes, resulting in a "dry" sensation or roughness.

In many individual tastings the judges were able to differentiate the two pairs of samples consistently but made the wrong choice as to which sample contained the added low level of tannin. This confusion was created by the tannin's effect on lowering the taste of acidity and making the sample seem milder or more dilute. It was also apparent that the judges in many instances picked the sample with the added tannin to be both

astringent and bitter at the same levels. This suggests that in many cases the judges, after noting one characteristic such as bitterness, reasoned that the sample should be astringent also. Since bitterness is usually associated with astringency in wines, this may indicate a bias in the wine judge or it may be related to the carry-over and aftertaste effects of tannin-protein reactions on the epithelium of the mouth.

The condensed tannin fraction is concluded (Table 3) to be, on a unit concentration basis, the most important group in contributing to the astringency, bitterness, and acid-taste interaction in a wine. The leucoanthocyanin and catechin groups are fairly similar in their effect on detectable bitterness and acid-taste interaction. The catechin group did not contribute recognizable astringency at any level likely to be found in wine, but was almost as bitter as the leucoanthocyanin fraction, and more effective in decreasing the apparent acidity. The leucoanthocyanin group gave about 1/10 the bitterness and 1/6 the astringency of an equal concentration of the condensed tannin fraction.

Since red wines have a total phenol level averaging about 0.2 g/100 ml, but as high as 0.5 g/100 ml, it can be seen that, even if only half of the phenols come from the seeds (8), this is 10 to 100 times the minimum detectable levels of these compounds in a white base wine. It is considered to be demonstrated by the data obtained in our studies that all three of the phenolic fractions have significant roles, different in both kind and degree, in red wine flavor. The levels likely in rosé wines still allow for significant effects on flavor, and, depending upon the vinification practices used, white wine flavor may also be affected by the amount and types of phenolics present.

A practical example of the effect of tannin on the taste acidity might be found in press wines. Press wines are generally considered to taste flat or less acid than the free-run wine of the same fermentation, yet the press wine has also been shown to contain more acid. The increased amount of tannin in the pressed wine, along with its taste interaction with acidity, should account for the flat taste.

More work is needed in this area to determine which, if not all, of the individual phenolic compounds within the groups are responsible for the effects on the taste of a wine. The threshold levels, even though in some cases 100-fold more intense than reported previously (2, 4), could be refined even more, and probably lowered by more detailed panel-study methods. Such study will be reserved for individual substances within the larger groups studied here.

Fining and adsorption reactions of the phenol fractions: The removal of phenolic compounds from wines by the addition of gelatin and casein has long been practiced as a means of decreasing the color and astringency of a wine and as an aid in wine clarification. Along with these fining agents, which largely affect the phenolic compounds, newer synthetic resins have been developed which should have somewhat similar adsorptive properties. Of these resins, nylon and PVP have shown

TABLE 3

Difference Threshold Concentrations of Grape Phenols Added to White Wine (in g/100 ml) for Bitterness, Astringency and Acidity

Phenolic fraction	Acidity	Bitterness	Astringency
Total extract	.008*	.008*	.008*
Condensed tannins	.0012*	.0012*	.002*
Leucoanthocyanins	.032*	.012*	.012*
Catechins	.016*	.020*	none

*Significant at the 19:1 confidence limit.

the most promise. The polymeric resins have a protein-like structure similar to casein and gelatin, namely polyamide linkages (Viz.):

$$\left(-\overset{O}{\overset{||}{C}} - \overset{|}{N} -\right)_n$$
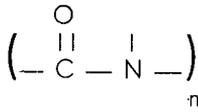
The functional groups of the fining agents interact with the phenolic hydroxyls, adsorbing them and removing the phenols from solution.

Based on the data of tables 4-7, the condensed tannin group is more effectively precipitated from solution than the leucoanthocyanin group, and gelatin is more effective than casein. No differences in the volume of precipitate could be shown when equal quantities of the different fining agents were used. The removal of phenolic compounds in most of the results was found to be more efficient in amount of phenol per unit fining agent at the lower fining agent concentrations. The Freundlich adsorption isotherm predicts this result.

The results with isinglass, a fish gelatin, were not appreciably different from those with ordinary gelatin, and are not shown.

Model-wine solutions of the catechin fractions were also prepared and fined in the same manner as the other phenolic groups, but the catechin compounds did not precipitate with gelatin, isinglass or casein. This was the anticipated result, since gelatin is reported to flocculate only with phenolic compounds of 500 molecular weight and larger. This also agrees with the tasting data in that the catechin fraction was not astringent and both tanning and flavor astringency correlate with increased molecular weight.

All the phenolic compounds were found to be adsorbed to some extent on both the nylon and PVP resins. Solutions of the total extract in synthetic wine were put through the resin columns. The effluent fractions from the column were chromatographed two-dimensionally on paper to determine which of the phenolic compounds in the extract were being held preferentially by the resin. The results of this breakthrough type of analysis showed all the leucoanthocyanin fraction and one of the compounds of the catechin fraction to be the most tightly adsorbed by both resins. The condensed tannins were adsorbed the least by the resins. The two remaining catechin compounds were adsorbed slightly more than the condensed tannins.

The qualitative paper chromatographic

TABLE 4

Gelatin Fining of the Condensed Tannin Fraction from Synthetic Wine[a]

Amount of gelatin added (mg/l)	Phenol content[b] (mg gallic acid/l)	Wt. of phenol removed ÷ Wt. of fining agent added
Control	287	......
25	256	1.26
50	228	1.17
100	173	1.14
200	83	1.02

[a] The phenolic content was entirely the condensed tannin fraction.

[b] Assayed after removing the precipitate.

TABLE 5

Gelatin Fining of the Leucoanthocyanin Fraction from Synthetic Wine[a]

Amount of gelatin added (mg/l)	Phenol content[b] (mg gallic acid/l)	Wt. of phenol removed ÷ Wt. of fining agent added
Control	748	......
25	720	1.12
50	698	1.10
100	662	0.86
200	598	0.77

[a] The phenolic content was entirely the leucoanthocyanin fraction.

[b] Assayed after removing the precipitate.

method of analysis was not the only means of evaluation, since polyamide resins were found in separate studies to degrade some phenolic compounds partially. In order to determine the quantitative affinity of the resins for the phenolic fractions, individual fractions of the phenolics in synthetic wine were put through the resin columns until the resin was saturated (i.e., effluent from the column contained the same phenolic content as the initial solution).

Using the Folin-Ciocalteu analysis (9) to assay the column effluents, calculations could be made to determine the capacity of the resin for the separate phenolic fractions. The results are summarized in table 8.

The quantitative capacities of the resins for the phenolic fractions (Table 8) agree with the qualitative results obtained with two-dimensional paper chromatography. The resins have a great affinity for the leucoanthocyanin fraction, followed by a lesser affinity for the catechin fraction and only a relatively weak affinity for the large condensed tannins. These results, along with those of the protein fining agents, show a considerable selectivity in affinity for the different types of phenolic compounds. They also indicate the effectiveness of the types of fining agent in comparison with each other; for example, PVP was 2.5 times as effective as nylon in

adsorbing the catechin fraction from solution. The results of these fining tests should enable a better understanding and control over the effects of the different types of phenolic compounds.

SUMMARY

Grape seed phenolics were extracted and separated into three distinct groups of compounds. The separated fractions were evaluated in turn for their sensory effects in water and in wine with respect to their thresholds for astringency, bitterness, and effect on taste interaction with acidity. It was found that the three frac-

TABLE 7

Casein Fining of the Leucoanthocyanin Fraction from Synthetic Wine[a]

Amount of casein added (mg/l)	Phenol content[b] (mg gallic acid/l)	Wt. of phenol removed ÷ Wt. of fining agent added
Control	916	------
150 ppm	796	0.80
300 ppm	734	0.61

[a] The phenolic content was entirely the leucoanthocyanin fraction.

[b] Assayed after removing the precipitate.

TABLE 6

Casein Fining of the Condensed Tannin Fraction from Synthetic Wine[a]

Amount of casein added (mg/l)	Phenol content[b] (mg gallic acid/l)	Wt. of phenol removed ÷ Wt. of fining agent added
Control	454	------
150 ppm	335	0.79
300 ppm	195	0.86

[a] The phenolic content was entirely the condensed tannin fraction.

[b] Assayed after removing the precipitate.

TABLE 8

Capacities of Nylon and PVP for Grape Seed Phenolic Fractions

Phenolic fractions	Resins	
	Nylon[a]	PVP[a]
Condensed tannins	2.8%	1.5%
Leucoanthocyanins	10.5%	25.0%
Catechins	7.5%	19.0%

[a] g of gallic acid equivalent per 100 g of resin.

tions have considerably different flavor effects, both qualitatively and quantitatively. The catechin fraction was bitter but not astringent at levels likely to occur in wine. The leucoanthocyanin fraction and the condensed tannin fraction were both bitter and astringent. All three affected the judges' reaction to acidity by moderating the apparent sour taste when added at levels near threshold. All three fractions appear capable of important effects on these quality factors at levels which may occur in white wines and certainly do occur in rosé and red wines. The condensed tannin fraction contributed the most intense flavor effects on a weight basis, and the evident flavor effects of the phenolic substances in wine were found to be 10 to 100 times as intense as previously noted. The levels present in a red wine are about 10 to 100 times the level barely detectable in a white wine.

A comparison was made of the adsorptive properties of casein, gelatin, isinglass, nylon, and insoluble polyvinylpyrrolidone with respect to these phenolic fractions. Large differences were found in relative affinity for the different phenolic fractions. The PVP had a greater capacity in phenol per unit weight than did nylon, and the proteins were greater yet. The synthetic resins had a greater affinity for the smaller molecules than for the condensed tannins, and the proteins were the reverse, under the conditions tested. The resins had considerable affinity for all the fractions in column tests, but, as fining agents, the proteins showed no affinity for the catechin fraction under our conditions.

ACKNOWLEDGMENTS

LITERATURE CITED

1. Berg, H. W., F. Filipello, E. Hinreiner, and A. D. Webb. Evaluation of thresholds and minimum difference concentrations for various constituents of wines. I. Water solutions of pure substances. Food Technol. 9(1):23-26 (1955).

2. Berg, H. W., F. Filipello, E. Hinreiner, and A. D. Webb. Evaluation of threshold and minimum difference concentrations for various constituents in wines. III. Sweetness, the effect of ethyl alcohol, organic acids, and tannin. Food Technol. 9:138-140 (1955).

3. Hinreiner, E., F. Filipello, A. D. Webb, and H. W. Berg. Evaluations of threshholds and minimum difference concentrations for various constituents of wines. III. Ethyl alcohol, glycerol and acidity in aqueous solution. Food Technol. 9(7):351-353 (1955).

4. Hinreiner, E., F. Filipello, H. W. Berg., and A. D. Webb. Evaluation of thresholds and minimum difference concentrations for various constituents of wines. IV. Detectable differences in wines. Food Technol. 9(10):489-490 (1955).

5. Joslyn, M. A. and J. L. Goldstein. Astringency of fruits and fruit products in relation to phenolic content. Adv. Food Res. 13:179-217 (1964).

6. Rossi, J. A., Jr. Studies on the nature of grape phenolics and their role in wines. Thesis, Master of Science in Food Science, University of California, Davis. 99 p. (1966).

7. Rossi, J. A., Jr. and V. L. Singleton. Contributions of grape phenols to oxygen absorption and browning in wines. Am. J. Enol. Vitic. 17:231-239 (1966).

8. Singleton, V. L. and D. E. Draper. The transfer of phenolic compounds from grape seeds into wines. Am. J. Enol. Vitic. 15:34-40 (1964).

9. Singleton, V. L. and J. A. Rossi, Jr. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic reagents. Am. J. Enol. Vitic. 16:144-158 (1965).

10. Singleton, V. L., D. E. Draper, and J. A. Rossi, Jr. Paper chromatography of phenolic compounds from grapes, particularly seeds, and some variety-ripeness relationships. Am. J. Enol. Vitic. 17:206-217 (1966).

11. Vuataz, L. and H. Brandenberger. Plant phenols. III. Separation of fermented and black tea polyphenols by cellulose column chromatography. J. Chromatog. 5:17-31 (1961).

Toxicity and Related Physiological Activity of Phenolic Substances of Plant Origin

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The toxicity to animals of plant phenols is reviewed; particularly, hydroquinone, salicylic acid, coumarin, dicoumarol, safrole, myristicin, urushiol, phloridzin, tangeretin, hypericin, psoralen, gossypol, rhein, sennoside, tetrahydrocannabinol, tremetone, dihydromethysticin, podophyllotoxin, rotenone, and tannin. General correlations and evolutionary significance are discussed. Phenols appear generally toxic if natural barriers or detoxification mechanisms are overloaded by amount, circumvented by the manner of administration, or foiled by uncommon compounds such as methylene diethers or isoprenoid structures. Frequently manifested features of phenol toxicity include synergism, bonding with body polymers, interference with metabolism of normal phenols (catecholamines, tyrosine, vitamin K), and involvement of the skin and liver.

It is rather difficult, perhaps impossible at this stage of our knowledge, to pull together into a satisfactorily coherent whole a subject as diverse and impinging upon as many disciplines as that indicated by the title of this report. It seems desirable, however, to avoid as far as possible the mere tabulation of a series of curiously toxic plant phenols. "Phenol chemistry" in living systems has not been as clearly organized as, for example, carbohydrate chemistry. Greater unification in understanding of the chemistry of natural phenols is finally emerging and perhaps toxicity and related physiological activity can serve as one key to furthering this trend.

The authors discuss the subject from several different aspects and, since complete literature coverage is impossible in the space available, cite primarily the most authoritative, provocative, or recent pertinent reports. The plant phenols to be considered do not include nitrogen-containing substances or triterperoids such as saponins or steroids, since the authors wish to focus on the compounds which owe their natural presence and perhaps their physiological effects to their phenolic nature. For convenience, the phenolic products of microorganisms and other lower plants have been excluded. On the other hand, substances are included which can be considered as derived from phenols such as chromones or coumarins whether or not they have additional free hydroxyls. Since toxicity is often manifested when "unusual" plant products are contacted by man or animals through ignorance or restricted access to normal food, discussion has not been limited to phenols likely to be present in foods or feeds.

Animals make very little direct use of the many phenolic substances of plants, with some outstanding exceptions such as tyrosine, the vitamin E tocopherols, the vitamin K naphthoquinones, and the ubiquinone benzoquinones. The physiological activity of these substances in a vitamin or desirable nutrient sense is outside our concern here. For the same reason discussion of the bioflavonoids (De-Eds, 1968) has been sharply limited.

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Phenolic substances are produced in animals for a few key functions, notably the catechol amines and phenolic indole amines involved in nerve action and associated effects, the tyrosine-dopa derivatives involved in melanin pigment formation, the phenolic steroidal estrogens, and the tyrosine of proteins. While the production of these compounds ultimately depends upon transformation of a benzenoid plant product, animals do not normally encounter these substances in ways which will disrupt their metabolism. It would seem evolutionarily essential that substances as potent in the bloodstream as norepinephrine or serotonin would not be readily taken up in active form from food plants which can contain them. Considering that one nettle sting may contain 10 times the 5-hydroxy-tryptamine of a bee sting and that 1 gram of banana may equal, in this particular regard, 50,000 bee stings (Ramwell *et al.*, 1964), it is fortunate that few plants are adapted for parenteral administration of toxins.

It would be reasonable to seek interference by "plant" phenols in the normal functions of phenols used by animals as a source of toxicity. However, potent analogs or inhibitors of essential "animal" phenols must be rare in common food plants, or animals must have effective "detoxification" mechanisms for common plant phenols. In the sense that there is no necessity to metabolize many plant phenols at all except to render them less toxic or hasten their excretion, detoxification is probably the proper term. However, as long as the detoxification system is efficient and not overloaded, toxic symptoms may never appear after ingestion of a potentially toxic phenol. Since many plant phenols are excreted partly unchanged, complete "detoxification" seems often unnecessary.

In contrast to the few phenols common in animals, plants produce many different phenols, vary them considerably plant to plant, and may contain them in small or very large amounts. A file based originally upon tabulations by Geissman and Hinreiner (1952) and by Karrer (1958) now lists about 800 different phenolic substances which fall in the group being discussed, not counting simple glycosidic variation, and is certainly not complete. If one selects from this group the phenols which are most

frequently present in plants as shown by chemotaxonomic examination (Bate-Smith, 1965, 1968; Bate-Smith and Swain, 1965; Swain, 1965), a familiar list of about 25 phenolics is almost universal in animal diets derived from plants. These include p-coumaric, caffeic, ferulic, sinapic, and gallic acids, and the common flavonoids with analogous variations of 5, 7, 3', 4', 5'-hydroxyl or 3', 5'-methoxy substitutions in the anthocyanidin, catechin, leucoanthocyanidin, flavonol, and flavone series. Ellagic acid and a few depsides and other derivatives would round out the list.

If, on the other hand, a list is prepared of the phenolic substances which have significant medical or toxic effects in animals and they fit our other restrictions (Stecher, 1968), about 150 are found. There is little overlap between the lists. None occurs if one eliminates the substances with reputed favorable effects such as the bioflavonoids and the nonspecific bitters and astringents and retains only those few dozen phenol-plant combinations which seem most significant from a toxicity viewpoint. Of course, a large portion of the total number of phenols reported from plants appear on neither the common nor the toxic list. Many of these have not been investigated from a toxicity viewpoint. A major factor shaping the list of the plant-phenol combinations with toxicity considered significant is a fortuitous combination of circumstances. If cotton had not been commercialized for its fiber, the toxicity of gossypol might have remained unknown and certainly would not have been as important. The practical toxicity of a phenol is also related to the amount present and the amount present is not necessarily related to the phenol's frequency of occurrence. Caffeic acid derivatives are commonly present but seldom exceed 5%; true tannin may range from 0 to over 30% of the dry weight in different plants or plant parts. Uncommon phenols are often present in their particularly notorious source in high amounts.

The plants which produce notably toxic phenols are often botanically isolated, divergent, or advanced. The unusual phenol's botanical distribution is a further clue, along with morphology and other composition, to the taxonomic relationships. Specific toxic phenols and phenols with closely related structures are often confined to certain families and not infrequently to certain genera, certain species, or even to certain varieties at certain times. Phenols are considered secondary substances because, except for tyrosine, they do not seem to be essential for life, at least at the cellular level (Neish, 1960; Ramwell et al., 1964). This concept is useful in that it helps explain why plants have been free to develop genetically controlled, chemotaxonomically significant, but highly variable qualitative differences between families. Similarly, the plant is relatively free to respond to environmental and physiological changes by quantitative variation in phenol synthesis. This "secondary" status of phenols, however, should not obscure the fact that they may have important roles in plants partly because they have been free to evolve and to adapt to environment.

Toxicity may, in fact, be a *function* of phenols in plants. There are examples of varieties of a plant species being more or less resistant to pests or pathogens depending upon their content of natural tannin or other phenols (Cruickshank and Perrin, 1964; Goodman et al., 1967; Herrmann, 1962; Kuć, 1966; Pridham, 1960; Rich, 1963). Attack by pathogens may be successfully resisted

by rapid production of higher than normal, inhibitory concentrations of phenols. In a few proved cases phytoalexins, so far mostly phenols, specifically inhibitory to plant pathogens are produced in response to attack (Goodman et al., 1967; Cruickshank, 1963). The effect which seems most significant, however, is the apparent employment of localized synthesis of high levels of usual phenols to kill certain plant cells themselves. According to this theory, which seems to have been demonstrated in some instances of hypersensitivity at least, the attack by a would-be parasitic organism triggers the production of chlorogenic acid or other phenol so rapidly and to such a high level that the plant cells quickly die and collapse ahead of the invading organism. A local necrotic spot develops which is walled off, and the would-be parasite dies or is confined to a saprophytic existence in the dead tissue while the rest of the plant remains relatively unaffected. Still other effects of plant phenols which might be interpreted as self-toxicity include the natural inhibitors of seed germination such as coumarins which must be leached or destroyed during a dormancy period before the seed will sprout (Berrie et al., 1968; Pridham, 1960). The ecological relationships which influence competition between root systems of adjacent plants and between roots and soil microflora seem to involve inhibitory effects of lignin degradation products and other plant phenols released into the soil (Flaig, 1967; Hennequin et al., 1967; Kefeli and Turetskaya, 1967; Wang et al., 1967).

The action of the simpler plant and synthetic phenols as antiseptics—i.e., their antibacterial effects as well as animal toxicity—is the most thoroughly studied aspect of phenol toxicology (Von Oettingen, 1949). Essentially every phenolic substance has some antibacterial properties (Jenkins et al., 1957). This generally holds true for both usual and unusual plant phenols although the activity may be of very low order. Commonly, a second hydroxyl group reduces the antibacterial activity. Introduction of alkyl substituents on the ring tends to reduce the animal toxicity and increase the antibacterial effect. Etherification generally decreases toxicity. In fact, animal detoxification of phenols may involve methylation of phenols as well as ethereal sulfate or glucuronoside conjugation (Booth, 1961; Fairbairn, 1959; Williams, 1959). The major point, however, is that phenols, including those of the plant type, are actually broad spectrum, if often weak, toxins to plants, microorganisms, and animals. At high levels even "desirable" phenols may be toxic (National Academy of Sciences, 1966). For example, tyrosine at 5% in a low protein diet for rats produces toxic symptoms in a few days (Bocter and Harper, 1968).

Any phenol may be toxic to any organism under some form of administration. Unadorned, this is basically a naive statement because the same might be said about water. It is true, however, of phenols at moderate levels and under relatively mild conditions of administration. The toxicity of a given phenolic molecule may be related to its reactivity in a nonspecific chemical sense, for example, membrane damaging activity by solvent power of phenol or by acidity of salicyclic acid. It may be related to acute interference with normal biochemical functions of a somewhat general nature such as uncoupling of oxidative phosphorylation, a capability of many phenols. Acute animal toxicity of certain phenols may devolve from interference with catecholamine metabolism and other nerve control mechanisms to produce hallucination, spasm, or convul-

sions. Chronic or long term animal toxicity may take the form of interference with vital substances such as vitamin E, vitamin K, and estrogens. It may be manifested as carcinogenic activity or liver damage of a limited degree of specificity. In any specific instance, operative toxic mechanisms of phenols are often speculative, but phenols do have several toxic capabilities. The particular effect of a given phenol may depend upon a particular stereochemistry and the phenolic hydroxyl may or may not be important. However, phenols have the possibility for more generalized toxicity, and differences in physiological effect may often relate to structure "ornamentation" which confers lipid solubility, prevents or delays detoxification, breaks down into toxic fragments after penetration to a vulnerable site, etc. In a series of substituted phenols, complex mathematical values relating molecular structure, ionizability, and relative lipophilic-hydrophilic character have been linearly correlated with relative toxicity to plants, relative toxicity to bacteria, and activity in uncoupling oxidative phosphorylation (Fujita, 1966).

Common food plants have a limited array of almost universally occurring phenols. Appreciable negative physiological activity of these substances would be surprising. Animals should have evolved means of tolerating them through frequent contact. This appears to be true; the phenols commonly present in plant foods are free of toxicity (Bate-Smith, 1954; Fairbairn, 1959). In view of the general toxic potential of phenols perhaps it would be better to say they are readily eliminated or detoxified by animals. To discuss detoxification mechanisms (Booth, 1961; Fairbairn, 1959; Ramwell et al., 1964; Williams, 1959) would take us too far afield, but since the flavonoids are commonly metabolized in animals to separate phloroglucinol and B-ring derivatives and since the toxic effect of these fragments is fairly well known, probable toxicities sometimes can be predicted. For example, since d-catechin, cyanidin, and mavidin are evidently nontoxic, the flavan-3-ol analog of malvidin (which is not known in nature) should be nontoxic.

Conversely, a plant phenol with unusual structural features is more likely to be physiologically active or toxic in animals. Particularly this is true if the unusual structure is such that it would interfere with or prevent the normal detoxification mechanisms or produce toxic fragments. Thus the presence of phenols in all plants and some unusual phenols in a few plants, would appear to have dietary and evolutionary significance when contrasted with, say, the alkaloids which are absent in most food plants and appear generally to be more toxic and not as easily handled by animals in their diet.

The idea that the unusual phenols of plants are more likely to be toxic because animals have had less time to evolve mechanisms to cope with them assumes that the unusual phenol is a relatively recent mutation. That this may not be true is suggested by the fact that the ginkgo tree, redwoods, cycads, and other "living fossils" tend to be "unusual" in their chemical makeup including phenols. One wonders if seed dispersal and other factors have given a competitive advantage in evolutionary ecology to those plants "pleasing" to animals. Certainly man's influence would have been in that direction even prior to development of agriculture. In a less deliberate sense, animals other than man and birds would distribute seeds in droppings and otherwise aid dissemination of plants which they frequent.

That the phenol content of plants influences selection by animals of their food cannot be doubted. Bate-Smith (Fairbairn, 1959) has emphasized that plant products chosen for exploitation as food are not only edible but also attractive, substantial, and convenient. Leafy foods consumed directly by man are low in lignin, therefore less tough. This choice tends to confine such foods to herbaceous plants with their associated constellation of phenols, notably a low content of condensed tannins, leucoanthocyanidins. Woody plants are a main source of fruit and the fruits are the only parts of such plants commonly eaten. Tannins may be high in the fruit and astringency, bitterness, color, and texture, which are all phenol related, will influence dietary choice among fruits or timing of harvest for a given crop. Certainly a trial food which produced even transiently uncomfortable effects or illness would be shunned as soon as the connection was realized if a choice was possible.

The normal intake level of phenols is quite variable according to diet but may be larger than is commonly recognized. Carnivores would ordinarily have a very low intake of phenols other than tyrosine. Omnivorous animals, like man, may consume considerable amounts of phenols depending upon the food they select or to which they are restricted. For example, red table wines contain about 1500 mg. per liter of total phenols and account for an annual per capita consumption of about 150 grams of "tannin" in some societies. Individuals are known to consume as high as 1000 grams per year from this source. For comparison, the minimum total essential amino acids for adult nitrogen balance is only about 7000 grams per year. Most fruits, some vegetables, and other plant-derived beverages such as tea are often relatively high contributors to the amount of phenols in the human diet.

Herbivorous animals consume massive amounts of phenols. If lignin is considered, phenols are probably second only to carbohydrates as dietary constituents and may approach 20% of the dry weight of the entire food intake. Although lignin is usually considered metabolically inert, recovery from feces and other tests of "digestibility" indicate often about 10% and sometimes more than half of the lignin fed is solubilized and may be absorbed in ruminants, rabbits, etc. (C.S.I.R.O., 1957; Lenz and Schürch, 1967). The lignin exiting from the gut is chemically different, generally with a lower carbon, hydrogen, and methoxyl content. The content of benzenoid compounds in the urine increases when purified lignin is fed even to dogs. Whether any of the lignin furnishes useful energy to the animal or whether the changes are dependent upon a particular microflora in the alimentary tract are beside the point that a sizable portion of the large amount of phenolic material passing through the animal may be solubilized and absorbed. Of course, lignin fragments and associated phenols as well as most of the flavonoids and other nonpolymeric phenols are able to pass animal membranes and can be absorbed from food.

Before considering some of the interesting specific examples of the toxicity of plant phenols to animals in more detail, let us compare some values for acute toxicities (Table I) (Jenner et al., 1964; Spector, 1956; Stecher, 1968). Such toxicity values are subject to considerable variation depending on the conditions of the experiments and can only be grossly compared when separate studies are involved. However a few interesting relationships seem to be evident. A few substances outside the group

being discussed are included for comparison. Firstly, the acute toxicity to rats of most plant phenols or similar synthetic phenols is rated as slight (LD_{50} in a single oral dose, 500 to 5000 mg. per kg.). A few are moderately toxic (LD_{50} oral, 50 to 500 mg. per kg.), but only a very few approach the highly toxic level (LD_{50} oral, 1 to 50 mg. per kg.), and none is extremely toxic (1 mg. or less per kg.) (Spector, 1956). The estimated comparable amounts for lethal doses in man would be about 250 grams for slightly toxic substances, 30 grams for moderately toxic, and about 3 grams for highly toxic substances. Most phenols are more acutely toxic than ethanol, man's most common intoxicant, much less toxic than strychnine or the "animal" phenols epinephrine or 5-hydroxytryptamine, and about in the range of toxicity of many potent medicinal substances.

The reputations of individual substances for toxicity appears considerably distorted by their individual frames of reference. Toxic phenols have tended to be considered on an individual, ad hoc, or curiosity basis. Rotenone as an insecticide or fish poison is noted for low toxicity to warm-blooded animals. Relative to its toxicity to cold-blooded animals and evidently to other insecticides this is correct, but it is still relatively toxic among the plant phenols for all animals. A synthetic vitamin K is about as toxic on an acute basis as other phenol derivatives some of which are banned from foods. Rutin, a bioflavonoid which is usually considered nontoxic and perhaps a desirable dietary constituent, appears nearly as acutely toxic as dicoumarol, which is noted for slow poisoning. The listed toxicity for quercetin seems unexpectedly high. More recent studies indicate that rutin produces an acute LD_{50} intraperitoneally to rats of 9110 mg. per kg. and no effects when administered orally for 3 months at 850 mg. per kg. per day (Radouco-Thomas et al., 1965). Toxicity in the form of eye cataracts results from contamination of rutin (Nakagawa et al., 1965), and other such problems may produce excessively high toxicity in individual instances. In general, flavonoids of the common types show no acute toxicity at oral doses of about 500 mg. per kg. and intraperitoneal doses of about 50 mg. per kg. (Böhm, 1960). Owing perhaps to the amounts of material required and the costs of obtaining the substances, relatively few studies of lethal oral levels of flavonoids have been reported.

Since microorganisms may not only modify phenols but may destroy them entirely, the microflora of the alimentary tract may considerably reduce the apparent toxicity of orally administered phenols. This would be particularly true of animals adapted to plant diets such as rabbits and horses. Ruminants should particularly profit from this form of assistance considering not only the extensive plant-adapted rumen microflora but also the extra fermentation time allowed in their system. Ordinarily the LD_{50} for rats or rabbits dosed intravenously is about one-tenth the LD_{50} dose orally (Spector, 1956). When the oral toxicity is less than this in proportion to the intravenous toxicity, either greater than usual barriers to absorption into the bloodstream or destruction in the gut, presumably by microorganisms, can be hypothesized. Some phenols fit this one-tenth "rule" and some do not. The high intravenous toxicity for epinephrine and tannin (Table I) are noteworthy. Conversely, some substances seem less toxic parenterally than would be expected from the oral data (eugenol, safrole), presumably owing to lipoid solubilization in the digestive tract.

Table I. Some Approximate Minimum Lethal Acute Toxicities[a] (Mg./Kg. Body Weight; Rat, Oral Except as Noted)

Substance	Toxicity		
Phenol	530	S.c.	400
o-Cresol	1350	S.c.	650
m-Cresol	2020	S.c.	900
p-Cresol	1800	S.c.	500
Anisole	3700	I.p.	500
Catechol	3890	S.c. (rabbit)	225
Guaiacol I.v. (rabbit)	3.7	S.c.	900
Vanillin	1580		
Resorcinol	450		
Hydroquinone	320		
Phlorglucinol		S.c.	1550
Pyrogallol (rabbit)	1100	S.c. (rat)	650
Gallic acid		S.c.	5000
Methyl salicylate	887		
Coumarin	680		
Dihydrocoumarin	1460		
Carvacrol	810		
Thymol	980	S.c.	1650
Anethole	2090	I.p.	70
Eugenol	2680	I.p.	900
Isoeugenol	1340		
Safrole (rabbit)	1950	I.v.	200
Dihydrosafrole	2260		
Methylenedioxy-benzene	580		
β-Naphthol		S.c.	2940
Cyclohexanol	2060		
Ethanol	13660	I.p.	5000
Chlortetracycline	3000	I.v.	118
Menadione (mice)	1000		
Serotonin		I.v.	30
Epinephrine	30	S.c.	7
	I.p. 10	I.v.	0.005
Strychnine	16		
Quercetin (mice)	160	S.c.	100
Rutin (mice)		I.v.	950
Lupulone	1800	I.m.	330
Tannic acid (mice)	6000	S.c.	200
		I.v.	80
Rotenone	133	I.p.	5
Dihydrorotenone	330–2500		
Dicoumarol	542	I.v.	52
Gossypolacetate (cat)		I.v.	75
Podophyllotoxin (mice)	90		
Cotoin S.c. (frog)	8		

[a] For clarity, no distinction has been made between LD_{50} (most of the values) and other estimates of typical or minimum lethal doses. S.c. = Subcutaneous, I.p. = intraperitoneal, I.v. = intravenous and I.m. = intramuscular administration.

Too few data were found for very firm conclusions, but a possible relationship between increasing toxicity of phenols to more strictly carnivorous animals can be inferred from acute toxicity data (Jenner et al., 1964; Spector, 1956; Stecher, 1968; Von Oettingen, 1949). The cat seems most susceptible, the dog nearly so, the rat or mouse intermediate, and the rabbit or guinea pig more tolerant. This is not just the effect of internal microbes since parenteral administration tends to show the same relationship. For example, phenol itself injected subcutaneously gave average fatal dose values of 85 mg. per kg. in the cat, 270 in the dog, 290 in the mouse, 300 in the frog, 385 in the rat, 470 in the guinea pig, and 620 mg. per kg. in the rabbit (Von Oettingen, 1949). Admittedly these are highly selected values, but they and many other less extensive comparisons do suggest some lesser resistance of carnivores to phenols of the plant types and may help explain some of the rather large differences in susceptibility to

phenols frequently noted between animal species. The high susceptibility of cold blooded animals like the frog to certain phenols does not carry over to all other phenols. Animal scientists are well aware of differences between domestic animals or even between breeds in susceptibility to toxic reaction from various forages, etc., but often these have not been clarified from a chemical or biochemical viewpoint.

Toxicity in a practical sense is much more involved than is indicated by simple acute lethal potency, of course, and there seems to be no completely satisfactory way to systemize the heterogeneous data available for a brief presentation. A botanical origin, pharmacological effect, biosynthetic relation, or chemical structure approach becomes cumbersome if made complete or adhered to rigidly. Probably selection of examples will be more useful than too great an effort to systemize the data available. However, all systems reach the same focus because a certain uncommon molecular feature produces a certain physiological effect and only certain plants can synthesize phenols with that feature.

Hydroquinone's para dihydroxylation is relatively unusual in plant phenols and is responsible for one type of fatal forage poisoning of livestock which has had considerable local economic impact. Cocklebur (*Xanthium* spp.) seeds contain toxin shown to be hydroquinone and although the spiny burrs are seldom eaten, seedlings still in the decotyledon stage retain toxic levels of the poison (Kingsbury, 1964). The seeds (separated from the burrs) are toxic at about 3000 mg. per kg. of body weight and the symptoms of anorexia, nausea, muscular weakness, convulsions, and death in about three days may be reproduced exactly by hydroquinone administration. All farm animals are susceptible except that poultry present few symptoms other than profound depression. Arbutin, hydroquinone glucoside, occurs in pear tree parts other than the fruit, but does not present a significant hazard. At one time arbutin, probably from bearberry, was used as an orally administered urinary antiseptic. Administration with tannin made it more effective by inhibiting β-glucosidase action on it in the digestive tract (Stecher, 1968).

Salicylic acid is the "plant" phenol responsible for the most actual deaths in humans, primarily through overdose of aspirin by children or suicides. About 200 deaths annually occur in England and Wales from this cause (Dixon *et al.*, 1963). Of course, poisonings from direct consumption of plant material bearing salicylates or related substances like salicin are not a real problem. Although the medical value of these compounds was learned through use of willow bark (*Salix* sp.) and these and *Populus* species produce many chemically interesting variations (Thieme, 1967), we do not usually think of salicylates as plant phenols. The physiological effects of salicylates have been studied in detail (Dixon *et al.*, 1963; Smith and Smith, 1966).

The acute toxic effects often are more related to the strongly acid carboxylic function and effects on acid-base balance than specifically to the phenolic nature. The hydroxyl substitution ortho to the carboxyl is, however, relatively rare in plants and it is significant that this uncommon substitution pattern is involved in these drugs. Vitamin K is helpful in treating salicylate poisoning.

Methyl salicylate exhibits analgesic properties when rubbed on the skin indicating penetration by that route.

The efficacy of the methyl ester and the acetate in aiding penetration to a desired cite of action without the strongly irritant effect of free salicylic acid indicates the importance of such factors as blocking groups or lipid solubility on producing different physiological symptoms. Salicylates bind to albumin in the blood and are no longer dialyzable (Moran and Walker, 1968). A strong ionic binding site with pK 8.5 to 9.0 is indicated plus weak sites which are three times as numerous. The mechanism of pain suppression by aspirin may be inhibition of oxidative phosphorylation at specific pain receptor sites (Smith and Smith, 1966).

Salicylic acid is now generally not permitted as a food additive; however, as wintergreen flavoring, methyl salicylate is still used. Chronic toxicity studies for period up to two years have shown no effect in rats fed 1000 p.p.m. of methyl salicylate in their diet or dogs given daily oral doses of 50 mg. per kg. (Hagan *et al.*, 1967). At higher dosages, liver fatty degeneration and weight loss or growth retardation were primary symptoms. With 0.3% acetylsalicylate in the diet of chicks, the growth rate was not significantly affected and anaphylactic response to bovine protein was reduced (Morgan and Glick, 1968).

Coumarin (I) is an ortho hydroxy derivative and might be considered as related to salicylic acid. However, it is also an α-β unsaturated lactone, a structure often physiologically potent. Since it is detoxified in animals by hydroxylation in the 3-, 7-, 8-, or sometimes 5-positions, it would be expected to be more toxic than the usual more phenolic coumarins (Williams, 1959). Open-ring homologs of salicylic acid occur among animal metabolites of coumarin, but analgesic activity of coumarins is doubted (Booth *et al.*, 1959; Soine, 1964). In plants, coumarin and some of its derivatives have inhibitory and regulatory interrelationships with plant hormones; and hydrogenation of the nonaromatic double bond considerably affects activity, at least in certain competitive reactions with gibberellin (Berrie *et al.*, 1968; Khan, 1967; Knypl and Rennert, 1967).

Coumarin itself has toxic action in many animals and the symptoms are manifested in several different ways. It has been considered a narcotic for rabbits, frogs, earthworms, and many other animals, a sedative and

I   II
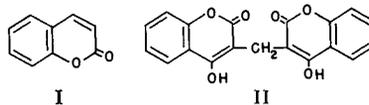
hypnotic for mice, and a toxin for man or dogs. About 5 grams may kill a sheep and 40 grams a horse, but about 4 grams is required to produce relatively mild toxic effects in humans (Dean, 1952; Sethna and Shah, 1945). Coumarin occurs in a number of natural flavoring materials. It is probably most concentrated in tonka beans, but a number of other odorous products contain it in significant amounts including lavender, lovage, and woodruff. Evidence of extensive liver damage at high dose levels in experimental animals has caused the use of coumarin and tonka beans to be banned as food additives since 1954 (Hazelton *et al.*, 1956; Jacobs, 1963; National Academy of Sciences, 1966). Diets containing 1% coumarin killed rats within about 8 weeks (Hagan *et al.*, 1967). Growth was suppressed and liver damage occurred at dosage lower than 1%. At 1000 p.p.m. of coumarin, in a diet including 3% corn oil and fed for two years, no effect on the rats was detected. Daily oral doses to dogs at 10 mg. per kg.

for nearly a year produced no definite effect; but at 25 mg. per kg., liver damage was marked in one dog, mild in two others. Dihydrocoumarin produced no effect at 1% of the diet fed for 14 weeks lending support to the unsaturated lactone function as at least part of the reason coumarins are unusually active. The sedative and hypnotic effect of coumarins seems particularly related to the unsaturated lactone feature (Soine, 1964).

The coumarins as a group are probably the most widely distributed phenols in plants which have important physiological effects on animals. The coumarins are present (in some species) in decreasing order of frequency in the families Umbelliferae, Leguminoseae, Rutaceae, Compositae, and Solanaceae (Duquenois, 1967). All of these families are considered evolutionarily relatively advanced. Coumarins occur in a few cases and usually with unusual structures in a few lower plants, aflatoxin, for example. Furocoumarins are found in the first three families above plus the Moraceae which are not so advanced, and there are some occurrences of coumarins in other botanically diverse plants. The more complex coumarins are more isolated in occurrence and more likely to be toxic.

The simpler coumarins have been cited as toxic agents in some poisonous plants. Esculetin, 6,7-dihydroxycoumarin, in the form of esculin its 6-glucoside, has been indicated to be the primary toxic agent in a number of buckeyes, *Aesculus* species (Hippocastanaceae) (Kingsbury, 1964). Cattle and other animals may die from consuming leaves or nuts and the nuts are reported to have killed children. Symptoms in cattle included incoordination, twitching, and sluggishness or excitability. However, other coumarins, notably daphnin in *Daphne mezereum*, are now believed not the significant toxin in poisonous plants although formerly so indicated. Esculetin is a potent inhibitor, especially in vivo, of phenylalanine hydroxylase and experimentally induces phenylketonuria (DeGraw *et al.*, 1968). This and other physiological effects including liver damage and capability of involvement with other specific enzyme systems suggest that coumarin in general can have significant toxic action (Dadak and Zboril, 1967; Degkwitz *et al.*, 1968; Feuer *et al.*, 1966). Soine (1964) tabulates 115 coumarins from natural sources including a few from microorganisms and lists 31 reported types of pharmacological and physiological effects on animals. Studies have generally noted a high incidence of physiological effects among different coumarins, but animal genera differ in symptoms and susceptibility. Particularly, high sensitivity exists among fish and snails to both simple and more complex coumarins such as the furanocoumarins (Dean, 1952; Sethna and Shah, 1945; Soine, 1964; Stanley, 1963).

Dicoumarol, 3,3'-methylenebis-4-hydroxycoumarin (II) is a poisonous phenol from a plant source which became a respectable medicine whose gross method of action, if not its specific mechanism, is clear. That prolonged feeding on improperly cured, spoiled hay from sweet clover, *Melilotus officinalis* (Leguminosae), causes a fatal hemorrhagic disease in animals, particularly cattle, and that the active principle is a reaction product derived by mold action from the natural coumarin is now well known (Kingsbury, 1964; Link, 1944; Soine, 1964). Dicoumarol interferes with the action of vitamin K and prevents the synthesis of prothrombin and associated blood clotting factors in the liver. The resultant hypoprothrombinemia inhibits clotting if controlled and produces fatal bleeding

if excessive. Initial therapeutic doses are of the order of 200 mg. in humans and reduction in prothrombin clotting time below 20% of normal can be corrected by vitamin K administration. Something more than simple competitive effect is indicated by the fact that relatively large doses of vitamin K are required to reverse the effect of dicoumarol. The drug is apparently stored only in the liver (Wosilait, 1968). It forms complexes with protein in blood (Nagashima *et al.*, 1968b) and the biological half-life increases markedly with increased dosage. Side effects other than bleeding are rare even with prolonged dosage, but owing to the liver storage, the effect on prothrombin level persists for some time.

The structural features which govern antivitamin K anticoagulant activity appear more complicated and interacting than once thought, and although considerable data are available from natural and synthetic analogs, precise conclusions are not yet possible (Soine, 1964). Ayapin, the methylenedioxy derivative of esculetin, is reported to have anticoagulant activity and the addition of aromatic methoxyl substitution to dicoumarol analogs tends to enhance but hydroxyl to decrease activity. Dicoumarol appears to have additional effect as a vasodilator particularly on coronary blood vessels. This effect is pronounced with a number of other coumarin derivatives such as those associated with khellin in *Ammi visnaga*.

Safrole, 1-allyl-3,4-methylenedioxybenzene (III), makes up about 80% of sassafras oil from the root bark of *Sassafras albidium* (Lauraceae). It is also a component in star anise oil, camphor oil, nutmeg, mace, cinnamon leaf oil, and some other essential oils. It was used in small amounts for many years as a flavoring, particularly in root beer. Evidence that it is carcinogenic has resulted in its being banned for food use in the U. S. since 1960 (Hagan *et al.*, 1967; Roe and Field, 1965). Rats died on diets containing 1% and malignant hepatic tumors were found at levels above 0.25% in the diet. Liver damage, including lipoid deposits, but not malignant tumors, decreased from slight to moderate with 0.1% safrole in the diet to very slight at 0.01% or 100 p.p.m. of dietary safrole fed for two years. Daily oral doses to dogs for prolonged periods produced drastic effects including fatty liver damage, but not carcinoma, at 40 and 80 mg. per kg. body weight. At 5 mg. per kg. per day for six years, liver damage was still evident but minimal.

Dihydrosafrole produced much decreased but similar liver damage to safrole. It also produced tumors in the esophagus in 20% of the rats and 5% of the tumors were malignant at a dietary dihydrosafrole level of 0.25% fed over a two-year period (Hagan *et al.*, 1967). In the same tests, *isosafrole*, the 1-propenyl analog, had similar toxic effects to safrole—i.e., liver not esophagus tumors—but required five times as much for comparable severity. Tests with several other essential oil components were more limited in dosage and length. Anethole, *p*-propenylanisole, showed slight microscopic changes in the liver of male rats only, after 15 weeks with 1% in the diet. Similar treatment with dihydroanethole indicated slight osteoporosis after 19 weeks. Eugenol, 1-allyl-3-methoxy-4-hydroxybenzene, or isoeugenol, the propenyl analog, at 1% in the diet of rats for 19 weeks, produced no effect.

The seriously toxic feature of the safrole molecule evidently is the methylenedioxy group, but the degree and locale of the toxic symptoms produced is also considerably influenced by the nature of the hydrocarbon sub-

III

IV

V
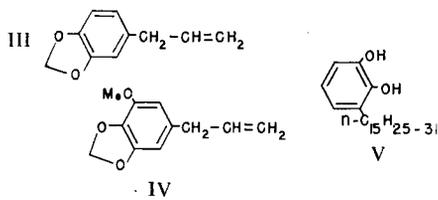
stituent. The only metabolite of safrole reported in animals is piperonylic acid (Williams, 1959). Piperonal produced no effect when fed to rats at 1% of the diet for 14 weeks (Hagan *et al.*, 1967). Other methylenedioxy derivatives, notably those in the sesame oil lignan series, have not shown as serious toxicity as safrole even in prolonged feeding (Ambrose *et al.*, 1958; National Academy of Sciences, 1966), although benign liver damage was noted at high levels.

Myristicin, 1-allyl-3,4-methylenedioxy-5-methoxybenzene (IV), is an important constituent of nutmeg oil, nutmegs, and mace from *Myristica fragrans* (Myristicaceae). Nutmeg is the seed kernel and mace the dried aril covering the seed. Consumption of nutmeg in (for a spice) large amounts produces drowziness, stupor, and even death. The volatile oil has much the same but reduced activity and will produce narcosis, delirium, and death. Some of the symptoms of the delirium produced in some people include fairly prolonged or recurrent hallucinations or stupor with short lucid periods between and distorted perception of sight and sound, etc., which resemble the effects of tetrahydrocannabinol or lysergic acid diethylamide. For this reason there has been considerable recent interest in myristicin and nutmeg out of all proportion to the 30 or so known poisoning cases and one documented fatality from this source (Green, 1959; Shulgin, 1966; Truitt *et al.*, 1961; Weil, 1965; Weiss, 1960). A whole nutmeg weighs about 5 grams and contains 5 to 15% volatile oil and 25 to 40% nonvolatile ether extractables. Two nutmegs have produced death when eaten by an 8-year-old child and toxicity requiring medical attention has resulted from smaller doses in adults. Smaller doses do not always produce appreciable toxicity, perhaps partly due to individual sensitivity (Truitt *et al.*, 1961), but also due to variable myristicin content and partial loss of volatiles from the spice.

Nutmeg essential oil has about 4% myristicin, 0.6% safrole, and the remainder is largely terpenes, particularly *d*-camphene. The myristicin fraction from nutmeg, however, is contaminated by about 30% elemicin, 1-allyl-3,4,5-trimethoxybenzene. If the volatile oil was removed, 10 grams of nutmeg residue did not produce hallucination, but still produced intestinal discomfort and affected sleeping (Truitt *et al.*, 1961). Myristicin in 400-mg. oral doses produced "cerebral stimulation" in some people, but much less than 15 grams of powdered nutmeg (which might be expected to contain about 60 mg. of myristicin). It seems clear from animal experimentation that myristicin or a precursor of it is the crucial component for the toxic effect of nutmeg, but it also appears that potentiating, synergistic, or at least additional factors are present both in the volatile oil and in the residue. Although participation by the terpenes and fatty material in absorption and distribution of myristicin is suspected, proof awaits further work.

Myristicin decreased the sleep-inducing effect of phenobarbital in rats. Doses of 50 mg. per kg. intravenously administered to monkeys produced incoordination and disorientation for about two hours. Prior administration of chlorpromazine masked this effect and the effects of morphine were increased in cats by myristicin. Man seems more susceptible to the disorientation effects of myristicin than are many animals, and doses which produce visible behavioral effects in cats appear to terminate in death within a few days from liver degeneration. Favorable effects of myristicin in a few cases of mental illness have been reported. Myristicin potentiates tryptamine induced convulsions, antagonizes the effects of reserpine, and increases brain serotonin, all effects suggesting action as a monoamine oxidase inhibitor. Addition of the elements of ammonia to the side chain of either elemicin or myristicin would produce amphetamines known to be psycotomimetic (Shulgin, 1966; Weil, 1965).

The obvious possibilities that myristicin would mimic safrole as a long-term, low-potency liver carcinogen and that safrole would be a hallucinogen have not yet been studied in detail. Small doses of oils containing safrole have been reported (Von Oettingen, 1949) to produce hallucination and psychic disturbance lasting several days. The trio found in nutmeg, safrole, myristicin, and elemicin occurs also in the oil from *Cinnamomum glandiferum* (Lauraceae) and myristicin occurs with different related compounds in several essential oils from Umbelliferae and a few from Labiatae. Apiol—the 2,5-dimethoxy, dillapiol—the 5,6-dimethoxy and the 2,3,4,5-tetramethoxy relatives of myristicin occur with it in oils from parsley, dill, fennel, or related sources (Toth, 1967). These and other variants with three or more phenolic ethers on the ring all, so far as they have been studied, produce toxicity at moderate levels (Weil, 1965).

Urushiol, the active principle of poison ivy, is a mixture of about 2% 3-*n*-pentadecylcatechol (V), 10% of the analogous Δ-8 monoolefin, 64% Δ-8,11 diolefin, and 23% Δ-8,11,14 triolefin (Dawson, 1956; Markiewitz and Dawson, 1965). Poison ivy and its variants, *Rhus toxicodendron*, belong to the largest genus (120 species) of the 60 in the Anacardiaceae, an interesting family from a utility and a phenolic viewpoint (Kingsbury, 1964; Morton, 1961). Most of the Anacardiaceae are tropical and many of them are noted for skin irritating effects. Nevertheless this family inludes the pistachio (*Pistacia vera*), mango (*Mangifera indica*), cashew (*Anacardium occidentale*), and lacquer-producing trees, particulaly *Rhus verniciflua*, which is used in making oriental lacquerware. The saps of many of these plants will oxidatively darken and can be made to polymerize to an inactive form which is no longer an irritant, but in the fresh or incompletely reacted form they are skin vesicants much like poison ivy.

Mango stem sap, which often contaminates the peel, and perhaps the peel itself, but not the fruit, produces a similar rash and allergically cross-reacts to a considerable degree with poison ivy (Keil *et al.*, 1946). The cashew "apple" or the fleshy receptacle bearing the nut is readily eaten out of hand, but the shell oil of the nut itself is very irritating, particularly before roasting. The constituents of cashew shell oil include anacardic acids, salicylic acid derivatives (2-carboxy-3-alkylphenols), with a series of C_{13}, C_{15}, and C_{17} side chains with 0 to 3 double bonds (Gellerman and Schlenk, 1968). Anacardic acids also are toxins in the leaves and nuts of *Ginkgo biloba* (not botanically related), but here the location of the unsaturation is evidently different from the 8, 11, 14 cashew pattern.

Anacardic acids make up about 90% of cashew shell "oil." During heating they decarboxylate to give a cardanol (3-alkylphenol) series of compounds. Cardol, analogous 3-alkyl-5-hydroxyphenols, and other substances such as 6-methylcardol are also present in cashew shell liquor (Murthy *et al.*, 1968; Tyman, 1967; Tyman and Morris, 1967). The commercial production of cashew nuts has made cashew shell liquor available in an estimated 15,000 tons annually from India alone (Morton, 1961), and it is used industrially to the degree that contact dermatitis in harvesters and processors must be a problem of some magnitude.

Skin reaction to urushiol and closely related natural and synthetic compounds has an important sensitization-allergy aspect. Babies and others who have never been exposed seem immune to the first exposure to amounts which produce typical reactions in sensitive people. However, a general toxicity also is involved and it has been reported that at elevated levels all persons can be made to react. Upon skin contact with urushiol the reaction does not appear for about four days and the familiar symptoms of redness, itching, blistering, etc., ordinarily subside in less than 14 days. About 1 μg. of urushiols will produce a typical reaction about 5 mm. in circumference on a sensitive individual. The systemic toxicity is relatively low, but serious gastric, intestinal, and nephritic irritation has occurred. Complications from dermal poisoning have produced numerous deaths.

A number of studies have investigated the structure-activity relationships in the urushiol-like series (Baer *et al.*, 1967; Byck and Dawson, 1967; Dawson, 1956) in both human and animal tests. For skin toxicity to nonsensitized guinea pigs an alkyl side chain of at least five carbon atoms is required with toxicity increasing to an apparent limit at about 15 carbons. Branching or cyclization is unimportant and lipoid solubility therefore is more important than shape. Cardol and another meta-dihydroxy analog are nontoxic. A monophenol derivative appears to be as toxic as a catechol derivative provided the structure is capable of being hydroxylated by tyrosinase. The relationship of toxicity and albinism has not been studied, but might explain difficulties in testing urushiol on laboratory rats or mice. Methylation of one phenolic group in 3-pentadecylcatechol reduced considerably the toxicity to nonsensitized guinea pig skin and the second still more but did not entirely eliminate it, presumably because of limited demethylation reactions in the skin.

Catechol, in terms of the percentage of guinea pigs becoming sensitive, was as effective in inducing sensitivity to a subsequent homologous contact as were urushiol analogs. The intensity and cross-reactions were affected by the length of the alkyl chains, however, and maximum intensity of sensitivity was produced with 3-undecylcatechol. The sensitivity and toxicity effects are relatively separate as shown by the production of compounds with high activity in one regard and none or little in the other. Urushiols bind to protein in vitro and are rapidly and evidently irreversibly bound very rapidly in skin. This appears to be primarily related to the sensitization phenomenon and represents an amino group covalently linking by nucleophilic addition to the ortho-quinone. This would appear to explain why analogs without activated substitution sites (4,5-dimethyl-3-pentadecylcatechol) and those incapable of direct oxidation to the quinone (6-pentadecylguaiacol) are quite toxic but very poor sensitizers.

Kernels of the "marking nut" (used to mark laundry and thereby causing dermatitis) *Semecarpus anacardium* have been reported to have psychopharmacological effects including improvement of memory, but this lacks satisfactory proof (King, 1957).

Phloridzin (VI), the 2-glucoside of the dihydrochalcone phloretin, occurs in apples and close relatives in parts other than the fruit. It has considerable interest from a botanical viewpoint (Pridham, 1960; Williams, 1966) and because it is one of the few dihydrochalcones known in nature. In early studies in connection with diabetes, it aroused interest from its toxic ability to produce glucosuria in man and animals (Lotspeich, 1960–1; McKee and Hawkins, 1945). Flavanones and chalcones are generally nontoxic like most common flavonoids. Therefore phloridzin is of interest as perhaps the simplest or most nearly "usual" flavonoid analog with generally recognized toxicity. The "toxicity" is of low order, however, 200 to 400 mg. per kg. being commonly used in experimental glucosuria production.

VI   VII
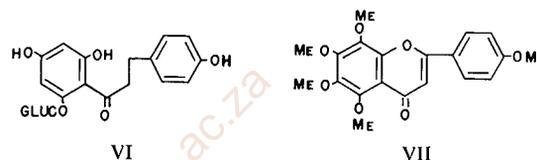
Phloridzin blocks reabsorption of glucose into the blood in the kidney by affecting the epithelial cells lining the proximal convoluted tubules. Prolonged administration leads to hypertrophy of the kidney, but apparently other effects are minimal. Absorption of glucose in the small intestine is also inhibited and the increased glucose absorption occurring in muscle following insulin injection is antagonized. Phloretin is essentially devoid of activity in kidney and intestine but affects glucose absorption by erythrocytes. Absorption of glucose in these cases requires active transport across the membrane. Phloridzin appears to compete by an adsorption process for active sites on the membrane or possibly carrier molecules and is actually extremely potent considering effective concentrations in the target organs.

Reabsorption of fructose or galactose is evidently not affected by phloridzin. The synthetic galactoside of phloretin is much less active, indicating that the 4-position sugar hydroxyl is important in the combining with the active site. If the phenolic hydroxyls are methylated the product is inactive. Uncoupling of oxidative phosphorylation at a specific tissue locus appears to be involved in phloridzin and phloretin action (Deuticke and Gerlach, 1967; Lotspeich, 1960–1).

Tangeretin (VII), 4',5,6,7,8-pentamethoxyflavone, is uncommon to the extent of the 6- and 8-methoxy groups and is found with nobiletin (additional 3'-methoxyl) in tangerine peel. It is toxic to embryo zebra fish at 0.25 mg. per liter which was only exceeded by podophyllotoxin at 0.1 mg. per liter among compounds tested (Jones *et al.*, 1964). Nobiletin was one-fourth as toxic or less in the same test. Tangeretin was considered nontoxic as were a series of other flavones and flavonones with the more usual hydroxylation pattern in various screening tests including intraperitoneal dosage at 500 mg. per kg. to mice and orally at 1000 mg. per kg. to dogs (Stout *et al.*, 1963, 1964). However, when administered at 10 mg. per kg. per day subcutaneously to rats during gestation, 83% of the litters were born dead or died within three days

without visibly apparent abnormality. Nobiletin is also fungistatic (Ben-Aziz, 1967). The toxicity of these compounds brings to mind the extra toxicity which seems to occur with extra alkoxy groups in the safrole-myristicin series. The toxicity of tangeretin evidently offers no significant natural hazard, but these findings narrowly prevented tangerine peel being introduced as a bioflavonoid source.

Grazing of sheep on subterranean clover (*Trifolium subterraneum*) produces "clover disease," a condition which has caused large economic losses in Australia. The toxic condition is the result of estrogenic isoflavones in the clover. The symptoms include production of lactation in unbred ewes and castrated males, death of castrated males from urinary obstruction, difficult labor, lambing percentages decreased from 80 to as low as 10%, and permanently decreased fertility even when the sheep are removed from the clover. Genistein (VIII) was the first phenol identified as involved in this condition, and other natural isoflavones now known to be estrogenic include biochanin A (the 4'-methoxy analog of genistein), prunetin (7-methoxygenistein), daidzein (5-deoxygenistein), and formononetin (5-deoxybiochanin A) (Bickoff, 1968; Fairbairn, 1959; Moule *et al.*, 1963; National Academy of Sciences, 1966).

VIII   IX
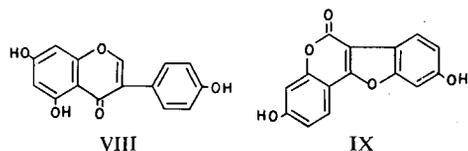
Among 100 species of clover (*Trifolium*), 14 had total isoflavone content comparable to subterranean clover or about 1% of the dry weight of the leaves (Francis *et al.*, 1967). Formononetin is estrogenic in sheep, but less active in mice, compared to daidzein, and the alimentary tract microorganisms are a factor in activation by demethylation and perhaps other mechanisms (Bickoff *et al.*, 1962; Braden *et al.*, 1967; Nilsson *et al.*, 1967). Investigation of other legumes and other estrogen-like responses of animals showed that coumestrol (IX) was an active estrogen from ladino clover, alfalfa, and subterranean clover (Bickoff *et al.*, 1957). It occurs to the extent of about 0.005% of the dry weight of alfalfa and ladino clover, and is about 10 to 30 times as potent as genistein (Braden *et al.*, 1967; Moule *et al.*, 1963; Soine, 1964), depending on the assay used. Other phenolic substances of plants which are estrogenic include miroestrol, a rare but very active pentacyclic monophenol (Cain, 1960) and psoralidin (*Psoralea corylifolia*), coumestrol with 6-C_5H_9 substitution.

The plant estrogens which humans might contact inadvertently seem limited to the isoflavones of soy beans (*Soya hispida*). The amount likely to be consumed in a human diet is too low to be appreciably active. If it were not for the high level in otherwise excellent forage plants and for domestic animals being restricted often to diets almost purely of a certain plant, the same probably would be true for them. Diethylstilbestrol, a synthetic stilbene derivative (but not similar enough to those from conifers to expect them to be active), has two hydroxyls at the extremes of the molecule. It is about 3000 times as active as coumestrol. It appears that activity usually depends on two phenolic hydroxyls or potential hydroxyls a certain distance apart and a relatively planar molecule. Known isoflavones and coumarins with estrogenic activity are limited to those with these features. Synthetic isoflav-3-

enes, which mimic diethylstilbestrol even more closely, approach its level of activity.

X   XI
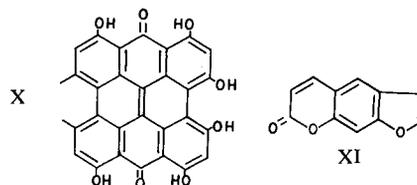
Hypericin (X) and psoralen (XI) represent unusual plant phenols, the former being a dianthrone derivative and the latter a furanocoumarin. Both phenols and their relatives are responsible for toxic reactions making animals hypersensitive to light. Hypericin occurs in species of *Hypericum*, notably St. Johnswort or Klamath weed (*H. perforatum*), while fagopyrin occurs in buckwheat (*Fagopyrum esculentum*). Fagopyrin is similar in structure to hypericin with other groups substituted for the methyl groups and a number of additional variations occur in the same plants (Brockmann, 1957; Clare, 1955; Fairbairn, 1959). The effect on animals may be severe and the invasion of St. Johnswort into grazing land in northern California was becoming a very serious problem until a beetle which selectively feeds on this plant was introduced (Huffaker and Kennett, 1959). The plant, a perennial herb 1 to 5 feet tall, contains hypericin in characteristic glandular dots visible in the foliage and petals (Kingsbury, 1964).

Hypericin remains intact through ingestion, digestion, absorption, and passage through the liver. It may be detected in skin and mucous membranes by direct spectroscopy or its characteristic red fluorescence. Commonly only white skinned animals are poisoned or in those with white spots only those spots are affected (Clare, 1955; Kingsbury, 1964). Unless dosage is very high, completely colored animals are unaffected. Typical photosensitization can be produced in white mice with subcutaneous doses of about 12 to 25 mg. per kg. Cattle or sheep having consumed toxic amounts and appearing well in subdued light are rapidly affected when placed in direct sunlight. Erythema, swelling, extreme itching, serous oozing, and, as the edema subsides, cracking of the skin and necrosis of the skin in spots are symptoms. The animals may appear crazed presumably from the itching and may refuse to eat. Starvation and secondary effects, such as infection from abrasion, lead to most of the deaths. The animals are hypersensitive to touch and to cold water; fording streams may lead to convulsions.

Although hypericin does not seem to be damaging to the liver, decreased effectiveness of liver function may lead to similar photosensitivity from chlorophyll and other substances in the diet. It therefore may be significant that *Hypericum* species seem to have a high level of chlorogenic acid and other phenols which might represent a detoxification load on the liver (Netien and Lebreton, 1964). Photosensitizers evidently act by broadening the wavelengths which produce sunburn and other damage from the normal below 320 mμ to longer ultraviolet and violet light. Free radical formation is involved and oxygen appears required. It is postulated that the localized production of free radicals overloads or circumvents the chain-terminating function of vitamin E and other free radical scavengers and produces cellular disruption and membrane damage, followed by more disseminated effects (Slater and Riley, 1966). Natural melanin granules

seem to *protect* by a similar light absorption-free radical generation mechanism, but the envelopment of these granules in phospholipid membranes apparently confines and dissipates the potentially damaging free radicals in a controlled manner.

Psoralen and related furocoumarins are being studied in some detail because either local application or oral consumption will produce photosensitization. Bergapten (5-methoxypsoralen) has produced a photosensitive dermatitis following applications of perfume containing it, and fowls have had vesicular dermatitis after eating seeds (*Ammi majus*) containing xanthotoxin (8-methoxypsoralen). Cases of dermatitis after skin exposure to celery and certain other juices and sun are believed to be due to this (Soine, 1964). Psoralens, however, have been used medically to stimulate melanin production in humans having vitiligo, a localized "albinism" (Schönberg and Sina, 1948; Trenchi, 1960). The furocoumarins are found in several plant families notably Umbelliferae, Rutaceae, Leguminosae, and Mimosae. Psoralen and very similar structures are the only natural furocoumarins which are photosensitizing, but other toxic effects, fish poisoning, etc., occur in the group.

The relative degree of photosensitizing ability of natural and synthetic compounds and their manner of action have been the subject of several studies (Biswas *et al.*, 1967; Caporale *et al.*, 1967; Pathak *et al.*, 1967; Soine, 1964). There is some disagreement as to the relative activity but it appears that psoralen is one of, if not the, most active, and the entire specific ring system is necessary. Substitution, which has much effect on conjugation or the electronic configuration reduces activity and maximum absorbance at 320 to 360 mμ, is characteristic for active compounds. These compounds emit fluorescence at maxima of 420 to 460 mμ. It has been suggested that emission of this specific light in close proximity to some sensitive component may be important. When we consider the losses involved this seems less likely than a more direct effect of the activation. Methyl substitution at the 3-position but not at others considerably reduces the activity according to some observations. Participation of the 3-position in binding at the active site is suspected. The 4'–5' double bond in the furane ring is required for activity. *In vitro* studies show a photocatalyzed oxidation of this bond in the presence of flavin mononucleotide. Psoralen combined in vitro, when irradiated, with the pyrimidine bases of DNA. The reactions may be more than localized effects in skin, however, because psoralen causes mobilization of copper from the liver to blood and interactions between the pituitary, melanin formation, and tyrosinase activity (Biswas *et al.*, 1967). Photodynamic action appears to be a more common reaction with phenols than has been generally appreciated; for example, flavones have such action (Nishie *et al.*, 1968).

XII
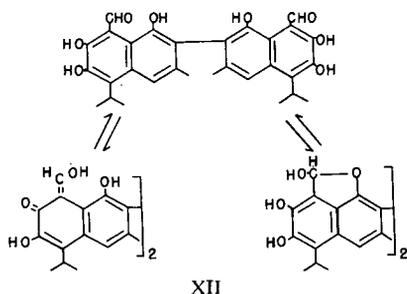
Gossypol (XII) represents an unusual toxic phenol of very limited occurrence being almost confined to the genus of cotton, *Gossypium* (Malvaceae) (Adams *et al.*, 1960; Bhakuni *et al.*, 1968; Kingsbury, 1964). Gossypol is the predominant yellow pigment which occurs as about 20 to 40% of the substances inside the "glands." These glands are spheroidal bodies about 100 to 400 microns in size, which are visible as dots in the cottonseed kernel. The amount of gossypol is proportional to the number of glands as it does not occur elsewhere in the seed. Cottonseed usually contains 0.4 to 1.7% gossypol, but glandless forms are being bred and *Gossypium* species have from 0.1 to 6.6% of the dry weight of the seed kernel.

The world's production of cottonseed is about 18 million tons and is estimated to contain 55,000 tons of gossypol. All of this is potentially poisoning because both the oil and kernel meal are eaten by man or animals. Fortunately, removal from the oil and causing binding in the meal renders both products nontoxic. If the oil is expressed from the raw kernel it is colorless, the glands are intact, and the meal is toxic to nonruminants. For ruminants it is an excellent feed. If instead the meal is treated with water and cooked with steam, the glands are disrupted and the gossypol partly dissolves in the oil. The oil is readily decolorized and the meal is found to now have most of its gossypol in a bound form which is no longer toxic to nonruminants. With modern processes at least 80% and often 95 to 99% of the gossypol is destroyed, bound, or removed from the meal (Phelps, 1966).

The toxicity of gossypol appears mild on a single oral dose basis; the LD_{50} is about 2400 to 3340 mg. per kg. in the rat (National Academy of Sciences, 1966). Pigs, rabbits, and guinea pigs are more sensitive, chickens about the same. The whole glands appear to be two to four times as toxic as pure gossypol but this may be only lesser inactivation of gossypol en route to a sensitive site. On repeated oral dosage, 10 to 200 mg. per kg. per day is fatal to the dog. With pigs, 0.02% in the diet appears to be the dividing line between toxic and nontoxic (Clawson *et al.*, 1961). This is approximately the level of free gossypol in commercial meal. For safety 0.01% or less free gossypol or not over 9% cottonseed meal in the diet is recommended. At a high level of food protein, gossypol is less toxic.

Pigs fed toxic levels may appear normal for a few weeks to a year, then abruptly begin to gasp for breath and die in two to six days with severe anemia and other complications (Adams *et al.*, 1960; Kingsbury, 1964; National Academy of Sciences, 1966). A common symptom with gossypol in the diet is loss of appetite and weight loss. Gossypol was briefly considered for use in human obesity (National Academy of Sciences, 1966). Hypoprothrombinemia has also been noted as a toxic symptom.

The structure of gossypol is unclassifiable in any usual plant phenol category, evidently because it arises biosynthetically from an isoprenoid route (Heinstein, 1967). The possible equilibrium forms appear to favor strongly the aldehydic form, but to be influenced by solvent and separable by gas chromatography (Raju, 1967). The aldehyde groups are able to react with aniline, ammonia, and more importantly with the free epsilon amino groups of lysine in protein. The bound, nontoxic gossypol is believed mainly this product. Although bound gossypol can be chemically liberated from its Schiff's base combination, this does not happen in digestion. Therefore the loss in toxicity is combined with loss in available lysine as

well (Adams *et al.*, 1960). Gossypol apparently may undergo other types of binding because cooking gossypol in test diets for humans reduced toxicity without reducing protein value or available lysine (Bressani *et al.*, 1964). Simple wetting and drying decreased free—i.e., toxic—gossypol and sugar seemed involved as well as iron and calcium.

The lack of toxicity of gossypol to ruminants results from indigestible binding of free gossypol to protein which would appear to result from gland mastication and time rather than microorganism effects (Reiser and Fu, 1962). Feeding of iron and of calcium salts with cottonseed meal decreases toxicity of gossypol. It now appears that gossypol binds iron and this is the cause of anemia. If sufficient ferrous ion is added to complex with all the gossypol and still meet the needs of the animal, normal blood conditions will appear, but toxicity will still result. If calcium alone is fed other aspects of toxicity may be prevented, but anemia will result. If both iron and calcium are added to the diet at correct levels normal animals result (Braham *et al.*, 1967). Ferric ion precipitates gossypol, but ferrous ion gives a soluble chelate which is rendered insoluble by calcium (Shieh *et al.*, 1968).

The most worrisome aspect of gossypol toxicity is that gossypol in the body is bound and retained particularly in the liver, kidneys, and spleen of both pigs and trout (Roehm *et al.*, 1967). The tissue-bound level was half as high after less than one-fourth the oral intake. After 12 months of feeding gossypol, shifting to a gossypol-free diet for 10 weeks gave little change in the total bound in the body of trout. Clearly a very difficult compound for the body to detoxify, or eliminate, perhaps because of the lack of acetate or shikimate "handles."

Most of the compounds discussed have been derived at least partly from the shikimic acid biosynthetic route. Anthraquinones of plants have been generally believed to be products of the acetate route, although it appears that that is not exclusively the case and shikimic acid can be involved (Leistner and Zenk, 1967). In addition to hypericin the compounds of this sort which are toxicologically most interesting are the cathartics from *Rhamnus* barks, casara sagrada and frangula; *Aloe* leaf juice, aloin; *Cassia* leaves and pods, senna; and *Rheum* roots, Chinese rhubarb. Cascara sagrada is probably most widely used and an estimated 4 million pounds of this "sacred" bark is collected on the Oregon coast each year, dried, and stored one year before use.

Rhein (XIII) may serve as a model for the anthraquinones which are the basis for the activity of these preparations. The most common structural variants have more reduced substituents in position 3 (aloe-emodin, CH_2OH; chrysophanol, CH_3) or extra hydroxyls (emodin, 6-hydroxychrysophanol). The total anthraquinone content is of the order of 1 to 3% in the natural dry drugs and typical doses would contain about 30 mg. of anthraquinone derivatives. However, anthraquinones from other sources are weak or inactive and gross anthraquinone content is a poor measure of cathartic power in these drugs. Quantitative assay of physiological potency has been a problem, and the mixture of substances turns out to be quite complex as is their chemistry (Fairbairn, 1959, 1964; Kinget, 1968; Lemli, 1967; Stoll and Becker, 1950).

The anthraquinones in these drugs occur as normal phenolic glycosides, and as an O-glycoside of the partially reduced oxanthrone or a C-glycoside of the anthrone form.

XIII   XIV
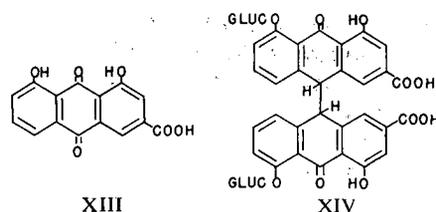
They also occur as homo- and less often heterodianthrones. Sennoside (XIV) is a rhein dianthrone glycoside. The phenolic hydroxyls appear important in activity from work with synthetic substances. In the alpha arrangement one free hydroxyl is inactive, two are active, and three reduce activity unless the third one is not alpha (Fairbairn, 1964). Acetylation causes loss of activity. Glycosidation is important because although anthraquinones produce peristalsis in the large intestine, they do not normally reach it in small dosage unless protected from metabolism by glycosidation. The reduced anthrone and dianthrone forms are more active than the anthraquinone forms. This evidently explains the practice of storage of cascara for a year which is observed to decrease the griping, cramping effects to more gentle action and presumably involves a slow oxidation.

There is, moreover, a synergistic effect when more than one anthraquinone derivative is present. It appears that the action is considerably more than just an "irritant" effect. The specific toxic action seems to be to initiate strong peristalsis in the large intestine. Since the threshold of peristalsis initiation appears to be controlled by 5-hydroxytryptamine (Ramwell *et al.*, 1964), interference with this substance or its oxidation would seem a possible mechanism.

Tetrahydrocannabinol (XV) is the primary active hallucinogen of marihuana or hashish. The chemistry and action of the toxic plant *Cannabis sativa*, hemp, have been reviewed (Mechoulam and Gaoni, 1967; Wolstenholme *et al.*, 1965) and the field is so active that this discussion is limited to a few observations. It is interesting that the plant is related to the nettles, noted for 5-hydroxytryptamine content and for bothering humans. The compound would appear to be a terpene derivative in the biogenetic sense. The structure which is hallucinogenic seems quite specific in that a number of similar compounds which occur with it or have been synthesized, including the unnatural 3,4-*cis* isomer, are low in this activity although sedative or weak antibiotic effect have been noted (Hively, 1967; Mechoulam and Gaoni, 1967; Razdan *et al.*, 1968). Equal activity is found with the double bond in either the 1- or 6-positions.

XV   XVI
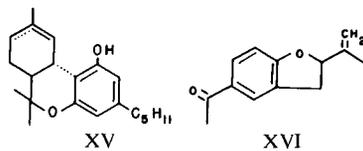
The hallucinogenic dose is about 3 to 5 mg. in humans. Motor incoordination in the dog and abolishment of the blink reflex in rabbits whose eyes are stimulated by a horsehair have been used for assays. The dominant neural effect appears to be impairment of impulse conduction along the presynaptic nerve fiber (Lapa *et al.*, 1968). Marihuana has been reported to increase 5-hydroxytryptamine in the brain, antagonize peripheral effects of 5-hydroxytryptamine, and interfere with peripheral response to acetylcholine and the epinephrines (Bose *et al.*, 1964).

Tetrahydrocannabinol is strongly analgesic at 20 mg. per kg. subcutaneously (Bicher and Mechoulam, 1968). The blink reflex is abolished by about 0.1 mg. per kg. and a natural resin contained the activity equivalent of 10% tetrahydrocannabinol (Valle *et a'.*, 1966). A plant resin sample had an acute LD_{50} intraperitoneally in rats of about 800 mg. per kg. and the sublimate produced by smoking only about 3000 mg. per kg. (Wolstenholme *et al.*, 1965). The minimum dose producing measurable incoordination in the same tests was 60 to 95% of the lethal dose in either form.

Tremetone (XVI) (Bonner *et al.*, 1964; Christensen, 1965; Kingsbury, 1964) is a poisonous constituent of white snakeroot (*Eupatorium rugosum*) and two other Compositae. The amount of this compound and its derivatives in the plant varies from near zero to about 0.2%. The fresh plant will kill animals when consumed at about 1% of their body weight per day. Human deaths are caused by drinking milk from affected cows. The plant is particularly common on partly cleared land in the east central United States. During the pioneer days in certain areas this plant was the primary cause of human mortality. President Lincoln's mother is said to have died from this "milk sickness."

The structure has some similarity to tetrahydrocannabinol, but the effect on animals has little similarity beyond a certain nervous involvement. Incoordination, apparent muscular stiffness, a reluctance to walk, and trembling spells when the animal is startled or forced to any exertion are symptoms. The compound is retained in the body, produces ketosis, extensive liver and kidney degeneration, and in apparently recovering humans, exertion may bring on fatal relapse even after considerable time.

Dihydromethysticin (XVII) is apparently the most active member of a series of related compounds which have been identified in the intoxicating complex of kava, the Polynesian drink prepared from a shrub, *Piper methysticum*, related to the black pepper plant (Keller and Klohs, 1963; O'Hara *et al.*, 1965; Sauer and Hänsel, 1967). The pharmacology of these compounds is complicated by the fact that they are nearly insoluble and there appears to be synergism and perhaps additional unidentified active components. Mastication, fine grinding, and emulsification increase effective activity, and close filtration is said to remove activity from the beverage. The crude drug produced tranquilization, deep sleep, apparently a pleasant mental outlook and "chaotic" dreams, plus other effects such as temporary limb paralysis.

XVII   XVIII
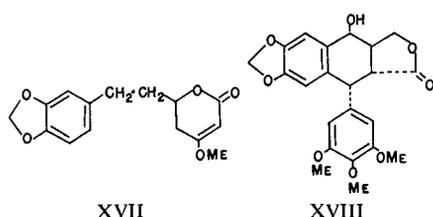
Dihydromethysticin in doses of 50 to 200 mg. per kg. orally in oil emulsion produces prolonged sleep and antagonizes strychnine convulsions in mice. The compound and several analogs, including a nonphenolic one, exhibit potent antiserotonin activity. The nonphenol had relatively high antifungal activity (Hänsel *et al.*, 1968). At doses of 500 mg. per day in humans, dihydromethysticin was not effective against schizophrenia, epilepsy, or as a muscle relaxant, but it was a mild tranquilizer. Dry,

scaly dermatitis developed in a high percentage of the patients upon continued use. The condition is present ("kavaism") in heavy drinkers of kava. Whether there is a photosensitive aspect to this condition apparently has not been investigated.

Podophyllotoxin (XVIII) is one of a number of very toxic lignan derivatives found in the roots of May apple (*Podophyllum peltatum*) and a small but growing number of other plants (Bianchi *et al.*, 1968; Hartwell and Schrecker, 1958; Kupchan *et al.*, 1967). Members of this group appear to be the most toxic natural phenols of all and it is interesting that other lignans which regularly appear in foods such as Sesamin (XIX) of sesame seed or the antioxidant nordihydroguaiaretic acid are essentially nontoxic. The alcohol-soluble root resin podophyllin was once used as a cathartic, but is too toxic for rational use for this purpose. The fruit of the May apple is mild but pleasing when ripe and so far as is known the plant represents no significant incidence of poisoning in man or animals. Podophyllin is a very powerful cytotoxin which is particularly effective on dividing cells (Di Giamberardino, 1966; Kelly and Hartwell, 1954). Although general toxicity has prevented use of these compounds against clinical cancer they are strongly inhibitory to cancer cells. Certain types of warts are cured by topical application of podophyllin under medical supervision.

XIX   XX
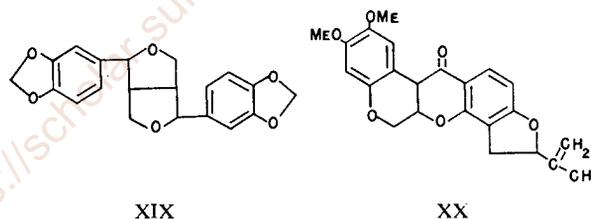
Rotenone (XX) is an isoflavone derivative (Crombie and Thomas, 1967), but represents a group of rotenoids which are of primary interest as insecticides and fish poisons (Crombie, 1963; Feinstein and Jacobson, 1953). They occur in roots of *Derris elliptica* and other related leguminous plants. The general property of higher toxicity to cold-blooded animals than to mammals seems also to operate with tremetone, which is said to be as toxic to goldfish as rotenone, with furocoumarins and with many other phenols. Since rotenone is one of the safest and least residual insecticides applied near or on humans it is often thought of as nontoxic. It is, however, rather toxic among phenols with an LD_{50} oral toxicity to rats of about 130 mg. per kg. and intraperitoneal 5 mg. per kg. Continued application in solution at about 150 mg. per kg. to the skin of rats produced toxicity. Breathing the dust may produce serious toxicity and consumption with oil increases the toxicity. Toxic doses in animals produce incoordination, convulsions, tremors, liver damage, and respiratory difficulties.

Sesamin (XIX), although nontoxic, acts as a synergist for the insecticidal activity of rotenoids. The methylene dioxy grouping is required for activity as a synergist, and a number of substances in addition to sesamin are active. Synergists have juvenile hormone properties in preventing adult metamorphosis in insects (Bowers, 1968), although sesamin has only slight activity.

Rotenone's toxicity appears to depend upon its ability to uncouple or prevent oxidative phosphorylation. This has been known for some time, but the details are now becoming clear (Jeng and Crane, 1968; Palmer *et al.*, 1968;

Papa *et al.*, 1967; Toth *et al.*, 1966). Rotenone evidently blocks NADH oxidation on the oxygen side of the nonheme iron of NADH-dehydrogenase. Under the proper circumstances, menadione opposes and dicoumarol augments the effect of rotenone in inhibiting respiration.

A number of other examples of physiologically active and potentially toxic unusual plant phenols could be cited, particularly if ferns and lichens were included. However, the list presented is believed to cover all but one of the type phenols important from food poisoning by higher plants or significant toxicity viewpoints.

It was indicated at the outset that we were looking for correlations on the theory that all phenols were likely to be toxic if they could breach the barriers and detoxification mechanisms of the animal. A number of such correlations can be seen. Phenols have an extraordinary solvent power and ability to penetrate the skin, particularly when their fat solubility is reasonably high. The skin toxicity of phenol itself and the topical effectiveness of methyl salicylate, urushiol, podophyllin, and psoralen come to mind. The importance of skin as a target organ for toxicity is indicated by dihydromethysticin as well as by hypericin, psoralens, and urushiols.

A number of suggestive relationships exist between toxicity of plant phenols and the nerve-regulating animal phenols. Cases in point are the proved or inferred mental effects of tetrahydrocannabinol, myristicin, kava constituents, marking nut, and safrole, for example. Also peripheral effects are indicated by salicylates, tremetone, the dianthrones, rotenone, and a number of the others in causing trembling, peristalsis, analgesia, etc. Significantly, one effect of bioflavonoids is to prolong epinephrine action by competitive inhibition of O-methyl-transferase (DeEds, 1968).

The mimic-interference which seems to exist between dicoumarol and vitamin K in prothrombin synthesis is also hinted at in gossypol and salicylate toxicity. Activity of genistein and coumestrol seems to depend on fitting a site awaiting natural estrogen. Phloridzin seems to combine its glucose and phenolic properties specifically to prevent glucose transfer. Less direct and perhaps incidental, but certainly intriguing, is the relation between hypericin, psoralen, and tyrosine-melanin metabolism. Even gossypol may affect hair color (Braham *et al.*, 1967), presumably a tyrosinase interaction.

The common observation of synergism or more effect than can be explained by the isolated phenols suggests both a little extra effect from a group of similar analogs, perhaps from the need for a series of adaptive detoxification mechanisms, and also nonspecific toxic aid from other plant phenols which often are present in relatively large amounts but inactive or much less active alone. This picture seems applicable with myristicin, dianthrone cathartics, dihydromethysticin, rotenone synergism, perhaps gossypol, and others.

Animals appear to have more trouble from toxic phenols which are not only unusual among plants but are made by less usual pathways (gossypol, tremetone, tetrahydrocannabinol). The "glands" of gossypol, the similar structures for hypericin, hop resins, and a number of other specialized idioblasts filled with "tannins" in plants suggest that the plant is required to make special arrangements to protect itself from high concentrations of phenols, particularly the more toxic ones. Hashish resin seems to be actually excreted from the plant.

The protection of phenolic groups from the animal's normal detoxification mechanisms until vulnerable sites are reached would seem to be a factor in the extra toxicity of parenteral administration, consumption in oil, probably the frequent but not universal association of methylenedioxy substitution with activity (safrole, myristicin, dihydromethysticin, sesamin), and perhaps the toxicity of the highly methoxylated substances (tangeretin, elemicin, dillapiol).

Finally, and perhaps most importantly, there is the ability of polyphenols to bind metals (gossypol anemia) and to bond by various mechanisms to the macromoles of animal tissue. This seems best illustrated with gossypol although the binding is not phenolic. The substance is not toxic if the binding to protein occurs outside the body, but is bound and "stored" and surely toxic inside the body at least partly because of this type of relatively permanent binding. One wonders if the protective effect of high protein diet is all related to nontissue-protein binding or may partly relate to effects on turnover of tissue protein already bound with gossypol.

Covalent bonding with protein at the activated sites of the urushiol ortho-quinone is evidently involved in poison ivy dermatitis. The acetophenone tremetone may bind by a reaction like gossypol's and tissue storage appears to be a severe aspect of its poisoning. The form of the binding is not clear but podophyllin and psoralen evidently act partly through combination with nucleic acids. The specific membrane adsorption affects of phloridzin may be hydrogen bonding or other weak multiple bonding, but like other phloroglucinol derivatives could involve facilitated covalent bond formation with an electrophilic agent such as a carbonium ion.

These examples seem to add up to fairly convincing presumptive evidence that many of the more toxic phenols act by more than one mechanism and that some of these mechanisms appear to be available to less toxic phenols if natural defenses can be evaded or overloaded. The remaining example of significant toxicity of higher plant phenols mentioned earlier, that of tannin, seems to illustrate this. The tannins of oak leaves, hydrolyzable tannins, are present at levels which lead to major toxic problems in livestock in certain areas. The toxicity of *Quercus havardi* has been most studied and is reported to cause annual losses in excess of $10 million (Camp *et al.*, 1967; Dollahite *et al.*, 1962, 1963). Tannic acid and other oak (hydrolyzable) tannins at high levels produce various toxic effects including intestinal, kidney, and liver damage and anemia. Substances such as iron salts, calcium salts, or high protein which precipitate, oxidize, or bind tannin, when added to the diet aid in preventing this toxicity.

The tannins are present in a number of plant materials at very high levels, often 10% or more of the dry weight. They may be significant in some common feedstuffs such as sorghum grains (Chang and Fuller, 1964) and rapeseed meal (Clandinin and Heard, 1968). The acute oral toxicity of tannic acid is very low and since it is about equivalent to gallic acid, at about 5000 mg. per kg. in rabbits, may represent only complete hydrolysis of gallotannic acid. Continued dosage lowers the toxicity, however, 10 days' dosage gave LD_{50} oral toxicity in rabbits of 3400 mg. per kg. per day. Tannin is much more toxic intravenously, about 80 mg. per kg. LD_{50} in mice (Spector, 1956). This would seem not surprising for a nondialyzable protein

precipitant like intact pentadigalloylglucose tannic acid.

Feeding experiments on chicks with tannic acid and other tannins have shown that at about 0.5% of tannic acid in the diet, growth is depressed and at 5% high mortality occurs (Vohna *et al.*, 1966). Condensed tannins were less detrimental than tannic acid. While these workers found no dietary relationship to methyl donors like methionine, Fuller *et al.* (1967) reported that supplementation with methionine, choline, and arginine reduced the toxicity of 1% tannic acid and completely alleviated the adverse effect of 0.5% tannic acid. This is believed to result from the need for methyl groups for the O-methylation of gallic acid derived from the tannic acid (Potter and Fuller, 1968) as had been shown previously for the rat (Booth *et al.*, 1961) and is a general phenol detoxifying mechanism (Williams, 1959).

The direct absorption of a whole hydrogen-bonding (Singleton, 1967), nondialyzable, protein-precipitating tannin macromolecule seems quite unlikely in the normally functioning animal. However, one of the symptoms of continued tannin feeding is gastritis as well as irritation and edema of the intestines. Under this circumstance it appears that tannin oligomers may be absorbed. The absorption of tannic acid through burned tissue has been known for many years along with the fact that condensed, nonhydrolyzable tannins were less toxic in this situation. The production of liver cancer by application of tannin to burns or by repeated subcutaneous injection has been demonstrated (Bichel and Bach, 1968; Kirby, 1960; Korpassy, 1961). The production of necrosis at the site of injection of condensed tannins but not of tannic acid indicates condensed tannin is more readily bound and being less mobile tends to be less active in liver carcinogenesis.

Tannic acid was introduced about 1946 for use in diagnostic barium enemas to clear away mucus and define colon walls for radiography. It was found in 1963 that this procedure was capable of producing death (McAllister *et al.*, 1963). The incidence of serious or fatal consequences was very low in comparison to the number of treatments, but was higher if the patient was juvenile, repeated enemas were given, or inflammation pre-existed (Janower *et al.*, 1967), and was particularly tragic because it was iatrogenic. The product was quickly banned, and alternative procedures were substituted. The rectal toxicity of tannic acid is about twice that of oral administration (Boyd *et al.*, 1965). The experimental effects of tannin administration include an apparent DNA binding effect in liver cells (Racela *et al.*, 1967) which may relate to carcinogenicity. There is experimental demonstration that hepatoxicity from tannin enemas is dependent upon the time of retention, tannin concentration, and preexisting irritation of the colon (Zboralske *et al.*, 1966).

These results with tannin suggest that on a milder basis some of the observed effects, even desirable physiological effects, of plant phenols may depend upon protein binding and "astringency" (Singleton, 1967) effects. The collagen strengthening, micropore shrinkage, sphincter activating effects of bioflavonoids on blood capillaries, for example (Aichinger *et al.*, 1964; Böhm, 1960; Fairbairn, 1959; Janower *et al.*, 1967), seem related. That the possibility of overloading detoxification mechanisms with nontoxic plant phenols exists, but that the margin of dietary safety is large is indicated by the fact that 2.5% bioflavonoids in chick diets for 8 weeks produced no detrimental effects, but 5% produced reduction in growth and feed utilization

without producing gross evidences of tissue damage (Deyoe *et al.*, 1962).

The potential importance of abnormal routes of administration of phenols as well as potentially important effects of degradation products of plant phenols is indicated by studies showing that many simple phenols repeatedly painted on skin increase the incidence of cancer, but only following triggering doses of carcinogens (Boutwell, 1967; Roe and Field, 1965). This would seem to have a bearing on foods and other products where pyrolysis of phenols and coproduction of carcinogenic hydrocarbons could occur such as in smoking (Boutwell, 1967; Fiddler *et al.*, 1967; Kaiser, 1967). Ferulic acid, for example, decomposes thermally to 4-vinylguaiacol and a number of other products.

In summary, plant phenols include a number of substances which are toxic and in certain circumstances do cause great losses. The symptoms and reactions involved seem to indicate a more coordinated picture than is commonly recognized. It appears that a number of the relatively common chemical and biochemical properties of phenols are involved in producing toxicity. While most common plant phenols are essentially nontoxic, their potential toxicity is worthy of consideration if many are combined, consumption is high, or unusual methods of administration are contemplated.

LITERATURE CITED

Adams, R., Geissman, T. A., Edwards, J. D., *Chem. Rev.* **60**, 555 (1960).
Aichinger, F., Giss, G., Vogel, G., *Arzneimittel-Forsch.* **14**, 892 (1964).
Ambrose, A. M., Cox, A. J., DeEds, F., J. Agr. Food Chem. **6**, 600 (1958).
Baer, H., Watkins, R. C., Kurtz, A. P., Byck, J. S., Dawson, C. R., *J. Immunol.* **99**, 365, 370 (1967).
Bate-Smith, E. C., *Advan. Food Res.* **5**, 261 (1954).
Bate-Smith, E. C., *J. Linnean Soc. London, Botany* **60**, 325 (1968).
Bate-Smith, E. C., *Mem. Soc. Bot. France* **1965**, 16.
Bate-Smith, E. C., Swain, T., *Lloydia* **28**, 313 (1965).
Ben-Aziz, A., *Science* **155**, 1026 (1967).
Berrie, A. M. M., Parker, W., Knights, B. A., Hundrie, M. R., *Phytochemistry* **7**, 567 (1968).
Bhakuni, D. S., Dhar, M. M., Sharma, V. N., *Experientia* **24**, 109 (1968).
Bianchi, E., Caldwell, M. E., Cole, J. R., *J. Pharm. Sci.* **57**, 696 (1968).
Bichel, J., Bach, A., *Acta Pharmacol. Toxicol.* **26**, 41 (1968).
Bicher, H. I., Mechoulam, R., *Arch. Intern. Pharmacodyn.* **172**, 24 (1968).
Bickoff, E. M., *Am. Perfumer* **83**, 59 (1968).
Bickoff, E. M., Booth, A. N., Lyman, R. L., Livingston, A. L., Thompson, C. R., DeEds, F., *Science* **126**, 969 (1957).
Bickoff, E. M., Livingston, A. L., Hendrickson, A. P., Booth, A. N., J. Agr. Food Chem. **10**, 410 (1962).
Biswas, N. M., Deb, C., Haque, M., Chakraborty, D. P., *Endokrinologie* **52**, 271 (1967).
Bocter, A. M., Harper, A. E., *J. Nutr.* **95**, 535 (1968).
Böhm, K., *Arzneimittel-Forsch.* **9**, 539, 647, 778 (1959).
Böhm, K., *Arzneimittel-Forsch.* **10**, 54, 139, 188, 468, 547 (1960).
Bonner, W. A., DeGraw, J. I., Bowen, D. M., Shah, V. R., "Symposium on Phytochemistry," H. R. Arthur, Ed., p. 123, Hong Kong University Press, Hong Kong, 1964.
Booth, A. N., *Plant Phenolics Group N. Am. Proc. 1st Symp.* **1961**, 72.
Booth, A. N., Masri, M. S., Robbins, D. J., Emerson, O. H., Jones F. T., DeEds, F., *J. Biol. Chem.* **234**, 946 (1959).
Booth, A. N., Robbins, D. J., DeEds, F., *J. Nutr.* **75**, 104 (1961).
Bose, B. C., Saifi, A. Q., Bhagwat, A. W., *Arch. Intern. Pharmacodyn.* **147**, 285, 291 (1964).
Boutwell, R. K., "Phenolic Compounds and Metabolic Regulation," B. J. Finkle and V. C. Runeckles, Eds., p. 120, Appleton-Century-Crofts, New York, 1967.
Bowers, W. S., *Science* **161**, 895 (1968).
Boyd, E. M., Bereczky, K., Godi, I., *Can. Med. Assoc. J.* **92**, 1292 (1965).
Braden, A. W. H., Hart, N. K., Lamberton, J. A., *Australian J. Agr. Res.* **18**, 335 (1967).

Braham, J. E., Jarquin, R., Bressani, R., Gonzalez, J. M., Elias, L. G., *J. Nutr.* **93**, 241 (1967).

Bressani, R., Elias, L. G., Jarquin, R., Braham, J. E., *Food Technol.* **18**, 1599 (1964).

Brockmann, H., *Progr. Chem. Org. Nat. Prod.* **14**, 141 (1957).

Byck, J. S., Dawson, C. R., *J. Org. Chem.* **32**, 1084 (1967).

Cain, J. C., *Nature* **188**, 774 (1960).

Camp, B. J., Steel, E., Dollahite, J. W., *Am. J. Vet. Res.* **28**, 290 (1967).

Caporale, G., Musajo, L., Rodighiero, G., Baccichetti, F., *Experientia* **23**, 985 (1967).

Chang, S. I., Fuller, H. L., *Poultry Sci.* **43**, 30 (1964).

Christensen, W. I., *Econ. Botany* **19**, 293 (1965).

Clandinin, D. R., Heard, J., *Poultry Sci.* **47**, 688 (1968).

Clare, N. T., *Advan. Vet. Sci.* **2**, 182 (1955).

Clawson, A. J., Smith, F. H., Osborne, J. C., Barrick, E. R., *J. Animal Sci.* **20**, 547 (1961).

Crombie, L., *Progr. Chem. Org. Nat. Prod.* **21**, 275 (1963).

Crombie, L., Thomas, M. B., *J. Chem. Soc.* **1967**, 1796.

Cruickshank, I. A. M., *Ann. Rev. Phytopathol.* **1**, 351 (1963).

Cruickshank, I. A. M., Perrin, D. R., "Biochemistry of Phenolic Compounds," J. B. Harborne, Ed., p. 511, Academic Press, New York, 1964.

C.S.I.R.O., Australia, Symp. "Lignification of Plants in Relation to Ruminant Nutrition," 108 pp. 1957.

Dadak, V., Zboril, P., *Collect. Czech. Chem. Commun.* **32**, 4118 (1967).

Dawson, C. R., *Trans. N. Y. Acad. Sci.* Ser. II, **18**, 427 (1956).

Dean, F. M., *Progr. Chem. Org. Nat. Prod.* **9**, 225 (1952).

DeEds, F., "Comprehensive Biochemistry," M. Florkin and E. H. Stotz, Eds., Vol. 20, p. 127, Elsevier, Amsterdam, 1968.

Degkwitz, E., Luft, D., Pfeiffer, U., Staudinger, H., *Hoppe-Seyler's Z. Physiol. Chem.* **394**, 465 (1968).

DeGraw, J. I., Cory, M., Skinner, W. A., Theisen, M. C., Mitoma, C., *J. Med. Chem.* **11**, 375 (1968).

Deuticke, B., Gerlach, E., *Klin. Wochenschr.* **45**, 77 (1967).

Deyoe, C. W., Deacon, L. E., Couch, J. R., *Poultry Sci.* **41**, 1088 (1962).

Di Giamberardino, T., *Corriere Farm.* **21**, 422 (1966).

Dixon, A. St. J., Martin, B. K., Smith, M. J. H., Wood, P. H. N., "Salicylates, an International Symposium," Little, Brown, Boston, 1963.

Dollahite, J. W., Housholder, G. T., Camp., B. J., *Southwestern Vet.* **16**, 114 (1963).

Dollahite, J. W., Pigeon, R. F., Camp, B. J., *Am. J. Vet. Res.* **23**, 1264 (1962).

Duquenois, P., *Quart. J. Crude Drug Res.* **7**, 1107 (1967).

Fairbairn, J. W., "The Pharmacology of Plant Phenolics," Academic Press, New York, 1959.

Fairbairn, J. W., *Lloydia* **27**, 79 (1964).

Feinstein, L., Jacobsen, M., *Progr. Chem. Org. Nat. Prod.* **10**, 423 (1953).

Feuer, G., Golberg, L., Gibson, K. I., *Food Cosmet. Toxicol.* **4**, 157 (1966).

Fiddler, W., Parker, W. E., Wasserman, A. E., Doerr, R. C., *J. Agr. Food Chem.* **15**, 757 (1967).

Flaig, W., *Qualitas Plant. Mater. Vegetabiles* **14**, 121 (1967).

Francis, C. M., Millington, A. J., Bailey, E. T., *Australian J. Agr. Res.* **18**, 47 (1967).

Fujita, T., *J. Med. Chem.* **9**, 797 (1966).

Fuller, H. L., Chang, S. I., Potter, D. K., *J. Nutr.* **91**, 477 (1967).

Geissman, T. A., Hinreiner, E., *Botan. Rev.* **18**, 77 (1952).

Gellerman, J. L., Schlenk, H., *Anal. Chem.* **40**, 739 (1968).

Goodman, R. N., Kiraly, Z., Zaitlin, M., "The Biochemistry and Physiology of Infectious Plant Disease," p. 187, Van Nostrand, Princeton, N. J., 1967.

Green, R. C., *J. Am. Med. Assoc.* **171**, 1342 (1959).

Hagan, E. C., Hansen, W. H., Fitzhugh, O. G., Jenner, P. M., Jones, W. I., Taylor, J. M., Long, E. L., Nelson, A. A., Browder, J. B., *Food Cosmet. Toxicol.* **5**, 141 (1967).

Hänsel, R., Weiss, D., Schmidt, B., *Arch. Pharmacol.* **301**, 369 (1968).

Hartwell, J. L., Schrecker, A. W., *Progr. Chem. Org. Nat. Prod.* **15**, 83 (1958).

Hazleton, L. W., Tusing, T. W., Zeitlin, B. R., Thiessen, R., Murer, H. K., *J. Pharmacol.* **118**, 348 (1956).

Heinstein, P. F., thesis, N. C., State University, 1967; *Dissertation Abstr.* **28**, 1342 (1967).

Hennequin, J. R., Juste, C., Lasserre, M., *Ann. Agron.* **18**, 545 (1967).

Herrmann, K., *Planta Med.* **10**, 29 (1962).

Hively, R. L., thesis, University of Delaware, 1967; *Dissertation Abstr.* **28**, 1421 (1967).

Huffaker, C. B., Kennett, C. E., *J. Range Management* **12**, 69 (1959).

Jacobs, M. B., *Am. Perfumer* **62**, 53 (1963).

Janower, M. L., Robbins, L. L., Wenlund, D. E., *Radiology* **89**, 42 (1967).

Jeng, M., Crane, F. L., *Biochem. Biophys. Res. Commun.* **30**, 465 (1968).

Jenkins, G. L., Hartung, W. H., Hamlin, K. E. Jr., Data, J. B., "The Chemistry of Organic Medicinal Products," Wiley, New York, 1957.

Jenner, P. M., Hagan, E. C., Taylor, J. M., Cook, E. L., Fitzhugh, O. G., *Food Cosmet. Toxicol.* **2**, 327 (1964).

Jones, R. W., Stout, M. G., Reich, H., Huffman, M. N., *Cancer Chemotherapy Rept.* **34**, 19 (1964).

Kaiser, H. E., *Cancer* **20**, 614 (1967).

Karrer, W., "Konstitution und Vorkommen den Organischen Pflanzenstoffen," Birkhaüser Verlag, Basel, 1958.

Kefeli, V. I., Turetskaya, R. Kh., *Fiziol. Rast.* **14**, 796 (1967).

Keil, H., Wasserman, D., Dawson, C. R., *Ann. Allergy* **4**, 268 (1946).

Keller, K., Klohs, M. W., *Lloydia* **26**, 1 (1963).

Kelly, M. G., Hartwell, J. L., *J. Natl. Cancer Inst.* **14**, 967 (1954).

Khan, A. A., *Nature* **216**, 166 (1967).

King, L. J., *Econ. Botany* **11**, 263 (1957).

Kinget, R., *Lloydia* **31**, 17 (1968).

Kingsbury, J. M., "Poisonous Plants of the United States and Canada," Prentice-Hall, Englewood Cliffs, N. J., 1964.

Kirby, K. S., *Brit. J. Cancer* **14**, 147 (1960).

Knypl, J. S., Rennert, A., *Flora (Jena)* **158**, 468 (1967).

Korpássy, B., *Progr. Exptl. Tumor Res.* **2**, 245 (1961).

Kuć, J., *Ann. Rev. Microbiol.* **20**, 337 (1966).

Kupchan, S. M., Hemingway, R. J., Hemingway, J. C., *J. Pharm. Sci.* **56**, 408 (1967).

Lapa, A. J., Sampaio, C. A. M., Timo-Iaria, C., Valle, J. R., *J. Pharm. Pharmacol.* **20**, 373 (1968).

Leistner, E., Zenk, M. H., *Z. Naturforsch.* **22**, 865 (1967).

Lemli, J., *Plant. Med. Phytother.* **1**, 201 (1967).

Lenz, F. J. Schürch, A., *Z. Tierphysiol., Tierernähr. Futtermittelk.* **22**, 236 (1967).

Link, K. P., *Harvey Lectures* **39**, 162 (1944).

Lotspeich, W. D., *Harvey Lectures* **56**, 63 (1960–1).

McAlister, W. H., Anderson, M. S., Bloomberg, G. R., Margulis, A. R., *Radiology* **80**, 765 (1963).

McKee, F. W., Hawkins, W. B., *Physiol. Rev.* **25**, 255 (1945).

Markiewitz, K. H., Dawson, C. R., *J. Org. Chem.* **30**, 1610 (1965).

Mechoulam, R., Gaoni, Y., *Progr. Chem. Org. Nat. Prod.* **25**, 175 (1967).

Moran, C. J., Walker, W. H. C., *Bioch. Pharm.* **17**, 153 (1968).

Morgan, W. Jr., Glick, B., *Poultry Sci.* **47**, 180 (1968).

Morton, J. F., *Econ. Botany* **15**, 57 (1961).

Moule, G. R., Braden, A. W. H., Lamond, D. R., *Animal Breed. Abstr.* **31**, 139 (1963).

Murthy, B. G. K., Siva Samban, M. A., Aggarwal, J. S., *J. Chromatog.* **32**, 519 (1968).

Nagashima, R., Levy, G., Back, N., *J. Pharm. Sci.* **57**, 68 (1968a).

Nagashima, R., Levy, G., Nelson, E., *J. Pharm. Sci.* **57**, 58 (1968b).

Nakagawa, Y., Shetlar, M. R., Wender, S. H., *Life Sci.* **4**, 753 (1965).

National Academy of Sciences, "Toxicants Occurring Naturally in Foods," Publication **1354**, 1966.

Neish, A. C., *Ann. Rev. Plant Physiol.* **11**, 55 (1960).

Netien, G., Lebreton, P., *Ann. Pharm. Franc.* **22**, 69 (1964).

Nilsson, A., Hill, J. L., Davies, H. L., *Biochem. Biophys. Acta* **148**, 92 (1967).

Nishie, K., Waiss, A. C., Jr., Keyl, A. C., *Photochem. Photobiol.* **8**, 223 (1968).

O'Hara, M. J., Kinnard, W. J., Buckley, J. P., *J. Pharm. Sci.* **54**, 1021 (1965).

Palmer, G., Horgan, D. J., Tisdale, H., Singer, T. P., Beinert, H., *J. Biol. Chem.* **243**, 844 (1968).

Papa, S., Lofrumento, N. E., Paradies, G., Quagliariello *Boll. Soc. Ital. Biol. Sper.* **43**, 1237 (1967).

Pathak, M. A., Worden, L. R., Kaufman, K. D., *J. Invest. Dermatol.* **48**, 103 (1967).

Phelps, R. A., *World's Poultry Sci. J.* **22**, 86 (1966).

Potter, D. K., Fuller, H. L., *J. Nutr.* **96**, 187 (1968).

Pridham, J. B., "Phenolics in Plants in Health and Disease," Pergamon Press, New York, 1960.

Racela, A., Grady, H., Svoboda, D., *Cancer Res.* **27**, 1658 (1967).

Radouco-Thomas, S., Grumbach, P., Nosal, G., Garcin, F., *Therapie* **20**, 879 (1965).

Raju, P. K., *J. Am. Oil Chemists' Soc.* **44**, 465 (1967).

Ramwell, P. W., Sherratt, H. S. A., Leonard, B. E., "Biochemistry of Phenolic Compounds," J. B. Harborne, Ed., p. 457, Academic Press, New York, 1964.

Razdan, R. K., Pars, H. G., Granchelli, F. E., Harris, L. S. *J. Med. Chem.* **11**, 377 (1968).

Reiser, R., Fu, H. C. *J. Nutr.* **76**, 215 (1962).

Rich, S., *Conn. Agr. Expt. Sta. Bull.* **663** (1963).

Roe, F. J. C., Field, W. E. H., *Food Cosmet. Toxicol.* **3**, 311 (1965).

Roehm, J. N., Lee, D. J., Sinnhuber, R. O., *J. Nutr.* **92**, 425 (1967).

Sauer, H., Hänsel, R., *Planta Med.* **15**, 443 (1967).

Schönberg, A., Sina, A., *Nature* **161**, 481 (1948).

Sethna, S. M., Shah, N. M., *Chem. Rev.* **36**, 1 (1945).

Shieh, T. R., Mathews, E., Wodzinski, R. J., Ware J. H., *J. Agr. Food Chem.* **16**, 208 (1968).

Shulgin, A. T., *Nature* **210**, 308 (1966).

Singleton, V. L., *Tech. Quart. Master Brewers Assoc. Am.* **4**, 245 (1967).

Slater, T. F., Riley, P. A., *Nature* **209**, 151 (1966).

Smith, M. J. H., Smith, P. K., "The Salicylates: a Critical Bibliographic Review," Wiley, New York, 1966.

Soine, T. D., *J. Pharm. Sci.* **53**, 231 (1964).

Spector, W. S., "Handbook of Toxicology," Vol. **1**, Saunders, Philadelphia, 1956.

Stanley, W. L., *Plant Phenolics Group N. Am. Proc. 3rd Symp.* **1963**, 79.

Stecher, P. G., Ed., "The Merck Index," 8th ed., Merck & Co., Rahway, N. J., 1968.

Stoll, A., Becker, B., *Progr. Chem. Org. Nat. Prod.* **7**, 248 (1950).

Stout, M. G., Reich, H., Huffman, M. N., *Cancer Chemotherapy Rept.* **36**, 23 (1964).

Stout, M. G., Reich, H., Huffman, M. N., *J. Pharm Sci.* **53**, 192 (1963).

Swain, T., *Mem. Soc. Botany France* **1965**, 176.

Thieme, H., *Planta Med.* **15**, 35 (1967).

Toth, L., *Planta Med.* **15**, 157 (1967).

Toth, L., Ferrari, M., Bruni, A., Santi, R. *Arch. Intern. Pharmacodyn.* **162**, 140 (1966).

Trenchi, H., *Avian Diseases* **4**, 275 (1960).

Truitt, E. B., Jr., Callaway, E. E., Braude, M. C., Krautz, J. C., Jr., *J. Neuropsychiat.* **2**, 205 (1961).

Tyman, J. H. P., *Chem. Commun.* **1967**, 982.

Tyman, J. H. P., Morris, L. J., *J. Chromatog.* **27**, 287 (1967).

Valle, J. R., Souza, J. A., Hyppolito, N., *J. Pharm. Pharmacol.* **18**, 476 (1966).

Vohra, P., Kratzer, F. H., Joslyn, M. A., *Poultry Sci.* **45**, 135 (1966).

Von Oettingen, W. F., Natl. Inst. Health Bull. **190**, 1 (1949).

Wang, T. S. C., Yang, T. K., Chuang, T. T., *Soil Sci.* **103**, 239 (1967).

Weil, A. T., *Econ. Botany* **19**, 194 (1965).

Weiss, G., *Psychiat. Quart.* **34**, 346 (1960).

Williams, A. H., "Comparative Phytochemistry," T. Swain, Ed., p. 297, Academic Press, New York, 1966.

Williams, R. T., "Detoxication Mechanisms," 2d ed., Wiley, New York, 1959.

Wolstenholme, G. E. W., Biol, F. I., Knight, J., "Hashish: Its Chemistry and Pharmacology," Little, Brown, Boston, 1965.

Wosilait, W. D., *Biochem. Pharmacol.* **17**, 429 (1968).

Zboralske, F. F., Harris, P. A., Riegelman, S., Rambo, O. N., Margulis, A. R., *Am. J. Roentgenol.* **96**, 505 (1966).

IDENTIFICATION OF THREE FLAVAN-3-OLS FROM GRAPES

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Abstract—Three phenolic substances present in sizable amount in grapes, particularly in underripe grape seeds, are relatively easily extracted into ether and were isolated and unequivocally identified as (+)-catechin, (+)-(2R:3S)-5,7,3′,4′-tetrahydroxyflavan-3-ol; (−)-epicatechin, (−)-(2R:3R)-5,7,3′,4′-tetrahydroxyflavan-3-ol; and (−)-epicatechin-3-gallate. These identifications clear up previous conflicting and incomplete reports and show that the grapevine synthesizes the catechin isomers to be expected from work with other plants.

INTRODUCTION

The phenolic substances of the grape berry have considerable importance as food constituents and reaction substrates.[1] The berry's total extractable phenols are present in only about 10 per cent or less in the pulp with the remainder about two-thirds in the seeds and one-third in the skin. The seeds are an important source of phenols in grape products, particularly red wines.[2] The catechin fraction extractable in ether is an important part of the phenols of grape seeds, red wines, and some white wines, and appears to be an important source of pigment in browned musts and white wines.[3] It also appears to be a significant source of bitter flavor.[4] Specific identification of the phenols in this fraction is therefore of considerable interest, particularly since the structural configuration of flavan-3-ols affects their rate of oxidation and other properties important in foods.[5]

Some workers consider that sufficient examples have been examined to conclude that only the 2R configuration occurs naturally in the flavanols of plants; that is, (+)-catechin, (−)-epicatechin, and the corresponding gallocatechins.[6] However, many reported identifications depended on paper chromatography and did not involve actual study of the optical activity. While it has been demonstrated that catechin epimers can be separated by adsorptive paper chromatography, the differences are small and identification by this means alone is considered uncertain, particularly in complex mixtures.[7]

In studies which did involve isolation of various fractions and measurement of their optical rotations,[8,9] the catechins of grapes or wine have been reported to be (+)-catechin,

[1] V. L. Singleton and P. Esau, *Advances in Food Research*, in press.
[2] V. L. Singleton and D. E. Draper, *Am. J. Enol. Viticult.* **15**, 34 (1964).
[3] J. A. Rossi, Jr., and V. L. Singleton, *Am. J. Enol. Viticult.* **17**, 231 (1966).
[4] J. A. Rossi, Jr., and V. L. Singleton, *Am. J. Enol. Viticult.* **17**, 240 (1966).
[5] J. W. Clark-Lewis, *Current Trends in Heterocyclic Chemistry*, p. 40, Butterworth's, London (1958).
[6] K. Weinges, *Phytochem.* **3**, 263 (1964).
[7] E. Haslam, *Chemistry of Vegetable Tannins*, 179 p., Academic Press, New York, (1966).
[8] S. V. Durmishidze, *Dubil'nye Veshchestva i Antotsiany Vinogradnoi Lozi i Vina*, 323 p., Izdatel. Akad. Nauk. S.S.S.R., Moscow (1955).
[9] S. V. Durmishidze, *Trudy Acad. Nauk. Gruz. S.S.R., Lab. Biokhim. Rastenii* 252 (1966).

($\pm$)-catechin, ($+$)-epicatechin gallate, ($-$)-gallocatechin, ($+$)-gallocatechin, and ($\pm$)-gallocatechin. Other investigations of these components of grapes have been largely made using paper chromatography. These reports are conflicting, some indicating the expected isomers others indicated the isomers not found in other plants or epimerized products.[1] Some appear confused (for example suggesting three separate paper chromatographic spots for ($+$)-, ($-$), and ($\pm$)-isomers) and proof of the true situation is considered insufficient.[1] It therefore seemed necessary to investigate more rigorously the specific identity of the major catechins naturally present in grapes.

RESULTS AND DISCUSSION

It was previously shown that the ether-extractable fraction from fresh grape seeds consisted of only three phenolic substances in appreciable amounts designated as E_1, E_2, and E_3.[10] Based upon paper chromatography, these appeared to be unspecified isomers of catechin (E_1), epicatechin (E_2), and epicatechin gallate (E_3). The latter substance tended to disappear from seeds of ripe grapes, so unripe grapes were the source chosen for further study.

Conditions designed to minimize epimerization or other reaction were used to isolate these three phenols in chromatographically pure form. Solvent extraction, partition chromatography, lyophilization, and other procedures were conducted rapidly, without heating, and with limited exposure to light or oxygen plus interim storage at low temperature. Recovery from underripe *Vitis vinifera* wine grapes (juice about 15 per cent sugar) was about 56 g of fresh seeds per kg of grape clusters and about 9·9 g of lyophilized extract containing all the extractable phenols per 100 g of fresh seeds. This dry solid extract was equivalent by Folin–Ciocalteu phenol assay[11] to about 610 mg of gallic acid per g of extract.

When this solid was subjected to partition column chromatography with mobile ether
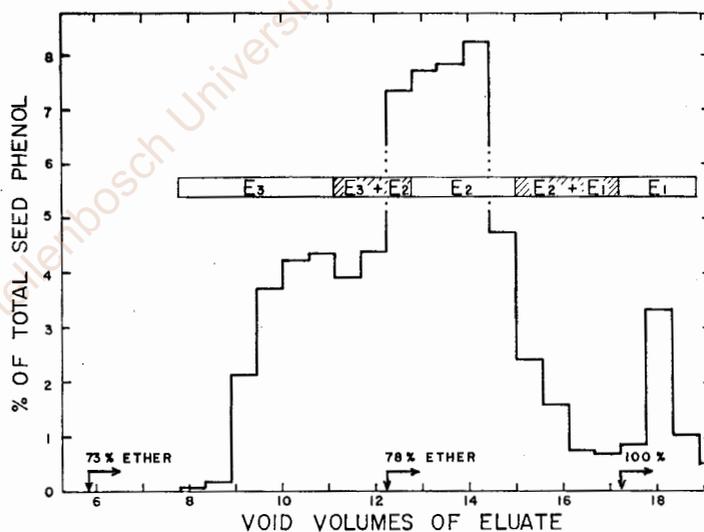
Fig. 1. Separation by column partition chromatography of E_3, E_2, and E_1 in pure form. The horizontal bar indicates the paper-chromatographically pure fractions and the overlapping fractions.

[10] V. L. Singleton, D. E. Draper and J. A. Rossi, Jr., *Am. J. Enol. Viticult.* **17**, 206 (1966).

[11] V. L. Singleton and J. A. Rossi, Jr., *Am. J. Enol. Viticult.* **16**, 144 (1965).

and aqueous stationary phases, the three phenols of interest moved as a group near the ether front. The combined ether eluate containing all three putative catechins typically assayed about 30 per cent of the total phenol extractable from grape seeds with a range of about 15 to 70 per cent in the limited number of diverse samples examined. By linear gradient methods it was shown that a relatively high ether content was required in ether–heptane mixtures for satisfactory chromatographic separation. Based on these studies a program of sequential development with 60, 73, or 78 per cent ether in heptane and 100 per cent ether was chosen and satisfactory recovery of a major portion of each of the three phenols in pure form was obtained (Fig. 1). The fractions found to contain similar components by paper chromatography were combined as shown by the horizontal bar in Fig. 1, and the respective combined fractions were E_3, 21·1 per cent; $E_3 + E_2$, 22·4 per cent; E_2, 40·9 per cent; $E_2 + E_1$, 7·9 per cent; and E_1, 7·6 per cent of the total ether-extractable phenol present in the original unfractionated "Zinfandel" seed extract.

A number of comparisons between the substances isolated from grapes, authentic catechin samples and values from the literature[12–14] are shown in Table 1. Chromatographically pure crystalline E_2 had the same melting point (Table 1), undepressed mixed melting point, and characteristic sintering at 145–155° as anhydrous (+)-catechin. Other properties of E_2 are essentially identical to those of (+)-catechin, notably specific optical rotation, acetate properties, and u.v. absorption (Table 1), as well as i.r. absorption spectra.[14] Further paper chromatographic comparisons also agreed with previous findings[10] that E_2 was indistinguishable from (+)-catechin.

Similarly, data from Table 1 and i.r. spectra plus paper chromatography show that E_1 is (−)-(2R:3R)-5,7,3′,4′-tetrahydroxyflavan-3-ol, ((−)-epicatechin).

Although precipitation of E_3 under conditions which had given crystalline products[13] gave nonbirefringent granules which fused near the reported melting point for (−)-epicatechin-3-gallate from tea, the acetate was nicely crystalline. Comparisons between E_3, E_3 acetate, (−)-epicatechin-3-gallate isolated from green tea and literature values for the latter[12, 13, 15] (Table 1) show essential identity between values for E_3 and for (−)-epicatechin-3-gallate.
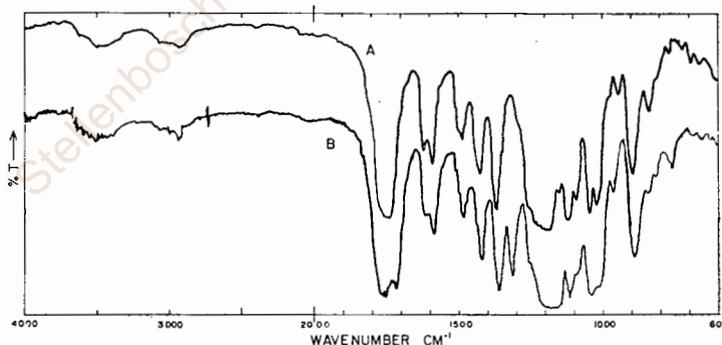
FIG. 2. INFRARED SPECTRA OF E_1 ACETATE (A) AND E_3 ACETATE (B). (RESP., (−)-EPICATECHIN PENTAACETATE AND (−)-EPICATECHIN-3-GALLATE HEPTAACETATE.)

[12] K. HERRMANN, Z. Lebensm. Untersuch. Forsch. 109, 487 (1959).
[13] L. VUATAZ, H. BRANDENBERGER and R. H. EGLI, J. Chromatogr. 2, 173 (1959).
[14] H. L. HERGERT and E. F. KURTH, J. Org. Chem. 18, 521 (1953).
[15] T. K. CHUMBALOV and M. M. MUKHAMED'YAROVA, Khim. i Khim. Teknol., Alma-ata, Sb 2, 209 (1964); Chem. Abstr. 64, 1011g.

TABLE 1. PROPERTIES OF ISOLATED AND AUTHENTIC SAMPLES OF FLAVAN-3-OLS

Substance	M.p. °		$[\alpha]_D$		λ_{max} nm		ϵ_{max}	
	Found	Lit.[12–14]	Found	Lit.[12–14]	Found	Lit.[12–14]	Found	Lit.[12–14]
E_1 (−)-Epicatechin	236–237	235–239	−58·3° (EtOH)	−69° (EtOH)	280	280	3,450	3,580
E_1 acetate (−)-Epicatechin pentaacetate	150–151	151–152	−13·2° ($C_2H_2Cl_4$)	−12° ($C_2H_2Cl_4$)	280	280	3,730	
E_2 (+)-Catechin	173–175 174	176–177	0° (EtOH) +16·1° (Me_2CO)	0° (EtOH) +17·1° (Me_2CO)	279	280	3,890	4,060
E_2-acetate (+)-Catechin pentaacetate	134–135 134–135	132–133	0° (Me_2CO) +16·0° (Me_2CO) +40·8° ($C_2H_2Cl_4$)	+40·6° ($C_2H_2Cl_4$)	280			
E_3 (−)-Epicatechin-3-gallate	236	253	−188° (EtOH)	−190° (EtOH)	279	279	13,595	14,000
E_3-acetate (−)-Epicatechin-3-gallate heptaacetate	122	119·5–120·5	−117° (Me_2CO)	−93·7 (benzene)[15]	280			

Mild hydrolysis of E_3 produced equal amounts of E_1 ((−)-epicatechin) and gallic acid as shown by paper chromatography. The i.r. spectra of the acetates of E_3 and of E_1 are very similar, indicating a close relationship (Fig. 2). Maxima at 1685 and 1315 cm^{-1} in the E_3 spectrum absent in that of (−)-epicatechin indicate that the gallic acid is ester-linked. The sharp maximum at 1315 cm^{-1} in the spectrum of E_3 acetate absent in that of E_1 acetate also strongly suggests E_3 is a gallate ester of E_1.

Authentic (−)-epicatechin-3-gallate from tea was compared with pure E_3 from grapes by one-dimensional paper chromatography in six systems. The respective R_f values (average of four replicates) were: 2% acetic acid 0·35, 0·34; 15% acetic acid 0·45, 0·44; 30% acetic acid 0·61, 0·59; butanol–acetic acid–water (4:1:5) 0·83, 0·83; butanol–acetic acid–water (4:1:2·2) 0·87, 0·87; and 2-propanol–water (3:2) 0·77, 0·77. In no case, including two-dimensional chromatograms, was any separation of mixed samples of the two substances suggested by the results. It therefore appears that E_3 is (−)-epicatechin-3-gallate.

EXPERIMENTAL

Grape Seeds and Their Extraction

"Emerald Riesling" white grapes (275·8 kg) from the University vineyard, Davis, harvested at a juice Brix of 15·8° were passed through a small winery-type stemmer and roller crusher. The rollers were so spaced that each berry was broken open but the seeds were not damaged. The crushed grapes were shaken in small portions in hardware-cloth baskets ($\frac{1}{4}$-in. mesh) beneath the surface of a tank of water so that the dense seeds escaped and settled to the bottom of the tank while the less-dense skins and most of the pulp was removed with the basket and discarded. The seeds obtained free of all other tissue by rinsing and rapid settling in water were drained and weighed 16·9 kg. The seeds were coarsely ground by passing them through a hand-operated wet mill (A. W. Straub & Co.), packed loosely into glass percolators and extracted exhaustively chromatographically with first 95% EtOH then 50% EtOH–water.[2] The combined extracts were freed from EtOH at low temperature in vacuo and lyophilized yielding 1830 g of dry extracted solids equivalent in phenol content[11] to 669·8 mg of gallic acid per g.

As another example, 192·9 kg of "Ruby Cabernet" red grapes at 15·2° Brix gave 9·6 kg of seeds and 874 g of extracted solids at 547·2 mg/g gallic acid equivalent. Since an unusually high proportion of E_3 was present in it, much of the work was done on a similar but much smaller sample from "Zinfandel" grapes, assaying 655 mg of gallic acid equivalent phenols per g.

Chromatography

A glass column 5 cm wide was packed with 5 g of dry Celite 545 diatomaceous earth, then 100 g of Celite 545 (wetted with 0·5 ml of H_2O per g containing 0·1% $K_2S_2O_5$ and 1% HOAc), and finally 12 g of Celite 545 (wetted with 6 ml of the same solution containing 3 g of the phenolic extract from grape seeds). The column was developed successively (each saturated with aqueous 0·1% $K_2S_2O_5$ and 1% HOAc) with 500 ml of heptane, 500 ml of 60% (V/V) Et_2O in heptane, 1300 ml of 73% Et_2O, 1000 ml of 78% Et_2O, and finally 300 ml of Et_2O free of heptane. Fractions (100 ml) were collected and analyzed by two-dimensional paper chromatography and for total phenol content.[11] Fractions qualitatively similar in composition were combined, concentrated in vacuo to a small volume of aqueous solution free of organic solvent and lyophilized.

Paper chromatography was conducted as previously described.[10] Fast blue V.B. salt, 0·05% in water, as a spray reagent gave rose color for E_1 and (−)-epicatechin, red for E_2 and (+)-catechin, and purple for E_3 without color changes when oversprayed with 20% Na_2CO_3 solution. Gallic acid gave purple becoming green with Na_2CO_3.

Crystallizations

E_1 was dissolved in MeOH, filtered, concentrated in vacuo until nearly saturated, and H_2O was added dropwise to induce crystallization. The crystals were filtered and dried in vacuo (P_2O_5) at room temperature for at least 1 week. E_2 was similarly crystallized as was a commercial sample of (+)-catechin. E_3 was precipitated from a concentrated MeOH solution by dropwise addition of CH_2Cl_2.

Acetates

Each known or isolated phenol (300 mg) was acetylated (K_2CO_3/Ac_2O) at 100° for 5 hr.

Instrumental Procedures

M.ps were determined in a capillary tube. Optical rotations were measured with sodium lamp, 1 dm micro cell, at 24°. Solvents and concentrations for polarimetry were: E_1, ethanol, 3·6 g/100 ml; E_2, acetone, 13·4 g/100 ml; E_3, 95% ethanol, 1·6 g/100 ml; E_1 acetate, $C_2H_2Cl_4$, 4·2 g/100 ml; E_2 acetate, $C_2H_2Cl_4$, 6·1 g/100 ml; E_3 acetate, acetone, 1·1 g/100 ml. U.v. spectra were determined in EtOH. I.r. spectra were determined in KBr wafers.

E_3 Hydrolysis

E_3 (20 mg) in 10 ml of 2 N HCl was refluxed for 5 hr. The flavanols precipitated as phlobaphenes, the solution was filtered, and the gallic acid was separated by paper chromatography. The amount of gallic acid produced was determined with a densitometer by comparing spot area and intensity from known amounts of gallic acid treated identically with $FeCl_3$–$K_3Fe(CN)_6$.[10] The gallic acid recovered was 0·65–0·91 moles/mole of E_3, averaging 0·75. If gallic acid alone was refluxed 5 hr in 2 N HCl, 10% of the gallic acid converted to degraded compounds immobile on paper in 2% acetic acid.

If hydrolysis of E_3 was carried out under milder conditions which avoided appreciable phlobaphene formation, E_1 was a major product as shown by paper chromatography and the apparent yield of E_1 was about equal to that of gallic acid.

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74

BROWNING OF WINES

Taken from a talk given by V. L. Singleton on May 28th, 1969, at the Viticultural & Oenological Research Institute, Nietvoorbij, to the Scientific Eno-Viticultural Association.

IT is proposed to outline some of the research conducted at Nietvoorbij this past vintage (Jan.-June, 1969) on browning of white wine and to discuss some of the implications of browning, as we understand it, in practical wine-making. Of course, many of the analytical results have only just been obtained and there has not yet been time to digest and interpret them in detail. Since it is expected that the results will be published in full in subsequent scientific papers, this report will be limited to preliminary findings and their apparent significance.

Before discussing the research it is a pleasure to acknowledge and thank a number of organizations and individuals who have co-operated in accomplishing our goals. I am greatly indebted to the K.W.V., to the Viticultural and Oenological Research Institute at Nietvoorbij, and to the University of California (Davis Campus). Particular thanks are due to Dr. C. J. G. Niehaus, Mr. C. B. Henning, Mr. F. J. van Zyl, Dr. J. A. van Zyl and Prof. H. W. Berg of these organizations. Professors C. J. van Wyk and C. J. Orffer of the University of Stellenbosch also gave indispensible assistance. Essentially the entire staff of the Institute at Nietvoorbij actively assisted at one time or another in this research (harvesting, sample preparation, taste panel, some analysis, etc.). I hope they will forgive me for not listing all their names and accept our thanks. Those who had very major parts in this work include P. de Wet, who was a co-worker throughout, and H. A. Sieberhagen, who supervised all the winemaking. Mr. C. S. du Plessis also was a very active co-operator during this research. A number of persons at co-operative and merchant's wineries not only were helpful in discussing mutual concerns in connection with browning, but also provided concrete assistance (sample bottles, etc.) in a number of instances. The universally enthusiastic co-operation which has been received is greatly appreciated and seems to me to typify a dynamic progressive wine industry such as is found here in South Africa.

The research and present understanding of browning in wine has been summarised in a review soon to be published (V. L. Singleton and P. Esau, "Phenolic Substances of Grapes and Wine and Their Significance", Adv. Food Research 17, Supplement 261 p.). From this work and active research at Davis and Stellenbosch, it seemed clear that the browning which occurs in wine exposed to air, is predominantly non-ezymatic oxidative browning of phenols. It was clear, however, that not all phenols had the same tendency or capacity to brown. Some phenols must brown more than others, since total phenol was an unsatisfactory measure of relative tendency to brown among a group of white wines. The evidence pointed to the monomeric flavonoids particularly catechins and perhaps leucoanthocyanidins as the most browning phenols. Singleton and Esau concluded that the phenols of white wine were predominantly derivatives of caffeic acid (and related non-flavonoids) with additional flavonoid compounds present depending largely on the degree of extraction from grape solids during crushing, pressing and fermentation. It appeared that flavonoid content would be a browning index.

Krammling and Singleton (Amer. J. Enol. Viticult., in press) quantitatively assayed the flavonoids and non-flavonoids by determining total phenol with the Folin-Ciocalteu reagent before and after precipitation of the flavonoids by reaction with formaldehyde in acid solution. They found the procedure reproducible and found about the same content of non-flavonoids in red wine as in white, but much more flavonoid in the red, of course. Further investigation of this method here has confirmed its value and it has been applied to determine the flavonoid content of a number of white wines specially prepared to give a range of browning potential. The measure of browning potential was also adapted from studies at Davis and at Stellenbosch. Forced browning at slightly elevated temperature in the presence of excess oxygen was used under standardised conditions.

Once these methods were verified, further objectives were to apply them to a series of white wines to see how well flavonoid content, so determined, correlated with browning, so estimated, and to correlate the findings with the vinification and variety used for the wine. Varieties selected were chosen for their importance in South Africa and their wide differences in tendency to brown. These were "Steen", "Green", "Riesling", "White French", "Colombard" and "Clairette blanche" varieties. When sufficient grapes were available (all but "Green" and a second harvest of "Steen") five lots of wine were made from each harvested variety. Immediately separated juice was settled and the clear top half formed one lot and the turbid bottom half the second. After these 2 lots were separated all 5 lots were inoculated with yeast and allowed to ferment at 16°C. The third lot was separated from its pomace 24 hours after inoculation, the fourth after 48 hours, and the fifth after 120 hours. The fermentation was continued to dryness with protection from access to oxygen in each instance and the wines were settled, racked, bottled and analysed after about a month.

In order to characterize the grapes obtained at each harvest, to compare the varieties, and to compare the phenol content of the grapes with that of the wine, berry samples were studied in some detail. The relative ripeness distribution was determined and the phenol content determined on the skins (husks), seeds, flesh pulp, pulp centrifuged from juice and clear centrifuged juice.

From a practical viewpoint it so far appears that the flavonoid content is a good estimate of a wine's tendency to brown with oxygen. The brown colour produced per unit flavonoid appeared more nearly constant within a variety than between varieties, but there appeared to be fairly good correlation in both instances. Wines made from the same grapes with more pomace contact have more flavonoid and therefore have a greater capacity to brown if allowed to do so. Varieties appear to differ in the amount of flavonoid and non-flavonoid their musts contain when prepared with the earliest possible juice separation and clarification. They also differ in the tendency to release further flavonoid easily from the solid parts into the must. The tendency to brown in these tests correlated well with general winemaker's observations that "Steen" and "Colombard" brown little or slowly, "Green" is a moderate browner, "White French" and "Riesling" are rather serious browners, and "Clairette blanche" has the highest potential to produce brown.

A preliminary sensory scoring of these wines by two tasters with the results averaged for all varieties is shown in the following table.

PRELIMINARY TASTING

Sample	Desirable Aroma (0—10)	Astringency (0—10)	Bitterness (0—10)	Quality (0—10)
Clear	6.6	3.6	4.6	13.5
Turbid	4.1	4.6	4.9	11.6
24 hr.	4.6	4.4	5.0	12.1
48 hr.	4.5	4.7	4.9	11.7
120 hr.	4.2	5.2	3.9	11.6

Several things are evident from these data and a consideration of the wines. The quality score is based on 13 to 16 being commercially acceptable wine with no noticeable defects and 12 to 9 being wines with defects which can be readily corrected to make them commercially acceptable. The wines as a group were judged as nearly free of defects and readily blended into commercial stocks as young dry white table wines, including those prepared by 5 days of fermentation on the pomace. The lack of a "red wine" character in these wines was quite surprising to those who had not examined such wines before. The colour was often somewhat darker in these "5-day" wines and of course they browned much more when exposed to oxygen, but some

were quite low in initial yellow colour and all were within the range of usual commercial white table wine colour. The "Riesling" samples tended to turn a faint pink, but this was less in the 5 day sample than in the others.

The most striking finding shown in the table is that the "Clear" samples had much more desirable aroma than the "Turbid" or other samples. The extra desirable aroma in the "Clear" sample was a delicate floral "blommetjie" character and is believed to account for the considerably higher quality scores for this set of samples. This aroma or its loss was evidently not directly related to phenol content, oxidation or browning potential because the wines from clear and turbid musts were nearly identical in these respects. The astringency was rated as slightly higher after the longest solids contact, as might be expected, and the bitterness was rated as slightly decreased, presumably owing to astringency masking the apparent bitterness. These wines are being retested by a 12-member panel. The results of this panel so far agree reasonably well with the preliminary tasting. The panels' results and the analytical details of this research will be the subject of scientific papers to be prepared together with the collaborators named earlier.

In the winery it is, of course, important to prevent enzymic browning by all the well known techniques of careful grape transport, SO_2 at the crusher, minimal oxygen contact with the must, etc. South African wineries are particularly aware and careful in this regard, it seems to me. It is also true that, considering brown colour only and not possible flavour changes, the yeast fermentation will remove much pre-existing brown by reduction to colourless products and perhaps also by absorption on the yeast cells. The minimal level of SO_2 to inhibit phenolase, the potential value of deliberate pre-fermentation browning, and other such subjects are poorly known and further research is indicated. However, enzymic browning is generally well controlled in South African wineries by the practices mentioned plus early removal of the insolubilized phenol oxidase enzyme through must clarification. The remaining problem appears to be prevention of nonenzymic browning after fermentation of the wine.

Our results indicate that lowered capacity to brown results from decreased contact with grape solids which gives lowered flavonoid content. However, there are indications from this and other research that more solids contact may be desired for other reasons. This means that we may have to accept a greater tendency to brown in return for more full, robust, less bitter flavour, for example. In our tests, heating (even of wines with high flavonoid content) under nitrogen gas gave very much reduced browning and as the elimination of oxygen or air contact was made more efficient, browning was further reduced. We believe that, if wine can be prevented from picking up oxygen from the air or if oxygen can be removed by efficient sparging with inert gas within a very short time after it is dissolved in wine, browning can be held to an acceptable level even in wine with a considerable capacity to brown. Browning of wines after leaving the winery in bottles can be minimised by making sure the wine is sealed in the bottle with nearly zero oxygen dissolved in the wine or in the headspace. The closure must be one which will effectively prevent entrance of oxygen for the period the wine is to be held before consumption.

The removal of precursors of brown by fining or adsorption is feasible and there is evidence in the literature suggesting that it can be done without removing astringent full flavour. More research will be required, but this research should now be easier, since it seems much clearer which fraction should be removed and its quantitative analysis is possible. Particulate, insoluble, protein-like polymers like polyamide (nylon) and PVP (polyvinylpyrrolidinone) tend to remove monomeric flavonoids in preference to either highly soluble chlorogenicacid-like or large astringent condensed tannin molecules (Rossi and Singleton, Amer. J. Enol. Viticult. 17, 231, 240 (1966)). Soluble fining agents like gelatin tend to remove the large tannins preferentially and leave behind the more brownable catechins. Further study along these lines appears promising from a practical viewpoint.

In closing let me thank again everyone who has aided me. It was a great pleasure to work with all my new-found friends in South Africa and although the period was short I believe it was quite productive for us all.

AN ANALYSIS OF WINE TO INDICATE AGING IN WOOD OR TREATMENT WITH WOOD CHIPS OR TANNIC ACID

V. L. SINGLETON, ANTHOULA RANDOPOULO SULLIVAN and CYNTHIA KRAMER

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ABSTRACT

American white oak, European white oak, cork, and redwood contribute predominantly nonflavonoid phenols to aqueous alcoholic extracts. Since the nonflavonoid content of wines by the assay presented is relatively constant, this assay can serve to monitor the progress of aging in wooden cooperage or certain other processes such as tannic acid removal in gelatin fining. It appears that treatments equivalent to about 2.5 g of American oak chips per liter of wine are near the recognition threshold for oakiness (but above the minimum difference threshold) and can be detected by this assay because they contribute about 45 mg/l of nonflavonoid. Analyses of wines with known aging in wooden cooperage generally agreed with the predicted effects and indicated that aging in small cooperage can give at least the equivalent of treatment with 15.5 g of American oak chips per liter of wine.

The progress of aging in wooden cooperage is followed almost exclusively by sensory judgment by the winemaker. Changes in composition have generally been so small or so subtle that methods of chemical analysis were inapplicable or promised to be very tedious. Although oak wood contributes extractable solids, phenols, acids, etc., to wine treated by chips or stored in barrels, in the range of wood treatment acceptable from a sensory viewpoint the amounts are small relative to the wine's original composition (1).

Singleton and Esau (2) suggested that formaldehyde precipitation could be used to determine flavonoid content of wine and noted that the tannins of grapes were condensed flavonoid tannins. Kramling and Singleton (3) developed such a method and applied it to analysis of wine. They verified that the nonflavonoid contents of all wines were similar because the nonflavonoid phenols are located almost exclusively in the juice of grapes. The large increases of total phenol in wines fermented in contact with pomace result from extraction of flavonoids from the grape solids.

The hydrolyzable gallic and ellagic tannins are not precipitated by formaldehyde in acid solution (2). Tannic acid as used in wine fining is hydrolyzable, and oak tannins from bark or heartwood are at least predominantly hydrolyzable tannins (2). It therefore occurred to us that an increase in nonflavonoid phenols could serve as an index of wood extract or residual tannic acid in wine and that an analysis should be possible which would be of practical use in monitoring the aging and processing of wine. This report gives the results obtained and conclusions reached in initial investigations of this possibility.

MATERIALS AND METHODS

The analytical procedure for nonflavonoids was the subject of further research which will be reported separately. The procedure used here was only slightly modified from that reported by Kramling and Singleton (3) so as to make the assay more convenient. This procedure is satisfactory for the type of comparisons reported, but the nonflavonoid

values are slightly high and slightly variable because the flavonoid-formaldehyde precipitate is slightly soluble in the analytical sample solution. Improvements in the analytical procedure to correct for this are being studied but do not invalidate the conclusions presented here.

The total phenol content of the sample was determined with the Folin-Ciocalteu reagent and the procedure of Singleton and Rossi (4) reduced in scale to a final volume of 20.0 ml (0.2 ml of sample or standard, 1.8 ml H₂O, 10.0 ml of Folin-Ciocalteu reagent diluted 1 ml plus 9 ml H₂O, and 8.0 ml of 75 g/l Na₂CO₃ aqueous solution). Samples and gallic acid comparison standards were prepared in duplicate and the duplicate values averaged. Samples, standards, and reagents were added by calibrated syringe-type diluters and dispensers. The color was developed for 2 hours at room temperature (23-25°C), and the absorbance developed at 765 nm was determined in reference to a blank sample in a Bausch and Lomb Spectronic 20 colorimeter with 1P40 phototube and red filter.

The formaldehyde precipitations were conducted by mixing 10.0 ml of the wine sample, 5.0 ml of 15% aqueous HCl, and 5.0 ml of 120 g/l aqueous formaldehyde and allowing the mixture to react and precipitate overnight under nitrogen gas at room temperature. A portion of the supernatant solution was then filtered through a cellulosic 0.45-µ membrane filter and the clear solution reanalyzed for phenol content as before except that the ½ dilution of the wine is compensated for by using 0.4 ml of sample solution plus 1.6 ml of water. The result is expressed as gallic acid equivalents (GAE) in mg/l and represents the nonflavonoid content. The flavonoid content is similarly expressed by subtracting the non-flavonoid value from the total mg GAE phenol per liter in the original sample.

Used for most chip-treated wines was a commercial sample of white oak heartwood chips (1). To compare variability among oak trees and for redwood, chips were prepared from individual staves and boards with a plane or jointer and then extracted with 55% v/v aqueous alcohol. The chips were weighed and immersed in the extracting wine or model solution at room temperature for various periods shown to give nearly complete extraction (1), usually 8 days. For correction of wood to dry weight, samples of the chips were dried to constant weight (6-10 hours) at 105-107°C. Moisture content of the original air-dried samples ranged between 3.06 and 8.40%, averaging 6.66%. Extracted solid was determined by drying a filtered sample of the extracts to constant weight (5-10 hr) at 50°C.

RESULTS AND DISCUSSION

The first point investigated was verification that the phenols extracted by aqueous alcohol from woody materials of interest to the winemaker were in fact largely nonflavonoid by our assay. Table 1 shows

TABLE 1

Analysis of Typical Woods Which Contact Wine to Demonstrate High Proportions of Nonflavonoid Phenols

Wood sample	Solids extracted g/100 g dry wood	Total phenols extracted mg GAE per g. extd solids	Nonflavonoid phenols extracted mg GAE per g. extd solids	% of phenols which are nonflavonoid
American oak				
Composite A	5.30	446.3	413.6	92.7
Composite B	6.00	375.0	373.1	99.5
Composite C	6.12	412.3	405.0	98.2
Stave 1	8.70	448.8	380.1	84.7
Stave 2	8.41	334.2	308.3	92.3
Stave 3	8.44	458.6	374.0	81.6
Stave 4	3.51	267.7	236.4	88.3
Stave 5	5.51	390.4	346.8	88.8
Stave 6	10.52	435.2	366.0	84.1
Stave 11	7.67	343.6	284.8	82.9
Stave 12	6.57	334.2	278.5	83.3
Stave 13	4.60	279.3	233.6	83.6
Stave 15	4.64	230.8	193.8	84.0
Stave 16	5.79	332.8	262.5	78.9
Stave 17	7.12	320.4	278.6	87.0
Stave 18	8.76	382.5	354.6	92.7
Stave 19	8.57	414.6	353.4	85.2
Average	6.45	365.1	320.2	87.7
Toasted chips B	6.98	363.2	360.7	99.3
Toasted chips C	6.84	417.0	363.7	87.2
European oak				
Limousin Stave	11.10	469.5		
Limousin A	10.37	613.2	520.6	84.9
Limousin B	14.07	655.3	547.3	83.5
Troncais	8.90	487.6	469.7	96.3
Troncais green	6.72	503.3	449.7	89.4
Nevers	11.42	616.5	542.9	88.1
Citeaux	10.13	579.7	501.7	86.5
Average	10.39	560.7	505.3	87.7
Redwood				
Board A	17.19	408.3		
Board B	19.03	456	438	96.1
Board C	17.03	685	685	100.0
Board D	12.54	603	592	98.2
Board E	13.93	686	648	94.5
Average	15.94	567.7	590.8	97.2
Cork				
Composite	2.44	141.4	137.9	97.5

that with various samples of American white oak heartwood from chips (composites) or individual barrel staves from unused barrels, an average of 87.7% of the extractable phenols were nonflavonoid and therefore should add to that fraction when taken into wine. The same high average percentage,

87.7%, was nonflavonoid among the phenols extracted from European oak of six samples of diverse French origins. Redwood samples and a composite granular sample of cork both had over 97% of the phenols as flavonoid.

Table 1 also shows that although individual wood samples vary considerably in extractable solid content, the total phenol content in that solid is less variable and the percentage of the phenol which is not flavonoid is nearly constant within woods of the same type. Toasting did not appreciably alter the characteristics in these respects.

The data of table 1 also confirm findings of Guymon and Crowell (5) that European oak usually contributes considerably more solids and more total phenols than American oak. In our tests (Table 1) the European oak gave, on the average, 161% of the solids extracted from American oak, and that solid contained 154% of the total phenol or non-flavonoid phenol content of the solids from American oak. Thus, the European oak usually contributes to an extract from a given weight of dry wood more than double (248%) the phenolic substances of American oak. In these brandy-like aqueous alcohol extracts as calculated from table 1, each type of woody material would typically contribute, at 1 g wood/l, the following respective amounts of total phenol and nonflavonoids, in mg GAE/l: American oak, 23.6, 20.6; European oak, 58.2, 52.5; cork, 3.4, 3.3; and redwood, 90.5, 88.0.

Having shown that most of the phenol of wood is nonflavonoid by our assay, the next study verified that, when added to wine, tannic acid, wood extracts, and fresh oak chips contributed measurably

to the nonflavonoid content of wine. The results are shown in table 2. Two different commercial samples of tannic acid were both essentially free of flavonoid components, and, when added to a white wine at only 150 ppm, both contributed about 85% of the amount expected from separate analyses to the total phenol and about 60% to the nonflavonoid. This result shows that tannic acid remaining in wine would be indicated by an increase in nonflavonoid content. Similarly, the addition of aqueous alcoholic extract of wood increased the total phenol and nonflavonoid analyses of a red wine nearly in proportion to the increases expected from separate analyses of the extract. Finally (Table 2), addition of white oak heartwood chips increased both the total and nonflavonoid content of wine progressively as more chips were added.

The recovery in wine analyses of the phenols and nonflavonoids from the wood chips (Table 2), was estimated from the analyses of an aqueous alcohol extract of the same lot of chips corrected for the difference in alcohol content (1). This is obviously only a rough estimate, and the apparent recovery of more than 100% is attributed to this cause. It appears significant, however, that the nonflavonoid fraction gives relatively uniform values regardless of the chip dose level whereas the total phenol recovery decreases with increased chip dose. This is believed to result from an effect of wood tannin to throw some of the wine flavonoids out of solution when present at high levels. That some of the wood tannin may also be thrown out of solution when added to wine is indicated by the 83-88% recovery by analysis of nonflavonoid. Since tannic

TABLE 2

Wood Extract, Wood Chips, and Tannic Acid Increase the Nonflavonoid Content of Wine

Wine	Treatment	Total phenols		Nonflavonoid phenols	
		mg GAE/l	% Recovery[a]	mg GAE/l	% Recovery[a]
	Tannic acid A, 150 mg/l	136		136	
	Tannic acid B, 150 mg/ml	121		118	
Dry white					
'Sauvignon blanc'	None	176		159	
'Sauvignon blanc'	Tannic acid A added	292	85	232	54
'Sauvignon blanc'	Tannic acid B added	282	88	239	68
Dry red					
'Carignane'	None	830		168	
'Carignane'	Wood Ext. B added	1015	97	320	83
'Carignane'	Wood Ext. 17 added	1000	89	314	88
'Carignane'	None	950		164	
'Carignane'	2.5 g Oak chips/l, 4 days	1000	151	203	126
'Carignane'	5.0 g Oak chips/l, 4 days	1034	127	256	148
'Carignane'	10.0 g Oak chips/l, 4 days	1040	68	318	125
'Carignane'	20.0 g Oak chips/l, 4 days	1204	95	477	127
'Carignane'	50.0 g Oak chips/l, 4 days	1504	83	884	117

[a] Estimated from previous analyses of wood extract B and Stave 17, and Composite Chips A, see also ref. 4.

acid would presumably represent a larger molecule than most of the wood tannin, it seems reasonable that its recovery as nonflavonoid is lower, 54-68% of the expected value.

Portions of a representative dry red wine were treated with 0.0, 0.5, 1.0, 2.5, 5.0, and 10.0 g of American oak chips per liter of wine in order to characterize more closely the minimal levels of oak chip treatment. Subsamples of each treatment level were taken at 2, 3, 4, 5, 6, 7, and 14 days of chip contact. The sets of subsamples were analyzed independently by two different analysts. The untreated sample of the wine was rated by the more experienced analyst as 961.6 mg GAE/l total phenol standard deviation 11.05, and 219.2 mg GAE/l nonflavonoid, standard deviation 8.7 mg/l. The less experienced analyst obtained similar values of 954.8 ± 37 and 224.8 ± 9.9. Similarly good agreement was obtained on the chip-treated wines.

It was found, in agreement with previous work (1), that the earliest samples had nearly complete extraction regardless of the chip dose, as shown by the combined data in figure 1. Thus, time was not a major variable and the data from both analysts and all dates was therefore combined to give the information on the total and nonflavonoid contribution of the chips to this wine, as summarized in figure 2. Note that the contribution by oak chips to the wine's nonflavonoid phenols was a linear function of chip dose, and, except for the lowest chip levels, so was the total phenol extraction. Note also, however, that the nonflavonoid content increased more rapidly than the total phenol content, again suggesting an interaction between wood phenol and wine flavonoids to cause some precipitation of the latter. For this reason and for the even more significant fact that the total phenol content of wine is more readily changed by processing effects, the nonflavonoid content is clearly much better than the total phenol content for monitoring the contribution of wood to a wine. Furthermore, if an original sample of the wine before wood contact is not available for analytical comparison, total phenol content would be valueless for estimating wood contact, whereas, since wines are relatively uniform in nonflavonoid content (3), nonflavonoid content could be informative even without a reference sample.

The average contribution of this composite and apparently typical sample of oak chips to the nonflavonoid content of this wine was 20.8 mg GAE/l per gram of chips at a chip dose of 0.5 g chips/l, and respectively 17.9, 16.4, 17.3, and 17.0 mg/l at 1.0, 2.5, 5.0 and 10. g chips/l for a grand average of 17.9 mg GAE nonflavonoid per liter per gram of chips. Since the standard deviation in nonflavonoid analysis in this wine was 9.9 mg/l even in the hands of a less practiced analyst, a statistically significant difference between single analyses should require 4 standard deviations, or 40 mg/l, in nonflavonoid content. That is, being 95% sure which of two samples of the same wine had been treated with wood chips would require analytical nonflavonoid levels at least 40 mg/l apart or replicated analyses.. Since 17.9 mg/l of nonflavonoid phenols were contributed by one gram of these oak chips this would indicate about 40/17.9 = 2.25 g of American oak chips per liter of wine as the minimum readily detectable level (or about 1.12 g/l of European oak).

Careful blind tasting of these chip-treated red table wines with a single experienced taster produced ratings of unrecognizable oakiness in the 0.0 and 0.5 g chips/l samples, doubtful to very slight oakiness at 1.0 g chips/l, slight to definite at 2.5 g/l, and very definite to strong at 5 and more g chips/l of wine. This indicates an approximate oak recognition theshold of 2.5 g American chips/l, essentially the same as the minimal analytical value. Separate tests some years ago (1), involving the same oak chips and the same taster, indicated a minimum *difference* threshold of 0.6 g chips/l in a
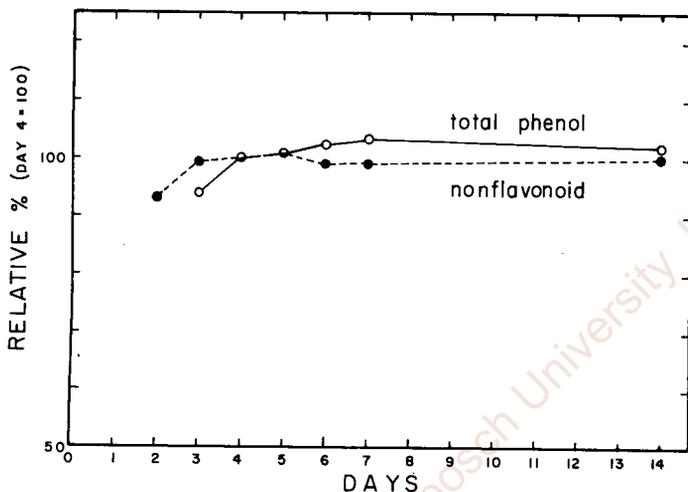
Figure 1. Extraction with time of phenols from oak chips relative to the value obtained after four days taken as 100%. Combined data for all 5 levels of chip dose.
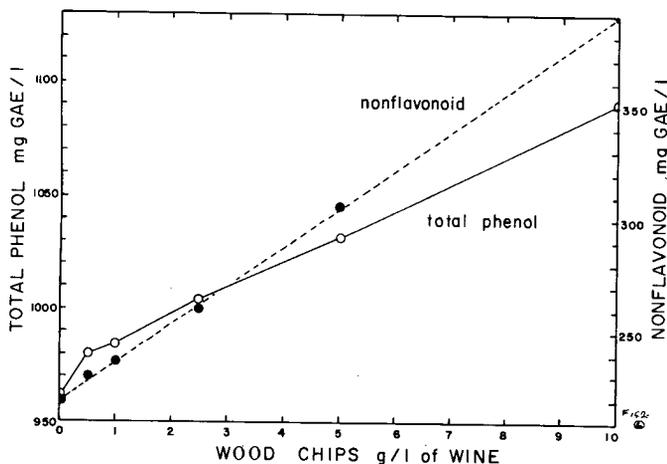
Figure 2. The contribution of total and nonflavonoid phenols to wine by oak chips at various doses.

port wine. The nonflavonoid contribution of these chips to wine at 2.5 g/l was 44.8 mg GAE/l, and one can therefore assume that 45 mg GAE of nonflavo-

noid of wood origin per liter of wine would indicate a sensorily recognizable level of oakiness.

To establish some basis for comparison, several

TABLE 3

Nonflavonoid Content of Rrepresentative Wines Without Any Wood Contact

White varieties	No. of samples	Nonflavonoid mg GAE/l		Red varieties	No. of samples	Nonflavonoid mg GAE/l	
		Avg.	Highest			Avg.	Highest
'Chardonnay'	18	168	200	'Pinot noir'	10	375	448
'Sauvignon blanc'	10	158	211	'Cabernet Sauvignon'	4	348	402
'Chenin blanc'	7	145	188	'Carignane'	3	206	216
'French Colombard'	7	158	172	'Refosco'	3	250	292
'Thompson Seedless'	2	158	168	'Calzin'	3	71	72
'White Riesling'	2	173	174	'Mission'	3	271	285
'Semillon'	2	128	130	'Napa Gamay'	3	249	284
'Flora'	2	168	168	'Mondeuse'	3	201	238
Average		157				271	

TABLE 4

Analyses of Wines with Known Storage in Wooden Cooperage

Wine	Wood treatment	Oak sensory rating	Total phenol mg GAE/l	Non-flavonoid mg GAE/l	Estimated[a] nonflavonoid from wood mg GAE/l	Chips[b] g/l equivalent
'Sauvignon blanc'	New Nevers bbl, 5½ mo.	3	419	384	184	10.3
'Dry red'	Used Amer. bbl, 6 yrs	5	1580	478	28	1.6
'Flor sherry'	Used Amer. bbl, 8-9 yrs	5	324	300	100	5.6
'Boal Madeira'	Used Amer. 10 gal, 4 yrs	0	344	268	68	3.8
'Port'	New Amer. 25 gal, 10 yrs	6	1432	664	214	12.0
'Barbera'	2740 gal redwood, 2 yrs	1.4	2307	350	0	—
'Barbera'	1900 gal Amer. oak, 1 yr	5.1	2250	358	8	0.4
'Barbera'	550 gal Amer. oak, 1 yr	4.1	2355	360	10	0.6
'Barbera'	Used Amer. bbl, 1 yr	5.4	2268	323	0	—
'Barbera'	Newly Shaved Amer. bbl, 1 yr	5.6	2162	323	0	—
'Barbera'	Used 60 gal Nevers, 1 yr	4.9	2304	395	45	2.5
'Chenin blanc'	German 340 gal, 6 mo	3	311	248	48	2.7
'Chenin blanc'	None	0	291	233	0	—
'Chenin blanc'	German 340 gal, 2 mo.	2	326	251	8	1.0
'Chardonnay'	New Nevers 60 gal, 6 mo	6	404	314	114	6.4
'Chardonnay'	New Nevers 60 gal, 1 yr	7	518	478	278	15.5
'Chardonnay'	New Limousin bbl, 5 mo	0	374	320	120	6.7
'Pinot noir'	Used Nevers 60 gal, 2 yrs	8	1490	606	156	8.7
'Merlot'	Used Nevers 60 gal, 2½ yrs	6	2014	320	0	—
'Cabernet Sauvignon'	None	2	1744	353	0	—
'Cabernet Sauvignon'	Used Nevers 60 gal, 3 mo	4	1798	352	0	—
'Cabernet Sauvignon'	Used Nevers 60 gal, 1 yr	4	1670	414	61	3.4
'Cabernet Sauvignon'	Used Nevers 60 gal, 3 yrs	5	1374	458	8	0.4
'Cabernet Sauvignon'	Used Nevers 60 gal, 18 mo	4	1596	404	0	—
'Cabernet Sauvignon'	New Nevers bbl, 3 wks	4	1752	286	0	—
'Cabernet Sauvignon'	New Nevers bbl, 8 wks	3	1792	300	14	0.8

[a]Minus 200 for white, 450 for red *or own untreated sample.*
[b]Estimated by dividing the previous column's values by 17.9, the average GAE mg contributed to wine by 1 g/l of the composite oak chips.

wines known to be free of wood contact and 100% from the grape variety named were analyzed for nonflavonoid content. The average and highest individual wine values are given in table 3. Analyses of a series of wines which had had known amounts of wood contact are shown in table 4. Some of the wines with considerable wood contact did show high nonflavonoid content and there is clearly a general tendency for newer wood, smaller cooperage, or very long time to give more nonflavonoid to the wine. The wines were rated (Table 4) for oakiness (by the single judge in the instances with integer values and by an 8-member expert panel with the decimal values) on an arbitrary 0-10 intensity scale where 1 g of chips/l was rated 3, 2.5 g/l was rated 4, and 5 g chips/l was rated 5 by the single taster.

From table 3 it was estimated that a nonflavonoid content of more than 200 mg/l in a white wine or over 450 mg/l in a red wine would indicate contact with wood (or tannic acid or other nonflavonoid source). On this basis, if no untreated reference wine was available or using the reference wine if it was, the nonflavonoid from oak was estimated and the American oak chip dose which would be required to produce this level was calculated as shown in table 4. With some exceptions the results agree with the known history of the wines. Even with these rather conservative estimates, natural aging in small cooperage apparently has given extract to wine equivalent to as much as 15.5 g of American oak chips/l of wine (nearly 130 lb of chips/1000 gal.) or perhaps 65 lb of European oak/1000 gal.

CONCLUSIONS

Although total phenol contributed by oak to wine would not be a satisfactory measure to monitor aging in wooden cooperage, it has been demonstrated that the assay presented for nonflavonoid phenols can be so used.

Further study will be required to refine methods and verify the estimates presented. It appears from these initial studies, however, that the method can certainly be useful in the winery to follow changes in a given wine during aging in wood and related processing. It even appears capable of estimating the kind and amount of wood aging that a bottled wine has had even when a reference sample of the untreated wine is not available, at least when the wood treatment is high or information is available on the typical nonflavonoid content of the wine type involved when it has had no wood or tannic acid treatment.

LITERATURE CITED

1. Singleton, V. L. and Diana E. Draper. Wood chips and wine treatment; the nature of aqueous alcohol extracts. Am. J. Enol. and Vitic. 12:152-158 (1961).

2. Singleton, V. L. and P. Esau. Phenolic substances in grapes and wine, and their significance. Academic Press, New York, p. 24, 67, 197 (1969).

3. Kramling, T. E. and V. L. Singleton. An estimate of the nonflavonoid phenols in wines. Am. J. Enol. and Vitic. 20:86-92 (1969).

4. Singleton, V. L. and J. A. Rossi, Jr. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. and Vitic. 16:144-158 (1965).

5. Guymon, J. F. and E. A. Crowell. Brandy aging: some comparisons of American and French oak cooperage. Wines & Vines 51(1):23-25 (1970).

EFFECTS ON RED WINE QUALITY OF REMOVING JUICE BEFORE FERMENTATION TO SIMULATE VARIATION IN BERRY SIZE

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ABSTRACT

Red wines were made from harvests of 9 grape varieties by removing or adding 10% free-run juice before fermentation on the pomace. This procedure was used to simulate variation in berry size without change in berry composition, and its justification is discussed. Berry size varied about ±60% among normal berries harvested from single plots of grapes of each of 5 varieties. Since two harvests of the same variety differed by 46%, the ±10% range in berry weight chosen for experiment appears to be within common variability. The wines produced covered a wide range of nondefective red wine composition. Significantly higher potassium content, pH, flavonoid content, anthocyanin content, aroma rating, red color rating, tannin (astringency) rating, and overall quality were found in direct correlation with decreased berry size or juice content. Comparison of chemical with sensory analysis indicated that in wines averaging about 1500 mg/l flavonoid (as gallic acid) a difference in flavonoid content of 268 mg/l did and 165 mg/l did not make a sensorily significant difference. Similar comparisons with anthocyanin pigment and visual color are discussed. Statistical analyses of the data are also discussed. Removal of juice apparently slightly lowered the efficiency of extraction of substances from the grape skin. Compositional changes were more nearly linear than sensory ratings of the resultant wines. The visual color of wines simulating large berries was higher than anthocyanin content would predict and was attributed to the lowered pH and other compensating factors. Also discussed are other sensory effects and some possibilities of capitalizing in vineyard management upon the increased red wine quality related to smaller berry size.

It is frequently stated that the varieties of grapes which give excellent table wine have small berries. This statement is often tied to the observation that premium varieties usually are shy-bearers, although many factors other than average berry size would affect yields in tons per acre or even pounds per vine. If it is true that, as a rule, premium wine varieties have smaller berries than do varieties making only ordinary wine, it would be likely that cultural, selection, or breeding practices that produce smaller berries without adversely affecting composition should improve wine quality. The author is unaware of data in the literature which would satisfactorily verify an inverse relationship between grape berry size and wine quality.

Anthocyanin pigment in the premier *Vitis vinifera* wine grapes is confined to the skin of the red or "black" berry. Since smaller berries would have more skin surface per unit of berry weight or volume, it seems clear that red pigment concentration and perhaps therefore red wine quality would be higher with smaller berries that are otherwise similar. It has been postulated (1) that this accounts for a historical but unrecognized tendency to select toward small berry size for red wine varieties but not for white wine varieties. A brief examination of several ampelographies gave an average berry diameter of 12.2 mm for 16 red and 12.8 mm for 15

Amer. J. Enol. Viticult., Vol. 23, No. 3, 1972

106

white varieties noted for wine production and grown in California. While the two sets of values overlapped and were so variable as to preclude statistical significance without much more data, the findings agree with the postulate. A number of the most prestigious red wine varieties, notably 'Pinot noir' and related varieties, have small berries, and none that come to mind have berries as large as the common fresh table grape varities. On the other hand some muscat grapes, Semillon, and other varieties capable of making high-quality white wines have large berries.

In view of these considerations, it appeared important to estimate whether a change in average berry size within the range viticulturally possible in a given variety would make a detectable or important difference in red wine quality. It has been said, particularly in the older and less scientific (though often astutely observant) literature, that a struggling vine makes the best wine. This is certainly not invariably true. It would seem contrary to physiological expectations and difficult to explain. A struggling vine would, however, tend to produce smaller berries, which might offer a reasonable explanation for the purported increase in red wine quality under certain types of vine stress. Furthermore, if quality effects can be shown to warrant it, it should be possible to develop practical agronomic procedures for decreasing berry size without, it is hoped, decreasing compositional quality or yield per acre.

The obvious way to investigate these questions would be to sort a population of berries by size and make the small and large berry portions separately into wine. The berries would have to be cut from the stems to enable sorting by size, however, which would entail a great amount of labor for the quantities necessary for winemaking. Furthermore, within a single harvested population from a single variety, berries which are smaller have fewer seeds and tend to ripen earlier or reach a higher Brix on a given date. Therefore, for the results to be meaningful specifically for berry size, it would be necessary to discard all berries that did not fall into a narrow density fraction (2) prior to sorting for berry size. To get sufficient berries to make a representative wine sample of two size ranges yet the same Brix, the amount of fruit and the labor was considered prohibitive, so another approach was sought.

Most of the readily removable free-run juice from freshly crushed red grapes is white and comes from the easily ruptured cells in the central pulp of the grape. The skin of a large grape is not obviously thinner than that of a smaller grape on the same cluster. It was therefore reasoned that removal of a portion of the free-running juice immediately after crushing would simulate to a satisfactory degree the composition of a must from smaller berries. By this approach, red wines were prepared from 9

grape varieties simulating the berry size as harvested, 10% smaller berries, and 10% larger berries. Chemical and sensory analyses were made of the resultant wines in an effort to estimate the importance of berry size variation to red wine quality.

Under some circumstances, particularly in other countries, red wines are made commercially after removal of a portion of the free-run juice from the must. Information was also obtained on the potential utility of that practice.

MATERIALS AND METHODS

The grapes were harvested between September 3 and October 22, 1971, from the University of California experimental vineyards: 'Carignane', 'Grenache', 'Malbec', 'Merlot', 'Napa Gamay', 'Ruby Cabernet', and 'Zinfandel' from Davis, and 'Cabernet Sauvignon' and 'Pinot noir' from Oakville plots. The grapes (about 400 lb) were crushed into a single container, treated with 75 ppm SO_2, inoculated with 1% of an actively growing Montrachet yeast culture, and mixed well. From this container 1-gal. portions were removed to other weighed containers (A, B, and C), and, with occasional mixing of the contents of the original container, transfer of 1-gal. portions in the sequence A, B, and C was repeated until all the must had been divided into three identical portions. Containers A, B, and C were weighed and mixed, and a portion of the free-run juice was removed from one by straining through a 2-mm-mesh screen and added to another so that the weight of the contents was decreased 10% in the one (A) intended to simulate 10% smaller berries, unchanged in the normal-berry (control) sample (B), and increased 10% in the portion (C) simulating increased berry size. The wines were then allowed to ferment side by side with as nearly identical treatment as possible. The cap was punched down twice or more per 24 hours and at the same time and in the same manner for each of the three lots of any one harvest. The wine was drained with only light pressing from the pomace after five days. After settling, the wines were racked once. They were then filtered with the addition of 25 ppm SO_2 in order of harvest within the period December 2-17, 1972.

The musts and filtered wines were analyzed by accepted chemical and sensory methods. All analyses except statistical were completed by February 3, 1972. The musts were analyzed for Brix and total acid, and the wines for pH, alcohol, potassium, volatile esters (as ethyl acetate) (3), anthocyanin (absorbance at 525 nm in 0.1 N HCl and a 1-cm cell times dilution), total phenol (4), and flavonoid (5). The sensory panel consisted of 9 trained tasters to whom the wines were each presented twice for a total of 18 judgments. The three wines constituting a set (i.e., the control, —10% and +10% juice for a single varietal harvest) were

presented together with the variety identified to the taster. The same amount of wine (about 50 ml) was presented in each sample in clear 9-oz. wine glasses under incandescent lighting with a white background. The tasters were asked to rate the wines for quality on the 0-20 scale long used in this department (6), where scores of 9-12 signify commercial but noticeably defective wines, and 13-16 indicate standard wines of increasing quality. In addition they were asked to rate (on arbitrary 0-low to 10-high scales) grape aroma (distinctiveness if varietal), color intensity, and tannin content (astringency) for each sample.

RESULTS AND DISCUSSION

Table 1 shows the average berry weights determined for 100-400 berries in several harvests. Also shown are the relative weights of the largest and smallest of the berries from the same sample which did not appear abnormal (excluding shot berries, water berries, etc.). Two harvests of 'Cabernet Sauvignon' taken the same day from plots in the same vineyard differed so much that the berries from one plot averaged 146% of the weight of those from the other plot. Within a single harvest of a single variety the large and small but normal berries averaged 170% and 42% of the average berry weight, and the values appeared fairly uniform among the 5 varieties in spite of a threefold difference in berry size. Thus, the ±10% variation in berry weight chosen for experimentation was well within the variation expectable in the field. Data collected previously in connection with other experiments also indicated that 20% or more variation in average berry weight is common among samples from different sources of ripe grapes of a given

variety. This served as a basis for the choice of ±10% experimental weight change, with the thought that it was small enough to be frequent and, if important, larger differences should be even more important.

Table 2 shows that the samples studied have a wide range of composition, 9.6 to 15.6% alcohol, for example, and collectively should cover most of the total compositional variation possible in sound red table wines. The data are consistent with expectations in several ways. The relatively high red color expected with 'Cabernet Sauvignon' and 'Ruby Cabernet' and the low color expected with 'Grenache' and 'Napa Gamay' are illustrated. The relatively high quality score for 'Ruby Cabernet' grown in climatic region IV and the high quality scores in 'Cabernet Sauvignon' and 'Pinot noir' particularly considering they were grown in a cooler region, are as expected. None of the wines were rated as seriously defective and were within the range considered commercial. The relatively high fruitiness of 'Grenache' and 'Pinot noir' is suggested by the volatile ester content.

TABLE 1

Variations in Berry Size

Variety	Av. berry wt. (g)	Large berry wt. (% of av.)	Small berry wt. (% of av.)
'Cabernet Sauvignon'[a]	0.99	160	38
'Cabernet Sauvignon'	0.68	242	31
'Grenache'	1.26	179	28
'Malbec'	1.86	163	41
'Merlot'	1.42	141	62
'Zinfandel'	2.10	137	55
Average		170	42

[a] This harvest was not otherwise used in these experiments. The rest were taken from the samples studied here.

TABLE 2

Composition and Quality of the Samples Studied

	Must		Wine			
	Brix	Acid (% Ta)	Quality[a] score	Color[bc] (A x diln.)	Ester[bd]	Alcohol[b] (%)
'Cabernet Sauvignon'	20.4	.5	14.0	21.2	45	12.8
'Carignane'	21.5	.9	12.8	9.0	90	12.7
'Grenache'	23.6	.7	13.0	4.6	110	13.3
'Malbec'	18.6	1.2	12.6	11.4	90	9.6
'Merlot'	21.0	.7	12.7	10.3	60	11.6
'Napa Gamay'	19.5	.9	12.4	5.8	70	10.6
'Pinot noir'	26.4	.7	14.0	11.0	170	15.6
'Ruby Cabernet'	22.8	1.0	13.9	22.5	110	12.0
'Zinfandel'	19.5	.8	12.7	8.4	70	12.7

[a] Average of all 3 treatments.
[b] Control wine value.
[c] Absorbance, 1 cm, 525 nm, times dilution in 0.1N HCl.
[d] ppm volatile ester as ethyl acetate.

For brevity, the complete data for the wine analyses are not shown. Table 3 summarizes some of the averages for the 9 varieties combined. The range of non-flavonoid phenol content in these wines in gallic acid equivalents (GAE) was 122-390 mg/l. The grape variety appeared to be the major source of

TABLE 3

Average Compositional Values[a] for Wines From 9 Grape Varieties

Analysis	"Small berry" (—10% juice)	"Normal berry" (control)	"Large berry" (+10% juice)
Nonflavonoid (gallic acid mg/l)	282	284	277
Volatile ester (EtOAc mg/l)	92.0	91.3	91.0
Alcohol (% by vol. at 60°F)	12.31	12.33	12.42
K (ppm)	1300	1275	1220
pH	3.35	3.34	3.32

[a] Values not underlined by the same line differ significantly at the 95% confidence limit by analysis of variance and the Duncan multiple-range test.

TABLE 4

Flavonoid and Anthocyanin Pigment Content of Wines Simulating Berry-Size Differences by Juice Removal or Addition

Variety	Flavonoid content (mg GAE/l)			Color (Absorbance at 525 nm times dilution in 0.1N HCl)		
	—10% juice	Control	+10% juice	—10% juice	Control	+10% juice
'Cabernet Sauvignon'	1405	1320	1227	22.95	21.25	19.25
'Carignane'	1138	1066	1038	9.35	9.05	8.75
'Grenache'	2070	1858	1705	5.05	4.65	4.23
'Malbec'	1354	1230	973	10.88	11.38	8.88
'Merlot'	1913	1571	1434	13.63	10.30	9.75
'Napa Gamay'	1018	916	857	5.80	5.75	5.53
'Pinot noir'	1479	1340	1211	11.70	11.05	9.35
'Ruby Cabernet'	3569	3537	3152	23.13	22.05	21.13
'Zinfandel'	765	689	574	8.75	8.45	7.38
Mean[a]	1635	1503	1352	12.36	11.65	10.47

[a] Values not underlined by the same line are significantly different by analysis of variance and the Duncan multiple-range test at 95% confidence limits or better.

variation, with 'Cabernet Sauvignon' being high and 'Napa Gamay' low in these samples. There were no consistent or significant differences related to juice removal or addition (Table 3). Since the non-flavonoid phenols of grapes reside primarily in the juice (5), this result would be anticipated.

Volatile ester content in the wines was affected very little by the treatment. It was very slightly higher in the wine simulating small berries and lower in the large-berry simulation; this trend was evident in 3 of the 9 sets and the overall average (Table 3). The differences were not statistically significant and appear too small for much sensory importance.

The tendency for higher alcohol content to be related to increased juice content (Table 3) was too small and variable to be statistically significant. It does appear correct from the known tendency for pressing of ripe but unraised grapes to yield lower-Brix must or lower-alcohol wines (7, 8). Similarly, skin juice or juice produced by pressing or longer extraction has higher potassium content and a higher pH (7, 8, 9). These differences were shown here also (Table 3), with the larger-berry simulation having slightly, though significantly, more acidic pH and less potassium content than the small-berry simulation.

Total phenol content of the wine was consistently higher in the small-berry simulation and lower in the large-berry simulation. However, since the total phenol values are the sum of the flavonoid and non-flavonoid fractions, only the flavonoid values are shown in table 4. Also shown in table 4 are the values for anthocyanin color. Note that with every variety the flavonoid content is less with the 10% added juice (large-berry simulation) and more with

the 10% removed juice (small berry simulation. The anthocyanin content values are almost as consistent, with only those for 'Malbec' not in the anticipated decreasing order of —10% juice, control, and +10% juice. In both flavonoid and anthocyanin content the average for each treatment for all 9 varieties is statistically significantly different from the other treatments.

The sensory analyses of the wines are shown in table 5. Since each listed value is the average of 18 judgments, an analysis of variance was possible for each variety as well as for the combined data. Significant differences from treatment are indicated in table 5. In some individual varieties and in the combined data, significant differences are shown indicating a negative correlation between berry size (by simulation) and aroma, red color, tannin, or overall quality of sufficient magnitude to be recognized by tasters unaware of the nature or identity of the samples. It thus appears that, other things being equal, a 20% decrease in average berry size and often a 10% one can be expected to produce a recognizable and therefore important increase in red wine aroma, color, tannin, and quality. Partial juice removal before pomace fermentation may be practical in some instances to accomplish the same

effects, but is unlikely to be widely useful in California under present conditions of high demand for red grapes and red wine.

Some interesting comparisons are possible between the chemical analyses in table 4 and the sensory differences detected (Table 5). Of course, there are reasons that comparisons must be made with caution. For example, the sensory analyses were made in some instances several weeks away from the chemical analyses. The anthocyanin estimate was made after dilution with 0.1 N HCl, whereas the visual color was estimated at the wine's natural pH. Owing to the Weber-Fechner relationship the sensorily detectable difference would depend upon the base concentration at which comparison was made. One would expect that the 'Ruby Cabernet' wines with high color and high tannin would require a larger difference in anthocyanin or flavonoid content to be detectable than, say, the 'Zinfandel' set. This does in fact appear to be the case (Tables 4 and 5). Other similar comparisons may be made which serve to estimate the magnitude of the compositional differences required to produce a sensory difference demonstrable by the panel of tasters.

The average of all the compositional differences between pairs of samples with a statistically significant sensory difference was 1.66 absorbance units for color and 268 mg/l flavonoid. Similar averages for the pairs of samples not sensorily different to a statistically significant degree in these tests were 0.89 absorbance units and 165 mg/l flavonoid. These mean differences indicate the relatively good agreement between the analytical and sensory data. Keeping in mind the cautions just mentioned, they give estimates of the minimum detectable differences to be expected in red wines.

It appears that the analytical color difference in terms of the tests used here is relatively large to produce a statistically significant difference in color rating when the wines are judged as a part of ordinary wine-quality ratings. It has been shown that the human eye is as capable as the spectrophotometer in finding differences in red wine color when panelists are asked to rate only color intensity and careful attention is paid to the lighting conditions, etc. (10). That much larger differences appeared to be required in these tests is interpreted as meaning that the judges were not focusing on barely detectable color differences, but were considering color differences of sufficient magnitude to have quality significance.

Differences in flavonoid content versus tannin rating seem more straightforward and important. It appears from the data presented that if red wines having about 1500 mg/l total flavonoid differ by about 268 mg/l in natural flavonoid content they

TABLE 5

Average Sensory Ratings (2 Ratings by 9 Judges) of Wines Prepared by Juice
Removal to Simulate Small Berries or Addition to Simulate Large Berries

Variety	Aroma (0-10) (distinctiveness if varietal)			Red color (0-10)			Tannin (0-10) (astringency)			Quality (0-20)		
	—10% juice	Control	+10% juice	—10% juice	Control	+10% juice	—10% juice	Control	+10% juice	—10% juice	Control	+10% juice
'Cabernet Sauvignon'	5.5	5.3	5.4	7.3	6.6	6.9	6.3	6.2	6.1	14.2	13.8	13.9
'Carignane'	4.0	4.1	3.7	5.4	5.3	5.1	4.6	4.7	4.6	12.9	13.2	12.3
'Grenache'	4.7	4.6	4.3	4.6	4.6	3.9	5.4	5.3	4.8	12.9	13.0	13.1
'Malbec'	4.8	4.2	4.4	6.1	4.9	5.1	4.8	4.7	4.6	12.9	12.3	12.4
'Merlot'	4.9	4.8	3.6	6.3	5.3	5.5	5.6	4.9	4.9	13.3	12.9	11.9
'Napa Gamay'	4.4	3.9	3.9	4.5	4.3	3.8	4.3	4.4	4.7	12.8	12.4	12.1
'Pinot noir'	5.8	5.8	5.4	5.7	5.4	5.2	5.1	5.3	4.9	14.2	13.9	13.8
'Ruby Cabernet'	6.1	5.8	5.9	7.6	7.2	7.1	6.6	6.5	6.4	14.1	13.8	13.7
'Zinfandel'	4.6	4.1	4.4	4.7	4.3	4.1	4.6	4.1	4.0	12.9	12.3	12.8
Mean	5.0	4.7	4.6	5.8	5.3	5.2	5.3	5.1	5.0	13.4	13.1	12.9

[a] Values not underlined with the same line differ significantly by analysis of variance and the Duncan multiple-range test at 95% confidence limits or better.

should be recognizably different in sensory tannin rating but that if the difference was only 165 mg/l they would probably not be sensorily recognized as different. The flavonoid group of compounds includes anthocyanins which, presumably, do not contribute to sensory effects recognized as tannin (astringency, etc.). Considering that anthocyanin differences did occur in these samples these flavonoid values necessary for sensory differences are expected to be higher than if only astringent tannin accounted for the difference. Nevertheless, the values are similar to those predicted from other studies (1, 11) and extend the comparisons with red wines.

Table 6 gives some details of the summary of analyses of variance. The high degree of significance in the differences caused by treatment (berry size simulations) is shown. The interactions between treatments and tasters or varieties were not significant, and it therefore appears that the varieties all responded similarly and the tasters reacted similarly to the difference produced by the treatments. The interaction between tasters and varieties is usual in wine studies because individuals commonly react differently to the varietal aspects of different wines and there are large differences related to the variety sampling in these wines. Similarly there is, as usual, a highly significant difference among tasters related to the parts of the rating scales they individually use as well as to differences in conclusion or detection by each taster. Note that if the taster x varieties interaction mean square is used as the
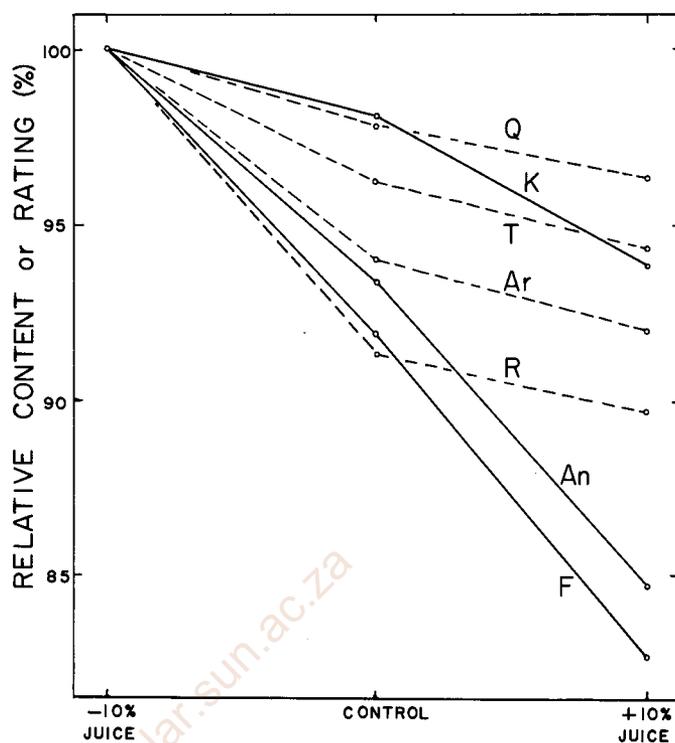
Figure 1. Relative mean contents (solid lines) of potassium (K), anthocyanin color (An), flavonoid (F), and sensory ratings (dashed lines) of quality (Q), tannin (T), aroma (Ar), and red color (R) for sets of wines prepared from 9 grape varieties by removal or addition of 10% juice prior to fermentation to simulate berry size differences.

TABLE 6

Statistical Significance of Sensory Ratings of Wines Simulating Berry Size Differences (F-Values and Error Mean Squares)

Source of variation	Degrees of freedom	Ratings (all varieties combined)				For significance	
		Aroma	Red color	Astringency (tannin)	Quality	F(95%)	F(99%)
Total	485						
Treatments	2	9.53**	22.86**	5.44**	12.03**	3.05	4.79
Tasters	17	56.07**	31.61**	75.65**	7.40**	2.00	2.19
Varieties	8	55.71**	89.59**	63.26**	28.46**	2.01	2.66
Treatment x tasters	34	0.94	0.92	1.77	1.08	1.82	—
Treatment x tasters	16	1.92	1.09	1.42	1.93	2.00	—
Tasters x varieties	136	5.46**	1.41*	5.95**	5.41**	1.35	1.53
Error	272	0.71	0.72	0.50	0.78		

* Significant at 95% confidence.
** Significant at 99% confidence.

denominator rather than the error mean square, the F-value for tasters becomes not significant for quality but remains significant for aroma, red color, or tannin ratings. This indicates that the tasters have had sufficient experience with the 20-point quality scale to tend to use the scale similarly. The other scales were evidently used less uniformly by different tasters, owing to less experience and comparison. The very large differences among wines from different varieties were highly significant even when compared with the tasters x varieties interaction.

The red-color visual ratings of the wines (Table 5) averaged much higher for the small-berry simulation samples than for the control or large-berry simulations. The difference in color rating between the control and large-berry simulation was unexpectedly small compared with the small-berry versus control difference. It might be postulated that this unexpectedly high color in the +10% juice samples was obtained because the addition of extra juice had enabled better extraction of color from the cap during fermentation owing to a larger fluid-to-solids ratio. That this is incorrect is shown in figure 1. The average flavonoid and anthocyanin color of the wines by chemical analysis were nearly 10% higher when 10% of the juice was removed, and 10% lower when an extra 10% juice was added to the must. The plots are not linear (Figure 1), but show that for anthocyanin, flavonoid, and potassium content the effect is opposite to the above postulation. The addition of juice to these red musts did not make extraction of these components more efficient, but, rather, removal of juice made it slightly less so.

The sensory ratings (Figure 1), however, all show effects opposite to those for the chemical analyses, the red color ratings being the most affected, and the quality ratings the least. It is believed that the relatively high visual color but low anthocyanin content in the +10% juice samples is explained by the more acidic pH of the wines having had juice added (Table 3). Small changes in acidity can have considerable effects on absorbance, equilibria with colorless forms, and polymerization of anthocyanins in wines (1, 12). Lowered potassium and increased alcohol contents in the +10% juice samples may also play a role, as may the relative content of flavonoid capable of polymerizing with the pigment.

The tannin sensory ratings for the large-berry simulation also average higher than would be predicted from the small-berry and control samples (Figure 1) assuming a linear relationship. This may be explained at least partly by a bias resulting from the tasters' knowledge that red-color intensity and tannin content often vary together. Red color is, of course, a factor in the overall quality of red wine, but only at low levels could we be certain that increasing red color would be considered an appreciable plus factor. Extra color above an acceptable

level may make the wine no better or even less attractive to the judges if the wine is very dark. Furthermore, increased tannin astringency may decrease overall quality rating. These facts may explain why, although the +10% juice samples averaged slightly higher in quality than would be predicted by linear extrapolation of the relative values for the small-berry and control or normal-berry simulations (Figure 1), the difference from linearity is less for quality than for the other features rated. The average aroma ratings also indicate that either the samples with +10% or —10% juice are rated better (higher) for aroma than would be predicted from the control and the other sample. Explanations for this would be speculative.

It appears clear from the data presented that a reduction in average berry size without berry composition change would improve the quality of red wine made from the grapes. Overcropping would decrease average berry size (13) but would also produce low sugar, low acid, and other undesirable compositional changes. For this reason it would be especially important to retain good Brix and acid levels when growing or buying red wine grapes of smaller average berry size. Growers, of course, would not want reduction in tonnage per acre either, without compensating increases in price per ton. After discussion with viticulturists in this department, two procedures appear to have the potential to lower berry size with minimal effects on yield or composition. Of course, other agronomic procedures may be found, and further possibilities are clonal selection or breeding.

Grapes, like most fruit, grow first by cell division and later by cell enlargement. The transition between the two stages occurs at veraison, the onset of ripening. If the vines are restricted during the early cell-division stage the berry's potential for enlargement during the cell enlargement stage is somewhat restricted. Late thinning of table grapes is known to have much less effect on enlarging berries than has early thinning (13). Experiments should therefore be tried involving pruning so as to set a potential overcrop and then thinning just after veraison. There is reason to believe that, properly timed, this could result in a normal crop yield of smaller but properly ripe berries (13).

A second promising possibility appears to be limiting the irrigation of the vine at certain stages. Withholding water early in the season will reduce berry size, and a slight water shortage just as maturity is approaching limits berry size and hastens ripening (13). Such observations probably tie most closely to the idea that a struggling vine makes the best wine. Further exploration of the practicality of these or other ways of capitalizing on the positive effect on red wine quality of reduced average berry size is out of place here. The only intent is to indicate that suitable methods appear possible and some may prove practical.

LITERATURE CITED

1. Singleton, V. L., and P. Esau. Phenolic substances in grapes and wine, and their significance. Adv. Food Res. Suppl. 1. New York, Academic Press. p. 1-282 (1969).

2. Singleton, V. L., C. S. Ough, and K. E. Nelson. Density separations of wine grape berries and ripeness distribution. Amer. J. Enol. Viticult. 17:95-105 (1966).

3. Libraty, V. Ester determinations and their application to wines. M. S. Thesis. University of California, Davis. p. 65 (1961).

4. Singleton, V. L. and J. A. Rossi, Jr. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Amer. J. Enol. Viticult. 16:144-58 (1965).

5. Kramling, T. E. and V. L. Singleton. An estimate of the non-flavonoid phenols in wines. Amer. J. Enol. Viticult. 20:86-92 (1969).

6. Amerine, M. A., E. B. Roessler, and F. Filipello. Modern sensory methods of evaluating wine. Hilgardia 28:477-567 (1959).

7. Benvegnin, L., E. Capt, and G. Piguet. Traité de vinification. Lausanne, Librairie Payot. 575 p. (1947).

8. Carles, J., A. Alquier-Bouffard, and J. Magny. De quelques variations apparaissant dans le jus de raisin au cours du pressurage. Compt. rend. Acad. Agr. France 48:773-80 (1962).

9. Ough, C. S. Substances extracted during skin contact with white musts. I. General wine composition and quality changes with contact time. Amer. J. Enol. Viticult. 20:93-100 (1969).

10. Berg, H. W., C. S. Ough, and C. O. Chichester. The prediction of perceptibility of luminous-transmittance and dominant wavelength differences among red wines by spectrophotometric measurements. J. Food Sci. 29:661-7 (1964).

11. Rossi, J. A. Jr., and V. L. Singleton. Flavor effects and adsorptive properties of purified fractions of grape-seed phenols. Amer. J. Enol. Viticult. 17:240-6 (1966).

12. Berg, H. W. Stabilisation des anthocyannes. Comportement de la couleur dans les vins rouges. Ann. Technol. Agr., Numero Hors Ser. 1, 12:247-59 (1963).

13. Winkler, A. J. General Viticulture. Berkeley, University of California Press. 633 p. (1962).

CHARACTERIZATION OF POPULATIONS OF GRAPES HARVESTED FOR WINE AND COMPENSATION FOR POPULATION DIFFERENCES

(Met opsomming in Afrikaans)
(Avec résumé en français)

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ABSTRACT

Single harvests of Clairette blanche, Colombar, Green, Riesling, White French and two harvests of Steen were sampled and each sample was segregated into specific density fractions by floating whole berries on sucrose solutions decreasing 1° Brix in the range 25–15° Brix. The resultant fractions were analyzed for berry mass, volume, juice Brix and pH. The juice Brix was very highly correlated with the Brix of the sucrose solution in which the berry just sank. The juice Brix was slightly lower than the average Brix of the sucrose solution Brix interval within which the berry just floated or sank. The difference was related to cultivar and different vineyards of the same cultivar and was attributed to the absence of appreciable air in the normal berry and density of the skins and seeds which were higher than that of the easily expressed juice. The utility of berry density segregation to characterize a given harvest, and to provide samples of precise juice Brix levels for comparisons among seasons, regions, vineyards and cultivars free of confounding differences in average ripeness is discussed and demonstrated.

INTRODUCTION

A recurring problem in viticultural and oenological research is the selection of fruit samples which truly reflect the vineyard harvest and the wine made from it with respect to characteristics and components which must, for practical reasons, be measured in small samples. Somewhat related is the larger problem of characterizing given harvests so that the product of different vineyard regions and different vintages can be compared in an efficient and meaningful way.

Sampling studies (Rankine, Cellier & Boehm, 1962; Roessler & Amerine, 1963) have generally been intended to provide a reliable estimate of field maturity in a given vineyard as measured by sugar or dissolved solids and pH or total acid contents. Analysis of the entire crop of a single vine has been shown to give a poor estimate of the vineyard as a whole because vine to vine variation is high. Variance of sample means is dominated by this vine to vine variation. It appears that the size of the crop on a given vine accounts for about half of the total variation in sugar and acid between vines (Rankine, *et al.*, 1962). On a given sampling date in a vineyard with typically similar vines of a single cultivar, the fruit on the vine with the lower crop tends to be riper with higher sugar and lower total acid. Although there is considerable variation among clusters on the same vine and among berries in the same cluster, random selections of clusters or of a sizeable number of individual berries from a group of vines give about equally good estimates of the composition of the total harvest from those vines. The sampling procedure generally recommended and that which appears most efficient yet effective in estimating average maturity (sugar and acid) of the entire crop of a group of vines, consists of collecting not less than 100 individual berries (and preferably 200-500), one or a uniform small number from each vine, from at least 10% or as many as possible of the vines chosen at random over the whole plot or vineyard.

Analysis of the juice from such a berry sample can characterize, for example, the average Brix of the juice to be expected if all the vines are harvested very shortly after the sample is taken. However, it cannot truly characterize the population of grapes because an average juice Brix of 20,0 could result from many different distributions of fruit above and below 20 Brix. Nelson, Baker, Winkler, Amerine, Richardson & Jones (1963) are believed to have been the first to apply density separation to intact grape berries. Singleton, Ough & Nelson (1966) showed that the Brix of the juice of grapes was closely similar to the Brix of the sucrose solution in which the intact berries would hover or barely sink or float. They used flotation of berries in a graded series of sucrose solutions to characterize populations of grape berries at various stages of ripening with two wine grape cultivars grown in two California vineyards. They suggested application of the technique to study fruit ripening free of weather effects, physiological limitation of maximum sugar storage in the berry, and differences which occur between wines prepared from single cultivars with the same average juice Brix.

The study to be reported here was undertaken to further develop and test the density segregation technique, to test it under South African conditions, to apply it to additional cultivars, to estimate its value in comparing regions and vintages, and to characterize the grape populations from which wines were prepared for detailed studies to be reported in subsequent papers.

PROCEDURE

The grapes were obtained from experimental plantings of the Elsenburg and Nietvoorbij vineyards. The cultivars, vineyard source and date (1969) of harvest were: Steen, head pruned 1,5 × 1,5m (5′ × 5′) planting Nietvoorbij, 25 February; Riesling, V-trellis 3,0 × 3,8m (10′ × 12,5′), Nietvoorbij, 10 March; Green, Elsenburg, 18 March; Steen, Elsenburg, 19 March; White French, Elsenburg, 24 March; Colombar, Elsenburg, 26 March; Clairette blanche, Elsenburg, 31 March. The grapes were usually picked in the afternoon, stored overnight in a 15°C cold room, and sampled early the next morning. In a few instances picking was early in the morning and sampling followed as soon as possible.

The harvests were intended to be about 220 kg (500 lb.) of fruit which was usually all that was available from the small experimental plots.

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Any cluster with appreciable mold or other damage in more than two or three berries was discarded. From each cluster the same number of berries, usually one, was snipped into each of two sample containers. The berries were snipped with small scissors so as not to break the berry skin and yet leave little or no pedicel. Ordinarily the cut was made between the torus and the berry and berries with cut skin or leaking juice were either replaced with another berry from the same cluster, or, if discovered later, discarded. The sampled berries were chosen at random over the clusters; damaged or abnormal berries (shot berries, etc.) were avoided.

One berry sample was used as a composite sample to represent the harvest average. The second berry sample was segregated by sequential dipping from 25,0 to 15,0° Brix sucrose solutions at one degree Brix intervals removing each time to a labelled container the berries which sank and passing on those which floated (e.g. 21-22° Brix = sank in 21° Brix and floated on 22° Brix). The sucrose solutions were prepared by mass-measuring commercial table sugar and distilled water. During dipping they were frequently rechecked by refractometer and readjusted by adding water (sugar to the 25,0° Brix) as required by the carry-over of sugar by berries from the previous higher Brix solution. Dipping from higher to lower concentration not only facilitated this adjustment, but also minimized possible water uptake by berries in solutions lower in sugar than their own juice concentration. After all the berries of the sample had been segregated, the Brix of the dipping solutions was again rechecked and adjusted if necessary and each fraction retested to ensure no errors had been made.

Prior to dipping, the berries were rolled gently without pressure back and forth in a dry towel to remove some of the surface wax so that air bubbles would not stick to the berries during the dipping. Following dipping, the sugar solution was removed from the surface by the same procedure. The total volume of each fraction was determined by covering the berries with a measured volume of water in graduated cylinders and subtraction to determine the water volume the berries occupied. This operation removed the residual sugar from the berry surface and was done rapidly to minimize water uptake. The berries were dried by rolling in a towel as before, counted, and the juice collected by crushing and firm squeezing by hand in cheese cloth. The juice was analyzed for Brix by refractometry and for pH by glass electrode *vs* calomel with a pH meter. The composite sample was similarly treated except that it was well mixed and then a 500-berry representative subsample taken and the berries were not segregated by dipping. The clusters after berry sampling were destemmed and crushed in the experimental winery and a sample of the free run must taken for comparison with the juice from the berry sample.

RESULTS AND DISCUSSION

The Brix of the juice obtained from each group of berries segregated by flotation from the seven populations sampled is shown in Table 1. It is apparent that, as expected (Singleton *et al.*, 1966), juice Brix increased as the maximum Brix of the sucrose solution in which the berry sank increased. A few results appear anomalous, notably the four least dense fractions of the berries from the cultivar Green. The berries of this harvest were more strongly bound by fibrous tissue to the pedicel and were much more difficult to snip off than was the case with the other cultivars. Further, it is believed that one person among several who helped snip only the Green sample, had not been sufficiently warned not to pull off rather than cut off the berries. It seems probable therefore that these anomalous results were caused by the introduction of an air bubble into some berries as the berry was pulled from the pedicel removing a "brush" of internal berry tissue with the pedicel. This conclusion is consistent with and seems substantiated by observations of physical and chemical composition of the Green population compared to the other cultivars of which will be mentioned later.

Self-correction for air-contaminated berries

Explanation of results which appear inconsistent is seldom worth much attention, but in this instance it seems important because it points out a self-correcting feature of flotation segregation. Introduction of an air bubble of the size of the pedicel brush evidently throws that berry into a considerably less dense category than would otherwise be the case. Since the air space in a hollow seed seems of the same order of magnitude and bird pecks, insect feeding, corkiness, rot, etc. often introduce appreciable air, these also should displace a berry into the low density fractions. This means that the more dense fractions should be free of such defective berries. As long as such defects are random in occurrence even the relative population distribution should be truly normal in the riper fractions if an adequately large number of mostly normal berries is segregated. Furthermore, since the defective, air-containing berries

TABLE 1 Brix of juice from grape berries segregated by density as determined by flotation in sucrose solutions of graded Brix
TABEL 1 *Brix van die sap van druiwekorrels wat d.m.v. flottering in sukrose-oplossings van spesifieke °Brix geskei is*

Sucrose solutions in which berries sank-floated (Brix) *Sukrose-oplossings waarin korrels gedryf of gesink het*	Cultivars							Average of all cultivars *Gem. van alle cultivars*
	Steen (I)	Steen (II)	Riesling	Green	White French	Colombar	Clairette blanche	
0–15	13,7	13,3	12,8	21,4	13,1	12,4	14,6	13,3
15–16	14,7	14,2	14,5	21,7	14,5	14,2	16,4	14,8
16–17	15,6	16,6	15,8	20,4	15,6	14,6	16,4	15,8
17–18	16,4	17,0	16,9	19,9	16,4	15,9	16,9	16,6
18–19	17,4	18,4	17,9	18,4	17,4	17,1	17,6	17,7
19–20	18,5	19,0	18,5	18,9	18,1	17,8	18,4	18,5
20–21	20,3	19,7	20,1	20,5	19,6	18,9	19,5	19,8
21–22	20,5	20,4	21,1	20,2	19,8	19,5	19,8	20,2
22–23	21,3	21,6	22,1	21,1	20,6	19,7	20,9	21,0
23–24	23,1	22,8	22,8	21,8	21,5	22,3	22,3	22,4
24–25	23,8	24,0	23,7	23,1	—	—	—	23,6
25–50	24,9	24,6	24,6	25,2	23,9	—	—	24,6

would have, on the average, a considerably higher juice Brix than that of the normal berries in the same low density fraction and the low-density fraction is a small portion of any nearly ripe grape population, the presence of abnormal air-containing berries would be indicated by an abnormally high juice Brix in the low-density berry fraction — as is shown by the Green variety samples in Table 1.

Relationships between juice Brix and segregation solution Brix

Regression equations and correlation coefficients were calculated for the juice Brix compared to the flotation solution Brix. Excluded from the calculations were the end fractions which included all berries which floated in 15° Brix or sank in 25° Brix sucrose solutions because these fractions might respectively be lower and higher in juice Brix than predicted from the intermediate fractions. All·other fractions were included if they in fact were represented in the samples except the anomalous fractions of the Green sample first sinking at 17° Brix or less. For convenience in comparison and calculation the nominal flotation Brix of the fraction was considered to be 0,5° Brix from the limit of the

fraction; i.e., if the grapes sank in 16° Brix and floated in 17° Brix sucrose solutions, the nominal flotation Brix of the fraction was considered to be 16,5° Brix. The results are shown in Table 2 arranged in order of increasing intercept value.

The direct correlation between flotation solution Brix and juice Brix is not unexpected, but the extremely high degree of correlation is gratifying. It also indicates that very precise juice Brix fractions of intact grape berries can be made by the flotation technique. No doubt reliable fractionation could be made at intervals of flotation Brix considerably smaller than 1° Brix. The fact that the correlation coefficient is only slightly lower for the Green sample than for the other samples shows satisfactory correction of the data by eliminating the anomalous values for the reasons discussed. That correlation is slightly lower for the combined data than for any one of the cultivars except Green (and is not raised by eliminating the Green data from the total) suggests that small but real varietal or environmental differences exist in the relationship between juice and berry density.
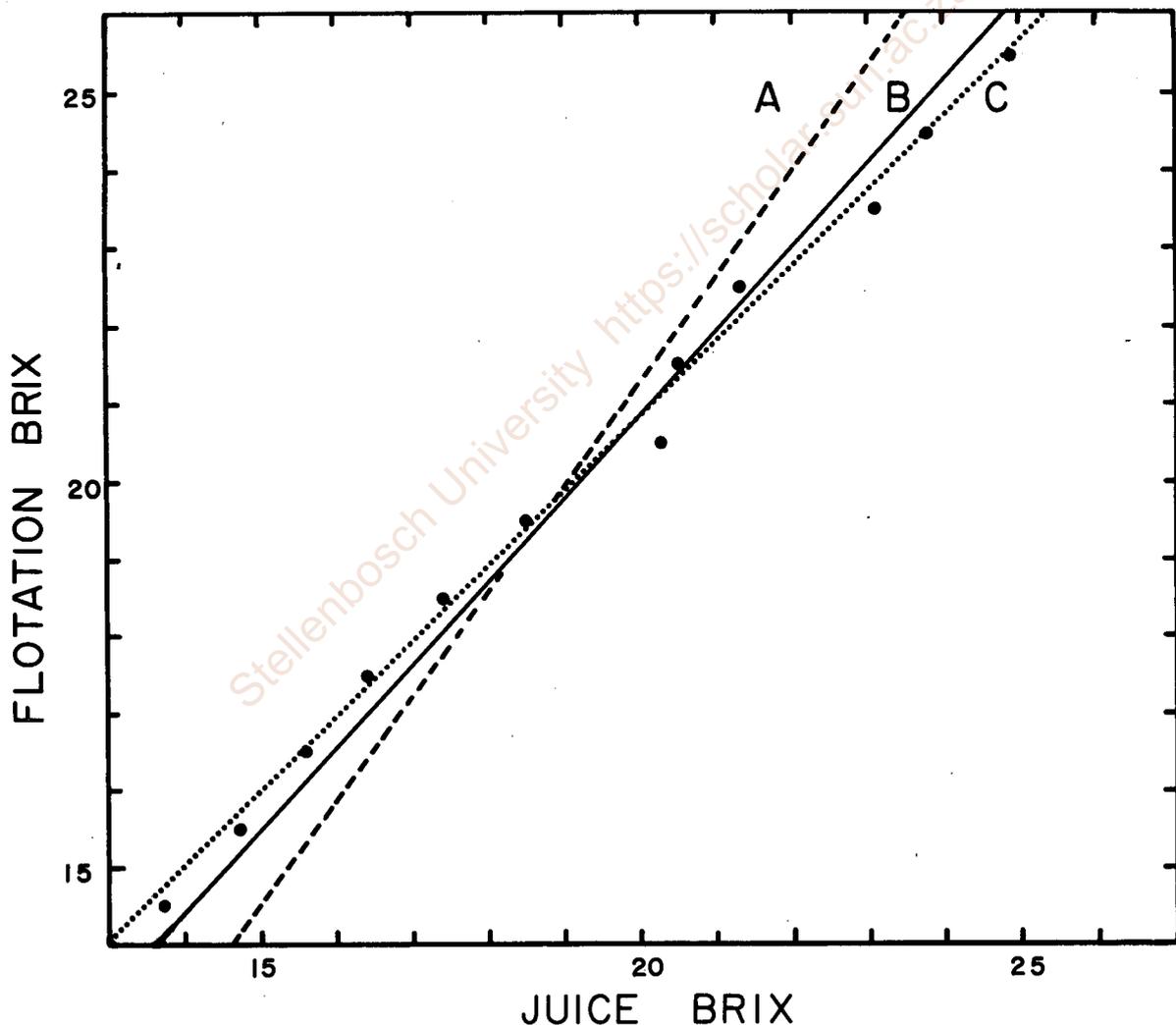
FIG. 1 The linear regression calculated by least squares method of the juice Brix compared to the nominal Brix of the sucrose solutions in which grape berries just floated or sank. A = Clairette blanche, the cultivar with the highest slope. B = combined data for all cultivars. C = Steen, first harvest; the cultivar with the lowest slope and for which the experimental values are shown by large dots.

FIG. 1 *Die lineêre regressie, bereken d.m.v. die kleinste vierkante metode, van die sap Brix vergelyk met die nominale Brix van die sukrose-oplossings waarin korrels net geflotteer of gesink het. A = Clairette blanche, die cultivar met die hoogste helling. B = gekombineerde data vir alle cultivars. C = Steen, eerste oes; die cultivar met die laagste helling en waarvan eksperimentele waardes deur swart punte aangedui word.*

3

TABLE 2 Correlation coefficients of juice Brix with flotation solution Brix and regression equations, for the harvested grape populations

TABEL 2 *Korrelasiekoëffisiënte van sap Brix met flotteringsukrose-oplossings se Brix en regressievergelykings vir die ondersoeksdruiwepopulasie*

Cultivars	*Regression equation values* *Regressievergelykingwaardes*		Correlation coefficient *Korrelasiekoëffisiënt*
	a = intercept *a = snypunt*	b = slope *b = helling*	
Steen (I) . .	— 1,55	+ 1,036	+ 0,996
Riesling . .	— 1,14	+ 1,024	+ 0,998
Colombar . .	— 0,52	+ 0,938	+ 0,985
Steen (II) .	— 0,18	+ 0,978	+ 0,990
White French .	+ 1,46	+ 0,857	+ 0,995
Clairette blanche · ·	+ 4,20	+ 0,743	+ 0,981
Green . . .	+ 4,66	+ 0,740	+ 0,974
All combined .	+ 0,57	+ 0,9327	+ 0,9794

*Regression equation: Juice Brix = a + b (nominal flotation Brix)

Regressievergelyking: Sap Brix = a + b (nominale flottering Brix)

The close correlation between juice Brix and flotation solution Brix is illustrated in Figure 1. The two complete harvests (not including the Green samples) with the highest and lowest slope (Steen 1 and Clairette blanche) from the regression equations (Table 2) are shown along with the data for all cultivars combined. Although the relationship is very similar, again differences appear (Fig. 1, Table 2) which indicate systematic varietal or population differences among the harvests. It is evident (Table 2) that the slope and intercept values calculated by the least squares, best linear fit method vary on both sides of an intercept of zero and a slope of 1,0 which would be a perfect 1-1 relationship between the two. However it is also clear that an increasing intercept value is associated with a decreasing slope in these data.

Consider now that a group of berries falling in a certain flotation density interval would in fact contain more berries on the high side of the density interval if it was on the low-Brix side of the bell-shaped population curve. Conversely, the fractions on the high-Brix side of the berry population would include more berries on the low side of the particular density interval. The juice from such fractions would therefore have a slightly higher Brix than that from berries at exactly the density of the middle of the flotation density interval if the fraction was below the mean juice Brix of the population and slightly lower if above the population mean. The degree of effect would be larger for the more closely grouped, narrow Brix-range populations and less for the low, wide form of the population distribution curve. The result of this effect would be to give a smaller change in observed juice Brix per unit change in nominal flotation Brix (lower slope) than if the berry density exactly equalled the nominal flotation solution density. This effect would be minimized if very narrow flotation Brix intervals were chosen. Since this effect would cause a consistent lowering of the slope of regression lines, regressions with higher slopes would appear less affected and more likely to be valid than those with lower slope.

This effect of the distribution curve of the population on the apparent relationship of juice Brix to flotation solution Brix is evidently a major contributor to differences among the regression equations (Table 2). Listing the samples in order of decreasing slope gives

Steen (I), Riesling, Steen (II), Colombar, White French, Clairette blanche, and Green. If one obtains graphically the span of juice Brix which includes the centre 85% of each population from a plot of juice Brix versus percentage of each berry population the respective values are in nearly identical order; respectively, they were 6,7; 6,6; 5,3; 4,4; 4,8; 3,1 and 3,5° Brix. No other factor such as mass of seeds per g of berry, berry mass, or mean population Brix appeared to correlate with this series and it is concluded that the relatively sharp, narrow distribution of Green, Clairette blanche, White French, and Colombar contributed in decreasing order to lowering the apparent slope of their regression equations of juice Brix on flotation Brix. A systematically low prediction of the slope with a pivot point near 19° Brix of the juice (Fig. 1) would also have the effect of increasing the intercept as is also seen in Table 2.

If one concludes therefore that a better general estimate of the rate of change of juice Brix with respect to flotation Brix would be obtained if a more disperse population were segregated, it is instructive to calculate the juice Brix which would have been obtained had the corresponding fractions of all seven harvests been combined after segregation. This calculation was made by summing the mass of the berries in each fraction times their juice Brix then dividing by the grand total of the mass of the fractions at the flotation interval. The calculated regression equation from the resultant 10 values (extremes at 14,5; 25,5 nominal flotation Brix again excluded) was: Juice Brix = 0,979 (nominal flotation Brix) - 0,86. The deviations of these values and of the experimental values from the nominal flotation Brix are shown in Table 3. Note that the juice Brix was invariably lower than the nominal flotation Brix and although the predicted trends (*i.e.* smaller differences on the rising and larger on the falling side of the population curve) appear, the data are more uniform than those from the individual cultivars as expected.

Factors other than juice Brix affecting berry density

It appears from these considerations that the best estimate of the slope of the juice Brix, versus nominal flotation solution Brix would be slightly higher than 0,98 or very near 1,0. This indicates that in relatively ripe (15-25° Brix) grapes of any given variety differences in the sugar content of the juice, control differences in the berry density. Assuming the regression equation slope is exactly 1,0 and calculating the intercept as the average deviation of juice Brix from nominal flotation solution Brix for the three most populous fractions for each harvest gives values of —0,6 to —1,6 (Table 4). The fact that in every instance the juice is lower in Brix than the respective flotation solutions shows that the content of low density substance and particularly air is essentially non-existent in the grape berry. This is in contrast to some other fruits which have large gas spaces (e.g. melons) or many tiny air pockets dispersed among the flesh cells (e.g. pineapples, apples). Since the flesh of a fairly ripe grape berry is very translucent, almost transparent, this lack of free air space in the fruit is perhaps no surprise, but this fact does appear very significant in considering gas exchange in the berry during photosynthesis, respiration, fumigation, etc.

As shown by the fact that juice Brix is lower than predicted from whole berry density (Tables 3, 4), the berry substance other than juice must be more dense than the juice. This was verified by experimentation. If the flotation solution's density was carefully adjusted so that a berry at the same temperature would just hover

4

TABLE 3 Deviations of juice Brix from the nominal Brix of the flotation solution
TABEL 3 *Afwykings van sap Brix van die nominale Brix van flotteringoplossings*

Nominal Brix of the flotation solution *Nominale Brix van die flottering-oplossings*	Steen (I)	Steen (II)	Riesling	Green	White French	Colombar	Clairette blanche	Average *Gemiddelde*	Weighted combined fractions of all cultivars *Geweegde gekombineerde fraksies van alle cultivars*	% of total mass *% van totale massa*
14,5	—0,8	—1,2	—1,7	—	—1,4	—2,1	+0,1	—1,19	—0,83	4,0
15,5	—0,8	—1,3	—1,0	—	—1,0	—1,3	+0,9	—0,75	—0,54	3,6
16,5	—0,9	+0,1	—0,7	—	—0,9	—1,9	—0,1	—0,73	—0,54	7,4
17,5	—1,1	—0,5	—0,6	—	—1,1*	—1,6	—0,6	—0,92	—0,95	12,8
18,5	—1,1	—0,1	—0,6	—0,1	—1,1	—1,4*	—0,9*	—0,76	—0,99*	16,8
19,5	—1,0	—0,5	—1,0	—0,6	—1,4	—1,7	—1,1	—1,04	—1,17	13,4
20,5	—0,2	—0,8	—0,4*	0,0	—0,9	—1,6	—1,0	—0,70	—0,84	13,1
21,5	—1,0*	—1,1	—0,4	—1,3*	—1,7	—2,0	—1,7	—1,31	—1,07	10,8
22,5	—1,2	—0,9*	—0,4	—1,4	—1,9	—2,8	—1,6	—1,46	—0,96	8,1
23,5	—0,4	—0,7	—0,7	—1,7	—2,0	—1,2	—1,2	—1,13	—0,71	5,4
24,5	—0,7	—0,5	—0,8	—1,4	—	—	—	—0,85	—0,80	3,2
25,5	—0,6	—0,9	—0,9	—0,3	—1,6	—	—	—0,86	—0,80	1,4

Ave./*Gem.*

| 15,5–24,5 | —0,84 | —0,63 | —0,66 | —0,93 | —1,33 | —1,72 | —0,81 | —0,975 | —0,850 | — |

*Most populous fraction
Fraksie met grootste aantal korrels

TABLE 4 Comparisons between the best estimate of the deviation of juice Brix from nominal flotation Brix and other factors affecting berry density
TABEL 4 *Vergelykings tussen beste benaderings van die afwyking van sap Brix van nominale flotterings Brix met ander faktore wat korreldigtheid beinvloed*

Cultivar	*Deviation juice Brix from flotation Brix Afwyking sap Brix van flottering Brix*	**Seed per g of berry *Pitte per g korrel*	**Surface per berry *Oppervlakte per korrel*	**Berry surface per 100 mg seeds *Korrel-oppervlakte per 100 mg pitte*	**Berry surface per seed *Korrel-oppervlakte per pit*	Berry mass *Korrel-massa*
		mg	cm²	cm²	cm²	g
Riesling	—0,60	41,6	7,52	8,6	3,8	2,0
Steen (I)	—0,80	34,5	6,48	11,2	4,3	1,6
Clairette blanche	—0,87	36,8	7,56	9,7	4,5	2,2
Steen (II)	—0,90	28,9	7,80	12,2	4,6	2,3
Green	—0,90	31,7	8,26	10,8	4,0	2,3
White French	—1,03	20,4	10,35	15,0	4,6	3,8
Colombar	—1,57	20,7	9,12	15,7	6,6	2,9

*Average of the deviation of juice Brix from nominal (average of the interval) flotation solution Brix for the fraction with the most berries and the two on either side of it. (Considered the best estimate for the sample)./*Gemiddelde van die afwyking van sap Brix van nominale (gemiddelde van die interval) flotteringsoplossing Brix vir die fraksie met die meeste korrels en die twee fraksies aan weerskante daarvan. (Beskou as die beste Benadering vir 'n monster).*
**Calculated from the data for the sink 20 - float 21° Brix sample, assuming spherical berries./*Bereken van die data van sink 20 - flotteer 21° Brix monster; korrels word as spheries beskou.*

within it without rising or falling and the berry was then squeezed to separate the juice into the flotation solution (a relatively large volume) while holding the berry beneath the surface, the skin invariably (among several berries tested) sank upon release. Of course, normal grape seeds are considerably more dense than the berry's juice and this has been used to separate seeds for other experiments (Su & Singleton, 1969). A total of 41 grape seeds from the cultivar Emperor all sank in 29° Brix and floated in 35° Brix sucrose solutions with an apparent average density equivalent to 32,1° Brix. It would appear therefore that differences among grape berry populations in the relationship between the berry density and juice Brix would depend on their proportions of dense seed and skin tissues relative to their juice content.

Since the seeds are the most dense it might be supposed they would have the greatest effect on the increase of the berry density over that of its juice. In fact, however, the mass of seeds per unit of berry mass decreased (Table 4) as the intercept value (assuming slope = 1,0) decreased (became a larger negative number). Increased berry mass and particularly increased surface per berry, calculated from the berry volume assuming spherical berries, did increase the more the juice Brix was below the flotation solution Brix. Thus, the skin tissues appear more important than the seeds in increasing berry density above that predicted from juice Brix. This is not unreasonable because the skins in these tests were about 4 to 6 times the total mass of the seeds. Further insight is gained by comparing berry surface per 100 mg of seeds or per individual

5

seed (Table 4) with decreasing intercept values. It seems clear that wine grapes from cultivars or harvests with increasing skin surface per berry (i.e., larger berries) tend to have juice Brix decreasing with respect to the berry density as measured by flotation solution Brix. This relationship is particularly strong if the berry size is large per unit of seeds and therefore the berry surface per unit mass or number of seeds is greater. The contribution of seeds to berry density, then, is complicated by the facts that increased number of seeds per berry gives increased berry size and more total skin surface (but less surface per unit of berry mass) within a cultivar, but may give more berry surface per unit of seeds in different cultivars.

The operation of these factors within a population at a single harvest of a single cultivar would evidently make the relationship between berry density and juice Brix deviate from linearity but apparently to a rather slight degree according to the data obtained. The berries with the greatest density as determined by flotation are the ripest (highest juice Brix) and yet are smaller berries and therefore must have fewer seeds per berry as shown in previous research (Singleton *et al.*, 1966; Singleton & Esau, 1969). Of course, the less ripe berries would be still enlarging and therefore the largest berries in a normal population would be those with intermediate Brix. Typical data are illustrated in Figure 2. Berry sizes relative to the respective 20 to 21° Brix flotation samples taken as 100% are shown for the

second harvest of Steen as a typical case, White French as the most different example, and the average of all 7 harvests combined. These data emphasize that the smaller berries ripen earlier, but they also show several other points. That occasional berries with empty seeds or air pockets from pedicel-removal damage etc. are thrown into the lowest density fraction or at least berries abnormally large but low in Brix are in the 15° Brix and less flotation fraction was shown by most of the samples and particularly the Green, as already discussed and Steen II in Fig. 2.

Possible indicator of overcropping

It is believed that the fact that the low Brix berries were relatively large in White French and to a lesser extent in Clairette blanche resulted from overcropping. This seems to have been proven for White French because not only was the amount of fruit per vine very high, but also for more than two weeks before this harvest the average juice Brix of these fruit measured in the field remained nearly constant and much below the cultivar's potential when the weather was such that adjacent vines of other cultivars were rapidly increasing their average juice Brix. Also the acidity of the White French was very low (0,48% tartaric) for the average Brix at harvest of 16,8. These are typical symptoms of setting too much fruit for the vine's capability to develop normally, i.e., overcropping. It therefore appears that density segregation which showed that the berries
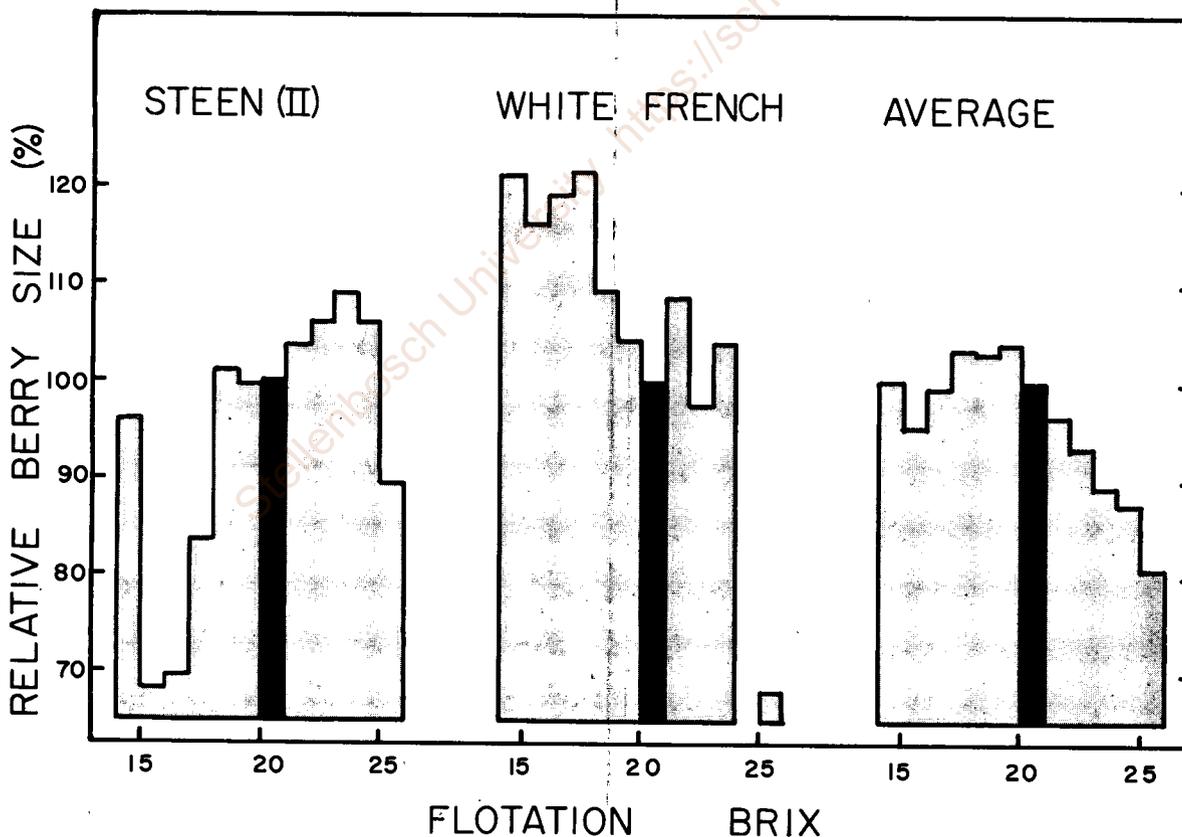
FIG. 2 The average berry mass in each fraction obtained by flotation segregation for density relative to the mass of an average berry in the 20–21° Brix fraction taken as 100% for two illustrative cultivars (Steen II and White French) and the combined data for all cultivars. The black sections represent the 20–21° Brix.

FIG. 2 *Die gemiddelde korrelmassa in elke fraksie, verkry d.m.v. flotteringskeiding t.o.v. digtheid, relatief tot die massa van 'n gemiddelde korrel in die 20–21° Brix fraksie wat vir twee illustrerende cultivars (Steen en Frans) as 100% geneem is, asook die gekombineerde data vir alle cultivars. Die swart gedeeltes dui die 20–21° Brix fraksies aan.*

had enlarged but still had not accumulated sugar (as in White French, Fig. 2) would be a new indicator of overcropping and potentially useful to judge pruning levels for the maximum crop of normal fruit.

Information from density segregation on other berry parameters and population characteristics

The data on juice Brix, population distribution, and pH for the Steen II harvest are shown in Fig. 3 as related to berry density by flotation fractionation. This harvest gave relatively complete and consistent data and appeared to develop the most normally in this rather rainy, cool harvest season. Note that the segregation by berry density which we have already shown gives good segregation for juice Brix also can give good segregation for ripeness-related variables such as juice pH and characterizes the population distribution, Fig. 3. Similar data for the other harvests with respect to population distribution are shown in Fig. 4. The fact that some populations were narrow, some wide, some high Brix, and some low Brix illustrates the power of the density flotation method to characterize these conditions.

The pH increases with increased ripeness as measured by sugar accumulation or juice Brix, Fig. 3, 5. Although this is well known in a seasonal progression sense, these data illustrate the same phenomenon within fruit harvested at the same time and therefore free of direct weather differences. By amplification of this sort of study it will be possible, for example, to determine the seasonal constancy or progression of pH or acid content within a single juice Brix or berry density fraction. A few apparently inconsistent pH values were obtained probably owing to variations in completeness of juice expression. The fact that higher pH juice is usually collected from stronger pressing of grapes is known and this is apparent in the Steen I data, Fig. 5. The

smaller samples (fewer berries) were readily expressed by hand squeezing and, since the 20 to 21° Brix fraction was separated into skins, seeds, juice, and pulp, comparably complete juice recovery and pH values were obtained. The fractions with large numbers of berries, however, on either side of the 20 to 21° Brix fraction were evidently not as completely expressed and the pH values were too low for comparison. This points up the necessity for attention to all details to prevent artificial variation in samples obtained by density segregation, if full advantage is to be taken of the minimization of natural variation produced by the technique.

The pH in the Green samples appeared (Fig. 5) too high in the low berry-density fractions and this was attributed to the effect of pockets of air shifting riper berries to low density fractions as previously discussed. The pH seemed to increase less with increasing berry density (juice Brix) for Clairette blanche and perhaps White French and this may relate to previous suggestions of wider population distributions and/or overcropping in these harvests. Of course varietal difference may also be involved.

It seems clear that density segregation can be used to characterize populations of grape berries harvested at a given time from a given vineyard of a single cultivar. It remains to be considered whether the properly recombined data are equivalent to unsegregated fruit and whether comparison of a single flotation fraction with the same fraction from another population is a better estimate of the intrinsic difference between the populations owing to vineyard, season, variety, etc. separate from average ripeness effects. For our purposes the berry fraction which sank in 20° Brix and floated in 21° Brix sucrose solution was chosen as the fraction for comparison among different populations. Perhaps other fractions would serve better for some purposes and of course detailed comparison of more than one fraction
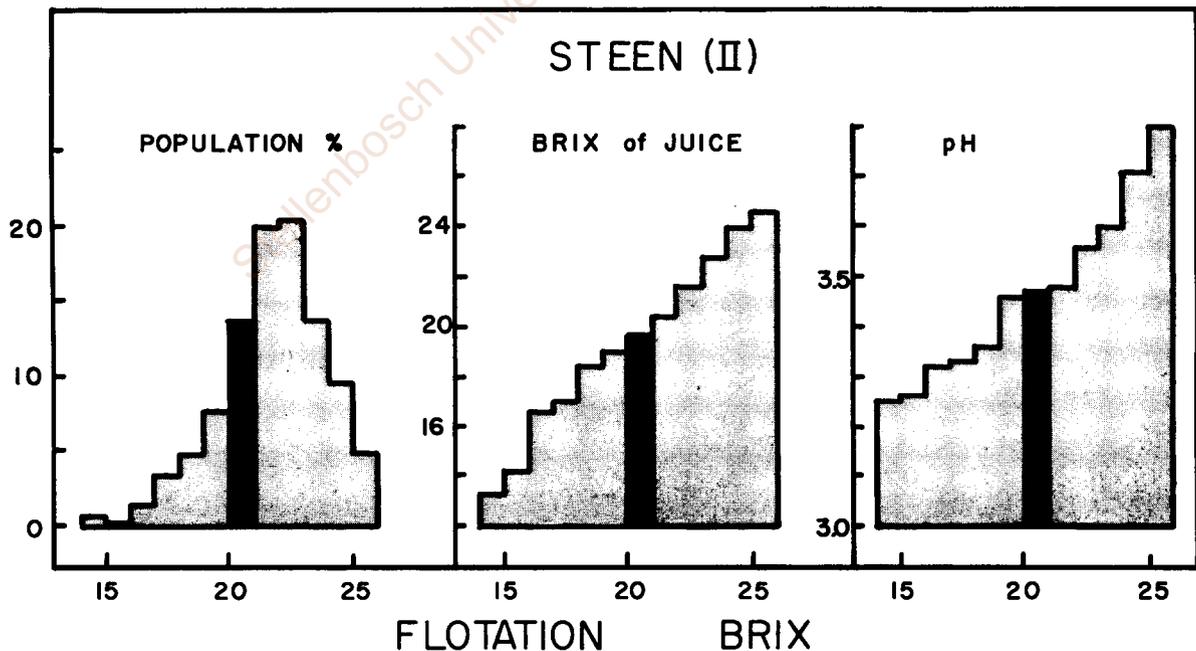
FIG. 3 For Steen, second harvest, the population distribution, Brix and pH of the juice of the berry density fractions obtained by flotation on sucrose solutions of 15–25° Brix. The black sections represent the 20–21° Brix fractions.

FIG. 3 *Die populasieverdeling, °Brix en pH van die sap van die korrel-digtheidsfraksies, wat verkry is d.m.v. flottering in 15–25° Brix sukrose-oplossings, van Steen II. Die swart gedeeltes dui die 20–21° Brix fraksies aan.*
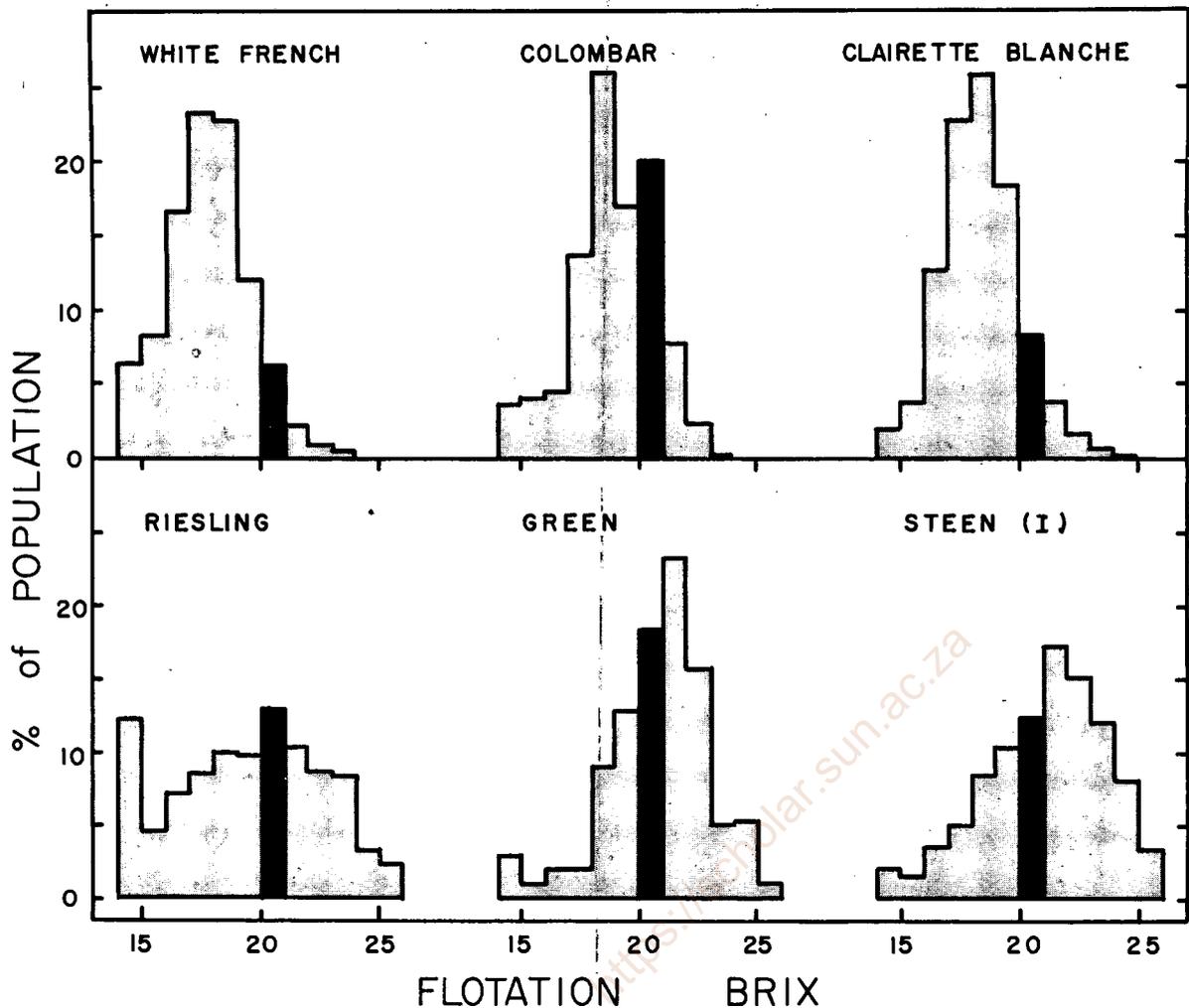
FIG. 4 The distribution of the berry population with respect to density for harvests of 6 grape cultivars fractionated by flotation on sucrose solutions of 15–25° Brix. The black sections represent the 20–21° Brix fractions.

FIG. 4 *Die verdeling van die korrelpopulasie m.b.t. digtheidsfraksies, verkry d.m.v. flottering in 15–25° Brix sukrose-oplossings, vir 6 cultivars. Die swart gedeeltes dui die 20–21° Brix fraksies aan.*

would be preferred. We chose the 20 to 21° Brix fraction because it was one which can be found when the average juice Brix is still relatively low for wine-making. It is still present in reasonable abundance when the grapes average above the preferred Brix for winemaking, and among grapes optimum for high quality table wines it would be one of the predominant fractions. The 20 to 21° Brix flotation fraction is emphasized for easy comparison in Fig. 2 to 5. Note that this fraction is riper than most of the fruit in the harvests of White French, Colombar and Clairette blanche, underripe in the two Steen and the Green harvests, and about average for the Riesling (Fig. 3 and 4).

In Table 5 data are listed for the 20 to 21 fraction, unsegregated samples, and values obtained by recombining the values for the density fractions weighted according to their proportion in the population. The appropriate values for comparison, columns B and C in Table 5, are most nearly identical and in all instances within the variation we have obtained on replicate 200 tot 500 berry samples properly chosen to represent

the vineyard population. It seems clear from these data that even for pH (which might be questioned on mathematical grounds - averaging logarithmic values) recombining the data in proportion to the contribution to the population gives a good estimate of the unsegregated population. This means that the extra information on population distribution with respect to sugar content etc. can be gained by this technique without sacrificing the usual representation of the average value for the sampled population. It is also shown that the berry sampling technique gave a good estimate of the must Brix and wine pH as obtained from the entire field harvest of 220 kg (500 lb) or so. With one exception the winery must tested slightly higher in Brix than did the recombined berry sample. This difference averaged 0,5° Brix. Several factors might have contributed to this slight difference including the fact the hydrometers were used for the winery value and a refractometer for the berry juice values. However, it is suspected that it resulted from the exclusion of all shrivelled and raisined berries in the berry sampling, but, of course, not from the large wine lot. Close agree-
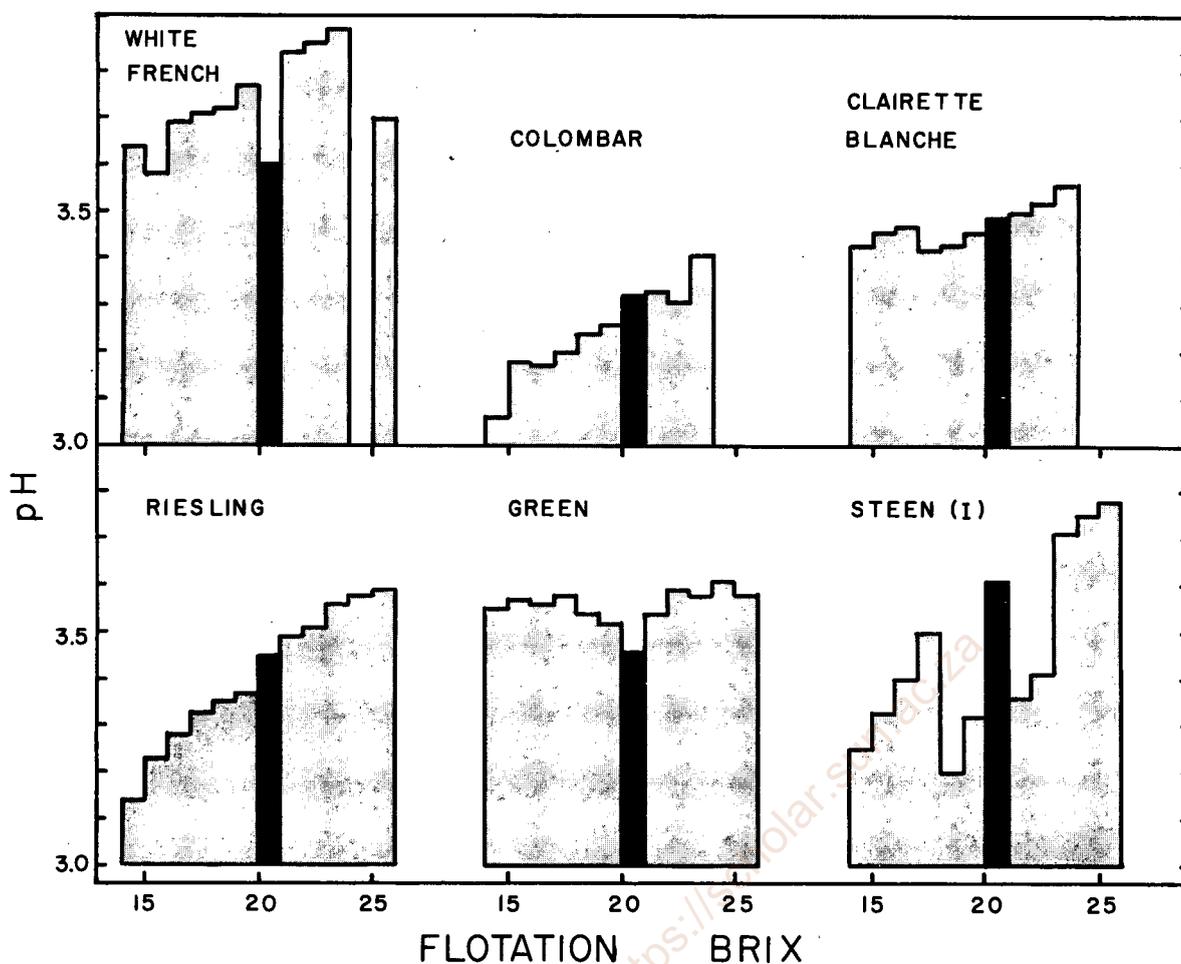
FIG. 5 The pH of juice from berry fractions segregated by flotation in sucrose solutions of 15–25° Brix. The black sections represent the 20–21° Brix fractions.

FIG. 5 *Die pH van sap van korrelfraksies geskei d.m.v. flottering in sukrose-oplossings van 15–25° Brix. Die swart gedeeltes dui die 20–21° Brix fraksies aan.*

ment was also found between the recombined and unsegregated berry sample's Brix, but complete data were not obtained.

Comparisons of the values in columns A show first that the berries of this fraction have a relatively constant juice Brix, 18,9 to 20,5, and therefore comparisons can be made with little or no interference from ripeness differences. The comparisons between the two harvests of Steen from two nearby but different vineyards are instructive. The masses of individual seeds in the two harvests were nearly identical in the 20 to 21° Brix fraction and this is known to be a relatively stable characteristic of a given cultivar. On the other hand, harvest II gave a notably larger berry with more seeds per berry than harvest I of Steen. Note that although there were fewer seeds per berry in harvest I it had a higher percentage of the fruit's mass as seeds than harvest II. Harvest I gave an appreciably higher pH in the juice of the 20 to 21° Brix fraction than harvest II. This was obscured and in fact reversed if either of the combined samples was used. It appears therefore for comparing different vineyards and vintages for a certain

cultivar that the use of a selected one or group of Brix segregated fractions will allow comparisons freed from or in addition to variations in ripeness and population distribution at the moment of sampling. This should be highly useful in simplifying comparison and clarifying evaluation of different seasonal, locality, vineyard, climatic and weather effects on grape composition separate from the confusion of ripeness differences, etc.

The comparative differences among cultivars can also be clarified by this technique of selecting a berry density fraction with nearly constant juice Brix. For example, White French is shown to have a relatively high juice pH in the 20 to 21° Brix flotation fraction and Colombar rather low pH (Table 5). The other cultivars, with the exception of Steen I, are more similar in juice pH, but this rather high degree of similarity is obscured if unsegregated fruit is compared. Comparisons of average berry mass, berry volume, seed mass and seeds per berry also appear more valid and the differences among cultivars more clearcut when the constant Brix fractions are compared rather than the unsegregated fruits' values.

9

TABLE 5 Comparisons of the 20-21° Brix flotation sample —A, the calculated data from recombination of the density segregated samples —B, and unsegregated samples —C

TABEL 5 *Vergelykings van die 20-21° Brix flotteringsmonster —A, die data bereken van hersamestelling van digtheidgeskeide monsters —B, en nie-geskeide monsters —C*

Cultivar	No. of Berries in sample *Aantal korrels in monster*			% of population *% van populasie*	Brix of Juice/ *van Sap*			pH of Juice/ *van Sap*		
	A	B	C¹	A	A	B	C²	A	B	C³
Steen (I)	126	1016	941	12,4	20,3	20,1	20,0	3,61	3,47	3,41
Steen (II)	122	881	500	13,8	19,7	21,1	21,7	3,47	3,53	3,47
Riesling	114	880	500	13,0	20,1	18,0	18,6	3,45	3,39	3,40
Green	78	424	500	18,4	20,5	20,4	21,6	3,46	3,56	3,56
White French	29	463	500	6,3	19,6	16,6	16,8	3,60	3,70	3,62
Colombar	183	908	500	20,2	18,9	17,2	17,8	3,32	3,25	3,18
Clairette blanche	81	976	500	8,3	19,5	17,6	17,8	3,49	3,45	3,50

Cultivar	Berry mass *Korrelmassa* g			Berry vol. *Korrelvol.* g			mg/Seed *mg/Pit*		Seeds/berry *Pitte/korrel*		Titratable acidity *Titreerbare suur* g/100 ml
	A	B	C¹	A	B	C¹	A	C¹	A	C¹	C²
Steen (I)	1,68	1,60	1,52	1,56	1,47	1,41	38,0	35,0	1,5	1,4	0,76
Steen (II)	2,21	2,26	2,29	2,02	1,95	2,12	37,9	39,2	1,7	1,8	0,85
Riesling	2,09	2,03	1,96	1,96	1,90	1,81	44,6	42,6	2,0	1,8	0,80
Green	2,41	2,26	2,22	2,27	2,12	1,93	37,4	37,4	2,0	2,0	0,65
White French	3,38	3,84	3,90	1,17	3,63	3,56	30,7	30,7	2,2	2,9	0,48
Colombar	2,80	2,89	2,87	2,62	2,71	2,69	41,7	41,7	1,4	1,5	0,99
Clairette blanche	2,11	2,22	2,27	2,07	2,19	2,08	46,7	44,7	1,7	2,0	0,63

Cultivar	Fresh mass, % of Berry mass *Vars massa, % van korrelmassa*							
	Seeds/Pit		Skins/Dop		Pulp/Prut		Juice/Sap*	
	A	C¹	A	C¹	A	C¹	A	C¹
Steen (I)	3,4	3,2	24,6	17,0	3,5	1,8	68,4	78,0
Steen (II)	2,9	3,1	12,9	16,1	3,2	4,3	81,0	76,5
Riesling	4,2	4,0	22,8	16,9	4,1	2,5	68,9	76,6
Green	3,2	3,4	11,4	17,5	3,6	3,9	81,9	75,2
White French	2,0	2,3	17,0	16,9	5,0	6,3	76,0	74,5
Colombar	2,1	2,1	13,6	19,4	7,3	7,7	77,0	70,8
Clairette blanche	3,7	4,0	14,1	22,8	5,6	5,1	76,6	68,2
Aver./Gem.	3,1	3,2	16,6	18,1	4,6	4,5	75,7	74,2

A = The berry sample which sank in 20° Brix and floated in 21°Brix sucrose solution./*Die korrelmonster wat in sukrose oplossings van 20°Brix gesink het en in 21°Brix geflotteer het.*

B = Data calculated by recombination in weighted proportions of all the density segregated fractions of berries./*Data bereken deur hersamestelling in geweegde verhoudings van al die digtheid geskeide fraksies van die korrels.*

C¹ = Unsegregated samples of the same berries./*Nie-geskeide monsters van dieselfde korrels.*

C² = Must analyses at the winery from the same harvest./*Mosontledings van dieselfde oes op keldermonsters.*

C³ = Analysis of wine from the same grapes shortly after fermentation./*Ontleding van wyne van dieselfde druiwe kort na gisting.*

*By difference/*deur verskil.*

CONCLUSIONS

It has been shown that there is little or no air in normal grape berries and that the tissues other than the easily expressed juice are more dense than the juice. Whole grape berries can be separated into narrow and precise fractions by testing their propensity to float or sink in a graded series of solutions of different sucrose content. Very high correlation between juice Brix and flotation solution Brix is shown and discussed. Juice Brix of intact berries can be approximated by an average regression equation with a slope of 1,0 and an intercept such that the juice Brix is 0,86° Brix below the nominal Brix of the 1° Brix interval at which the berries float and sink. Relatively small but definite variations in this regression relationship appear a varietal property affected by skin and seed content of the berry in relation to the juice content.

Data are presented indicating that characterizing the population of grape berries present in a given vineyard at harvest by density segregation clarifies the components making up the average Brix, pH, etc. of the must and can help explain differences among vintages with the same average juice Brix, etc. Ripeness progression can be studied separate from weather by segregating a single harvest. Furthermore selection for detailed analysis of a narrow berry density fraction (and therefore a relatively constant sugar content and degree of ripeness) provides a new and clarified means of comparing grape cultivars and populations of the same cultivar grown in different seasons in different places. Further development and application of the techniques presented should enable the answering of such questions as "does vineyard A truly produce more acid in berries at the same Brix than vineyard B or is it only that the grapes tend to ripen faster in vineyard B?", or "what are the specific compositional responses related to weather, climate, or soil fertility separate from ripeness differences?"

An indicator of overcropping appears to be relatively large berries in the low Brix fractions compared to similar or smaller berries in the intermediate Brix fractions. Segregation by berry density appears to a degree self-correcting in that berries injured in such a manner as to introduce air into them are displaced into the very low density fractions.

Opsomming

KARAKTERISERING VAN POPULASIES VAN DRUIWE VIR WYNBEREIDING GEOES EN KOMPENSERING VIR POPULASIEVERSKILLE

Enkel oeste van Clairette blanche, Colombar, Groendruif, Riesling en Frans en twee oeste van Steen is gemonster. Elke monster is in spesifieke korrelfraksies volgens digtheid geskei deur die heel korrels in 'n reeks sukrose-oplossings (25-15° Brix) wat met intervalle van 1° Brix verskil te dompel. Die verkreë fraksies is ontleed vir korrelgewig, korrelvolume, °Brix en pH van die sap. Die °Brix van die sap was hoogs betekenisvol gekorrelleer met die °Brix van sukrose-oplossings waarin die heel korrel net gesink het. Die °Brix van die sap was effens laer as die gemiddelde °Brix van die sukrose-oplossing-interval waarin die korrel net gesink of gedryf het. Die verskil het verband gehou met die cultivar en verskillende wingerde van dieselfde cultivar, en is toeskrywe aan die afwesigheid van lug in die normale korrel en aan die digtheid van die doppe en pitte wat hoër is as die van die maklik uitpersbare sap. Die bruikbaarheid van korrelskeiding, m.b.v. van digtheid om 'n gegewe oes te karakteriseer en om monsters met sap van presiese °Brix peile te verkry, vir die doel van vergelyking tussen seisoene, gebiede, wingerde en cultivars, sonder die verwarrende verskille wat by gemiddelde rypheidsdata voorkom, word bespreek en gedemonstreer.

Résumé

CARACTÉRISATION DE POPULATIONS DE RAISINS RÉCOLTÉS POUR LA FABRICATION DE VIN ET COMPENSATION POUR LES DIFFÉRENCES DE POPULATIONS

Des récoltes uniques de Clairette blanche, Colombar, Green, Riesling, White French et de deux récoltes de Steen furent échantillonnées et chaque échantillon fut classé en fractions de densité spécifique par flottement des baies entières sur des solutions de sucrose décroissant de 1° Brix dans la bande de 25-15° Brix. Les fractions résultantes ont été analysées pour le poids des baies, le volume, le jus Brix et le pH. Le jus Brix s'est révélé être en haute corrélation avec le Brix de la solution de sucrose dans laquelle les baies allèrent au fond. Le jus Brix se montra légèrement moins élevé que le Brix moyen de la solution sucrose Brix; interval durant lequel les baies flottèrent ou coulèrent. La différence fut apparentée aux cultivars ainsi qu'aux différents vignobles d'où le même cultivar provenait et ceci fut attribué à l'absence d'une quantité d'air appréciable dans la baie normale, la densité des pelures et des graines qui fut plus élevée dans le jus facilement extrait. L'utilité de faire une discrimination dans la densité de la baie afin de caractériser une récolte donnée, et de fournir des échantillons de niveau de jus Brix précis pour des comparaisons entre les saisons, les régions, les vignobles et les cultivars, libre de confondre les différences dans la maturité moyenne est discutée et démontrée.

REFERENCES

NELSON, K. E., BAKER, G. A., WINKLER, A. J., AMERINE, M. A., RICHARDSON, H. B. & JONES, FRANCES R., 1963. Chemical and sensory variability in table grapes. *Hilgardia* 34; 1–42.

RANKINE, B. C., CELLIER, K. M. & BOEHM, E. W., 1962. Studies on grape variability and field sampling. *Am. J. Enol. Vitic.* 13, 58–72.

ROESSLER, E. B. & AMERINE, M. A., 1963. Further studies on field sampling of wine grapes. *Am. J. Enol. Vitic.* 14, 144–147.

SINGLETON, V. L. & ESAU, P., 1969. Phenolic substances in grapes and wine, and their significance. *Adv. Fd. Res.,* Supplement 1, 1–282. 115–116.

SINGLETON, V. L., OUGH, C. S. & NELSON, K. E., 1966. Density separations of wine grape berries and ripeness distribution. *Am. J. Enol. Vitic.* 17, 95–105.

SU, CATHEY TSAI & SINGLETON, V. L., 1969. Identification of three flavan-3-ols from grapes. *Phytochemistry* 8, 1553–1558.

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11

CATECHOLASE ACTIVITY IN GRAPE JUICE AND ITS IMPLICATIONS IN WINEMAKING

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ABSTRACT

Catecholase (polyphenoloxidase) activity was measured in grape juice as related to parameters and variables of direct interest to the wine industry, including grape ripeness, degree of pressing, and the effect of bruising. Berries of sixteen grape varieties were separated according to sugar content, and the resulting musts were assayed for enzymatic activity. Enzymatic activity in the juice was maximum before the grapes reached full maturity, and increased with pressure in pressing. Bruising of grapes under controlled conditions prior to juice separation greatly increased catecholase activity in the musts, but the increase was prevented when bruising was in the absence of oxygen.

Polyphenoloxidase activity in grapes has been recently characterized primarily as catechol oxidase, or catecholase, since it has a relatively higher affinity for this substrate (4). However, as it occurs in other plant tissues, this enzymatic activity shows a relatively low specificity and will oxidase a wide range of phenolic compounds. The best known effects of polyphenoloxidase activity are the enzymatic browning reactions that occur in fruits and vegetables peeled and exposed to air. These enzymatic browning reactions in plant tissues have been divided into two distinct categories, referred to as "functional" browning and "adventitious" browning (7). "Functional" browning occurs during normal development of the plant; "adventitious" browning follows cell damage. In the wine industry, enzymatic browning in musts is generally an undesirable feature, falling in the category of "adventitious" browning since it is caused by cell damage resulting from physical treatment during crushing, pressing, and must separation. This study was made to determine some parameters of the polyphenoloxidase activity present in grapes and musts that might be of importance in practical aspects of winemaking.

All of the enzymatic determinations were done directly on juice samples, using catechol as substrate. No purification was attempted since the subject of study was enzymatic activity under the conditions that are more likely in the process of winemaking. Note that absolute original enzyme content is not claimed since it is well known that such reactions as tannin-protein complexing affect activity (12). Catecholase activity was measured in musts from different grape varieties at different stages of maturity. Also studied were the effects on catecholase activity from the degree of pressing in juice separation and from bruising of the grapes.

MATERIALS AND METHODS

Separation of berries and juice sample preparation: Berries of different sugar content were obtained by the method of flotation in sugar solutions (8,15). Grapes for varietal studies were picked when close to commercial ripeness and then separated by this method prior to juice preparation.

The difference in sugar concentrations between the solutions was one degree Brix, ranging from 15° to 25°Brix. The berries, wrapped in cheesecloth, were crushed by hand using only light pressure to get the must samples. Small aliquots of the musts were immediately cooled to 0°C on an ice bath and were promptly centrifuged at 3,000 x G for 10 min (Servall refrigerated automatic centrifuge). The pH values were determined with an expanded-scale potentiometer (pH meter model 26, Radiometer, Copenhagen).

Amer. J. Enol. Viticult., Vol. 24, No. 3, 1973

103

Phenolic content determinations: Total phenolics were determined by a colorimetric method using phosphomolybdic-phosphotungstic acid reagents and a 20-ml final solution volume as described by Singleton and Rossi (14).

Enzymatic assay: The enzyme activity was determined by measuring the catalytic oxidation of catechol into o-benzoquinone. This was done by following the increase of absorbance at 420 nm. The reaction mixture was 1.9 ml of 0.05 M NaH_2PO_4 adjusted to buffer at pH 6:5 plus 1.0 ml of 0.05 M catechol in buffer, to which the must aliquot (0.1 ml) was added last, to a total volume of 3 ml. The cuvette was then inverted twice and immediately placed in the spectrophotometer (Carl Zeiss Spectrophotometer PMQ II). The cuvette compartment was temperature-controlled at 30°C.

The increase in absorbance per minute was recorded on a paper-chart recorder (Varian Model G-2000), and the slope of the increase in absorbance per minute was obtained from the initial linear response, which lasted for 2 to 8 minutes in all the experiments. One unit of enzyme activity is herein defined as the amount of enzyme that would cause an increase of 0.001 in absorbance per minute, at 420 nm, pH 6.5 and 30°C.

Bruising conditions: The grapes were cut from the cluster at the pedicel, flush with the skin. They were bruised in 250-ml Erlenmeyer flasks with no more than two layers of berries. The flasks were shaken in a microbiological culture shaker (Gyrotory Shaker, New Brunskick Scientific Co.) at 300 rpm in a temperature-controlled room at 28°C. The action of the shaker produced vigorous bouncing of the berries against each other and the glass but did not break the berry skin except on a few discarded individual berries.

Pressing with a Carver press: The grapes were separated from the stem as described, above, and were wrapped with a heavy canvas in order to squeeze them with a Carver press provided with two pressure gauges (Carver Laboratory Press, Fred S. Carver Inc., New Jersey). The berries (900 g) were pressed by applying a steadily increasing pressure. The first 220 ml were considered as free-run juice, since little pressure was necessary (0 to 220 lb/sq. inch (Figure 7). All the juice samples were subjected to refrigerated centrifugation at 3,000 x G for 10 min, to attain a uniform state of partial clarification.

RESULTS

Determination of catecholase activity: The catecholase activity was determined in musts from berry fractions ranging from 15° to 25°Brix. The results from 16 grape varieties are summarized in table 1.

TABLE 1

Catecholase Activity of Musts. Musts of Different Brix Degrees were Prepared from Sixteen Grape Varieties, and Analyzed for Catecholase Activity Immediately after Centrifuging. The Table Shows only the Maximum Activity Value for Each Variety and the Brix Degree of the Must in which it was Found.

Grape Variety		Brix at max. activity	Max. activity (units/ml)
'Refosco'	(R)*	23°-24°	300
'Thompson Seedless'	(W)	20°-21°	360
'Aligote'	(W)	16°-17°	360
'Mission'	(R)	22°-23°	385
'Calzin'	(R)	19°-20°	705
'Emerald Riesling'	(W)	20°-21°	720
'Semillon'	(W)	17°-18°	800
'Pinot blanc'	(W)	21°-22°	900
'Napa Gamay'	(R)	18°-19°	1020
'Sauvignon blanc'	(W)	17°-18°	1500
'Muscat of Alexandria'	(W)	20°-21°	1750
'Pinot Chardonnay'	(W)	21°-22°	1775
'White Riesling'	(W)	16°-17°	1850
'Gamay Beaujolais'	(R)	20°-21°	2150
'Gerwurtztraminer'	(W)	21°-22°	2450
'Clairette Blanche'	(W)	17°-18°	2850
Average		19.75	1243

*(R) = red variety. (W) = white variety.
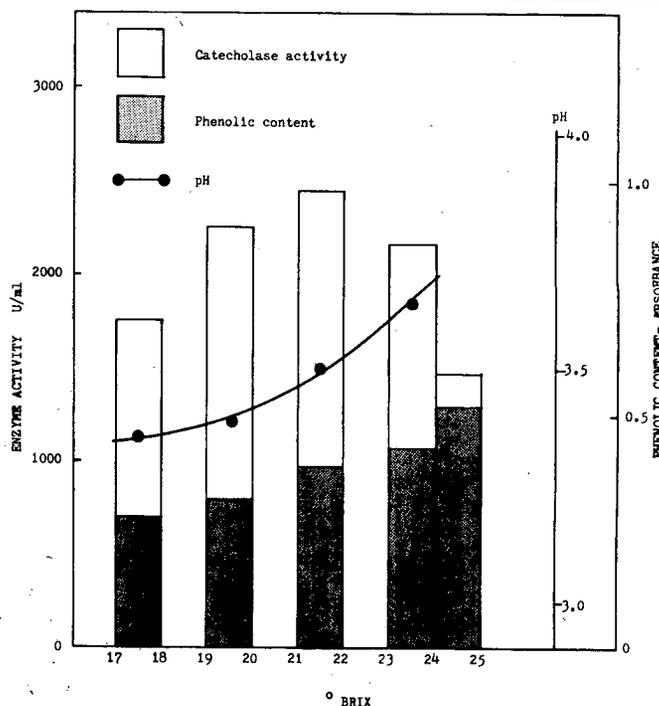
Figure 1. Correlation of catecholase, phenolic content, and pH in musts of different Brix degrees. The empty bars show the catecholase activity of juice from groups of berries segregated within one degree Brix. The shaded bars indicate the phenolic content of the corresponding sample. The pH values are represented by a continuous curve (—). The grape variety is 'Gewurtztraminer'.
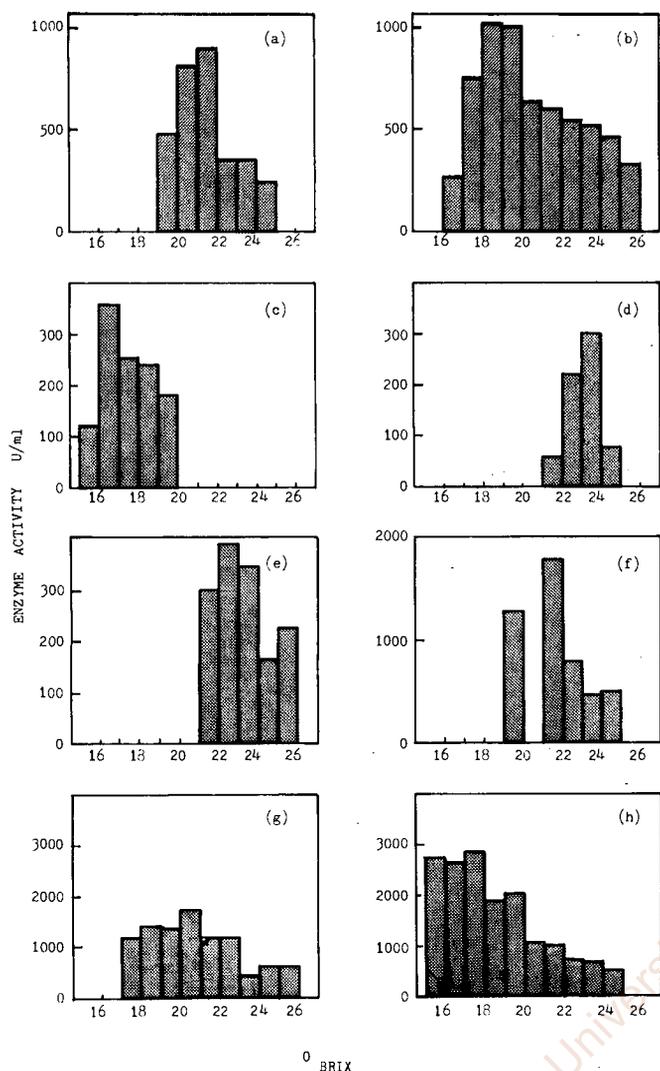
Figure 2. Changes in catecholase activity in musts of increasing sugar content. The histograms represent the result of the analysis of catecholase activity in musts of eight different grape varieties: a) 'Pinot blanc'; b) 'Napa Gamay'; c) 'Aligoté'; d) 'Refosco'; e) 'Mission'; f) 'Chardonnay'; g) 'Muscat of Alexandria'; and h) 'Clairette blanche'.

Figure 3. Catecholase activity in musts from 'Gamay Beaujolais' grapes. This experiment shows the increase of enzymatic activity caused by the presence of bruised and shriveled berries between 25° and 26° Brix. The activity found in a must obtained from shriveled grapes from the same source is indicated by the empty bar, whereas the shaded bar represents the activity found in the must from sound berries (as were used in all other tests).

Figure 1 shows the phenolic content, the pH, and the catecholase activity for the 'Gewürztraminer' variety. The catecholase activity was maximum between 20° and 23°Brix, whereas the pH and phenolic content increased steadily with sugar content of the musts. The observation that catecholase activity in the centrifuged must did not increase concomitantly with berry maturity was consistent in all varieties studied, as shown in the histograms in figure 2 for eight varieties. Although these histograms are slightly different in shape among the different varieties they exhibit a maximum activity in the range between 16° and 24°Brix, with an average of 19.75°Brix as calculated for table 1. Enzyme activity was maximum at a lower maturity than the physiological maximum of sugar accumulation (15) and well before appreciable dehydration of these berries.

Since berries in late maturity are more susceptible to physical damage by handling, it was suspected that the increase in catecholase activity in musts of higher Brix values could be caused by some shriveled and bruised grapes in the samples. In the experiment shown in figure 3, the berries from the sample corresponding to a value of 25° to 26°Brix were separated into two groups prior to juice extraction. Accordingly, it was shown (Figure 3) that the berries damaged by handling had an activity 6.6-fold greater than that of sound berries.

Effect of bruising on catecholase activity: Figure 4 shows the effect of bruising berries of 'Sauvignon blanc'. Thirty minutes of vigorous bruising gave a 3.65-fold increase in catecholase activity in the musts. However, the enzymatic activity did not increase further in musts of berries that were allowed to stand at room temperature after the mechanical bruising was halted (see also Figure 5). Control berries standing at similar conditions of
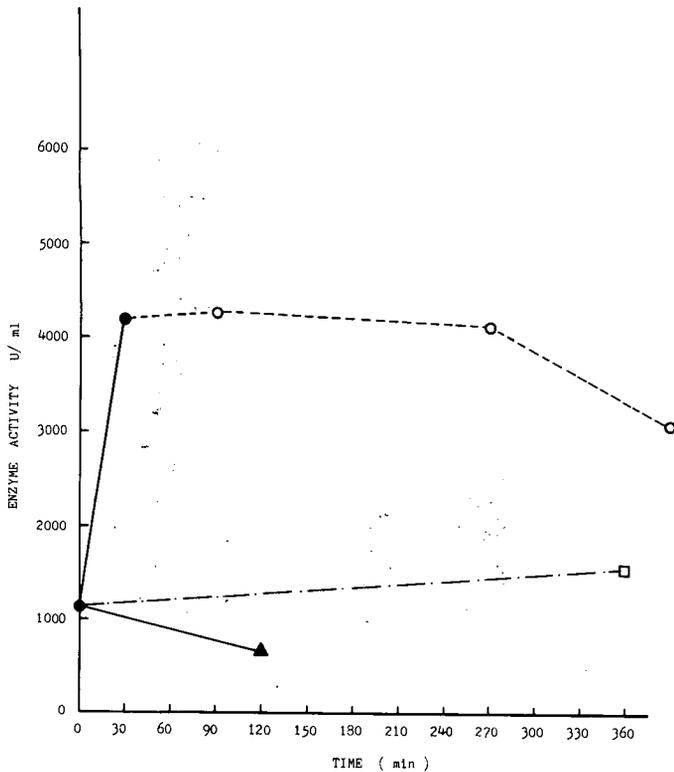
Figure 4. Effect on catecholase activity of bruising in the presence of air or nitrogen. This experiment shows the increase of enzymatic activity in musts produced by bruising the berries in air. The berries were bruised continuously for certain periods, and activity was then measured on the corresponding musts (—). After the bruising was halted, some samples of berries were allowed to stand at room temperature before must separation to determine the activity (●---○). Control berries were not bruised at all, and were allowed to stand at room temperature prior to measurement of the activity in the resulting must (●—·—□). Another portion of the same berries was bruised continuously for 30 min under nitrogen gas, then allowed to stand for 90 min before being squeezed to assay the activity in the resulting must (●—▲). The grape variety used was 'Sauvignon blanc'.
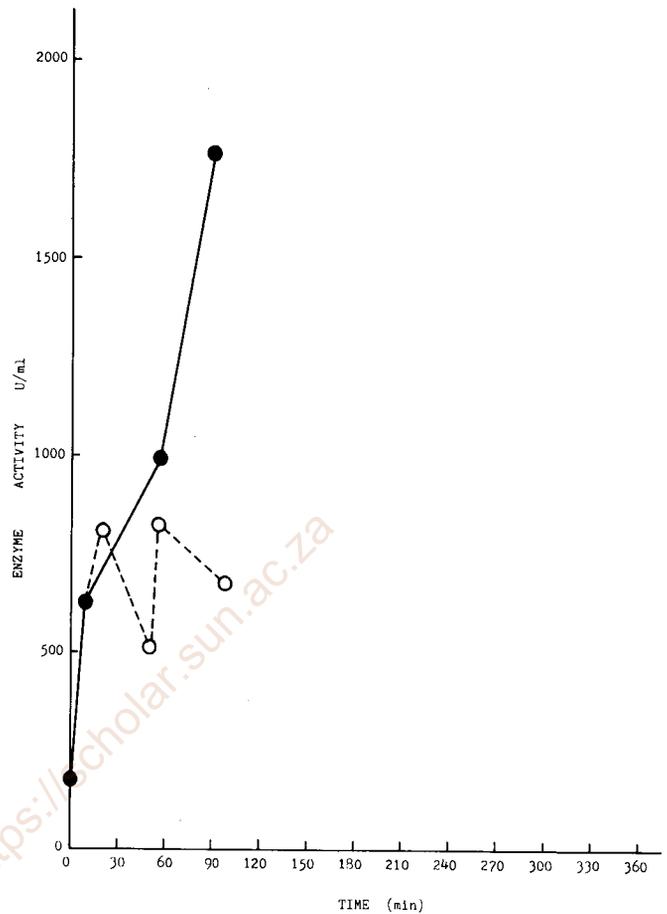
Figure 5. Effect of continuous bruising. Berries of the 'Chenin blanc' variety were bruised continuously for 90 min (—). Other samples of the same berries were bruised only for 10 min and then allowed to stand at room temperature before portions were squeezed to obtain the must for the assay (●---○).

time and temperature but without being bruised gave no significant increase of catecholase activity in the resulting musts. Berries bruised for 30 min under nitrogen gas gave a surprising result: no increase at all in enzymatic activity in the must even though the berries were squeezed in the presence of air in juice extraction.

Continuous bruising of berries up to 90 min gave an almost linear increase in enzymatic activity of the corresponding musts, as shown in figure 5. Ninety minutes of continuous bruising gave a 9.8-fold increase in catecholase activity in the must. In an experiment with a variety with low catecholase content, however, catecholase activity did not increase further after a certain period, and it even began to decline after long periods of continuous bruising (Figure 6).

It appears that more intense bruising has greater effect in proportion to the percent of the cells disrupted and therefore reaches a maximum when all cells are affected.

Catecholase activity as affected by pressing: Figure 7 compares catecholase activity in musts extracted with different degrees of pressure. Approximately 60% of the juice was extracted with only small variation in catecholase activity. Thereafter an increase was evident, and when approximately 80% of the total juice was extracted a maximum of catecholase activity appeared, being 5-fold greater than the value present in the free-run juice (Figure 7). Note, again, that this increased catecholase activity in the hard-pressed samples was in the juice clarified by high-gravity centrifugation.

Stability of catecholase activity after SO₂ addition: A fresh sample of must ('French Colombard' variety) was obtained in the University winery and treated with SO₂ (approx. 100 ppm) as in a normal winery operation. The sample was found to have an inactivation that was 94.3% that of a fresh sample of must prepared in laboratory conditions from the same grapes.
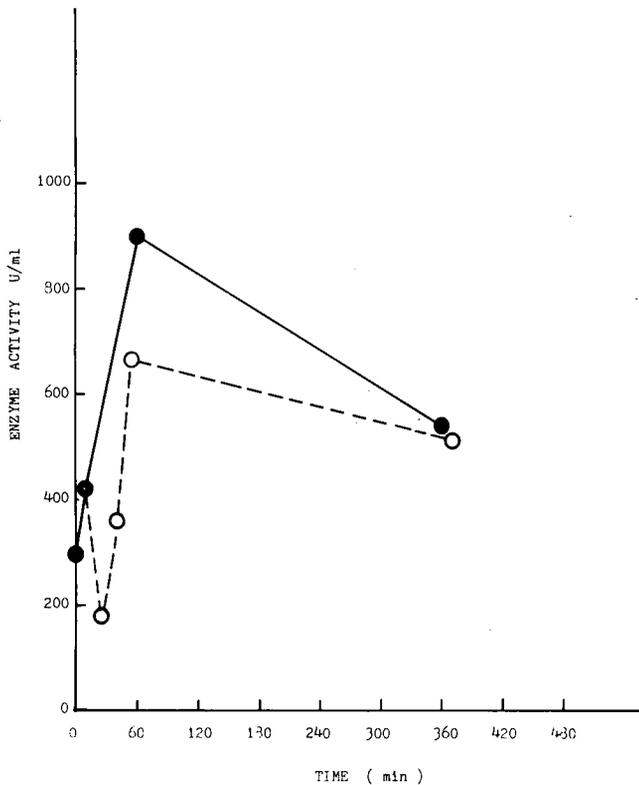
Figure 6. Effect of prolonged bruising. A sample of the 'Sultanina Marble' variety was bruised continuously for 6 hours (—●). Another sample was bruised only for 10 min and then allowed to stand at room temperature for 6 hours (●---○). This variety was reported previously (11) to contain no catecholase.

Figure 7. Catecholse activity levels in musts extracted with increasing pressure. The histogram shows the activity level on volumes of must extracted with increasing pressure. The curve (●—●) indicates the pressure applied to obtain those volumes.

DISCUSSION

The study indicates that the enzyme content does not increase proportionaly with ripening. Rather, the activity increases in early stages and decreases at high Brix. For most varieties, however, the catecholase activity appears to be at a maximum in musts produced from grapes that are near the maturity at which they are usually picked for making table wines. These results are consistent with results reported for 'Muscat rouge', 'Dimiat', 'Riesling', and 'Aligote' (5) indicating that polyphenoloxidase activity was higher early in ripening and decreased thereafter.

Information about the relative levels of the enzyme activity in different grape varieties does not necessarily imply a direct correlation with more or less susceptibility to browning, although it is an important indication. The variation of substrates for the polyphenoloxidase activity in musts from different grape varieties requires a more detailed study. It has been mentioned (12,13) that individual phenolic substances do not brown equally. Chlorogenic acid for example, is readily oxidized by polyphenoloxidase, but the products are not intensely colored (12,13). However, the direct products of enzymatic oxidation might proceed by further chem-

ical oxidation into brown condensation products (12,13) even after the disappearance of the enzyme activity, and therefore might have an effect on browning during the aging of wines. The catechin fraction, on the contrary, immediately yields brown compounds under the action of polyphenoloxidase activity, indicating that catechin compounds are important enzymatic browning substrates (12). However, these oxidation products show a tendency to be entrained on the particulate matter (12), and therefore might be sedimented after the completion of fermentation.

Another result reported herein that has an important bearing on practical considerations is the effect of increased enzymatic activity in musts from bruised or shriveled grapes. Two main factors that account for this increase should be distinguished: bruising of the berry flesh itself, and exposure of the injured tissue to air. It was shown that approximately a 10-fold increase of the enzymatic activity in the musts can be produced by maximal bruising of the berries, but it was also demonstrated that air must be present during the bruising for such an increase to occur. Bruising done in the absence of air had no such effect.

The mechanism that accounts for the increase in catecholase activity with prolonged bruising of grapes (see Figure 5) is not known in detail. Never-

theless, it is reasonable to conceive more cells are disrupted by longer bruising, and therefore more enzyme is released and exposed to the oxygen. Beyond a certain degree of bruising, however, the enzymatic activity does not increase further, presumably because cell disruption is already 100%. Catecholase activity then starts to decrease (see Figure 6), probably because of the interaction of the enzyme with phenolic compounds which are also being released from the grape tisues by the bruising. It is known that the products are inhibiting to polyphenoloxidase. A most interesting observation is the one regarding the "stimulatory" effect of oxygen (see Figure 4), which indicates that the amount of catecholase in the musts depends on an unknown mechanism only a part of which is mechanical extraction of enzyme by cell disruption. The fact that oxygen is required for this increase excludes the possibility that it would be just a matter of changes in the enzyme solubility in the grape juice, such as produced by pectinolytic activity, which could degrade pectic substances that might otherwise help in sedimenting the catecholase activity.

At the time the bruising experiments were done it had been suggested that an oxidative enzyme activity, such as catecholase, could likely be regulated by the presence of oxygen (16). That hypothetical regulation by oxygen was suggested to be either: a) a direct modification of the active site itself, making it available for the other substrate; b) an effect on the conformation of the protein molecule (a sort of allosteric activation); or c) an effect in helping to keep possible monomeric forms of the enzyme in an aggregation state, or oligomeric form such as required for optimum enzymatic activity. These hypotheses were further supported by experimental work since published by other workers (6). In purified fractions of grape catecholase, evidence was found indicating that oxygen may have an activation effect *in vivo* (6). This control of activity, depending on the level of oxygen could apparently be related with conformational changes near the active site of the enzyme, resulting in a considerable increase of the rate of reaction. Those same workers (6) also suggested that the grape enzyme also undergoes monomer-oligomer transitions and that the structure of the aggregate is dependent on pH, in addition to the oxygen control effect. The experimental evidence presented here is in accord with those results.

It is generally conceded that an important, or perhaps the only, proven role of polyphenoloxidase in plants is in mobilization of the plant's defenses upon injury. It seems highly significant that our data show that a cell bruised in the presence of air (equivalent to cutting or pest invasion) responds with an immediate and large increase of catecholase activity. Contrarily, a cell similarly disrupted under anaerobic (and not similar to pathological) conditions does not react in this manner. The increase is too large and too rapid to result from new protein synthesis and must come from an activation of a proenzyme, disaggregation, or other conformational changes.

In addition, the results shown here suggest some new ideas in the practical management of enzymatic browning. It is already accepted that minimizing physical damage to grapes during harvest, transport, and handling prior to crushing can be of critical importance in producing wines (such as white table wines) in which minimum browning is desired. These data, however, emphasize that it is not just injury which is important, but injury in the presence of air or oxygen. Exclusion of air by CO_2 or N_2 during transport of grapes may be useful in preventing browning not only by preventing oxidative reaction but also by preventing activation of polyphenolase activity. The recognized need for immediate application, upon crushing of phenolase inhibitors such as SO_2 is made even more evident, along with other desirable practices such as immediate pressing and juice clarification. These facts are further reasons in favor of mechanical harvest, including crushing, at the vineyard in order to keep grape handling minimal while avoiding unnecessary exposure of injured grapes to the air. The well-known inhibitory effect of H_2SO_3 on grape polyphenoloxidase (1,2,3) was also shown in this report. Others have indicated the decrease of this enzymatic activity during fermentation (10) and storage (2). Consequently, special care must be taken to avoid the detrimental effects caused by the polyphenoloxidase activity in early stages of the production (prior to addition of SO_2) since thereafter it almost disappears, although, as previously said, the substrate may continue to oxidize by chemical mechanisms, and render brown products non-enzymatically. A part of this research not reported here was a study of the loss of added phenolase activity with time in wine. These data indicated that under some conditions and without SO_2, the enzyme could play a role in wine browning for a limited period. In any case, our results further emphasize the need to protect wine from air and use in oxidation inhibitor like SO_2.

The higher extraction of enzymatic activity with more pressure applied in the Carver press suggests that the enzymatic activity is not equally located in the berries, with more enzyme apparently associated with the skins and pulp rather than with the free-run juice. Although these conclusions were not obtained from a physiological or histological experimental approach, they are quite valid to be applied in production. The importance of controlling the degree of pressing and the time of contact with the skins is strongly indicated. It has been reported that during ripening the skins had the greatest polyphenoloxidase activity (5). Other reports confirm this (1), and the major activity was found in the skins and pulps.

REFERENCES

1. Bayer, E. F. Bonn, and K. H. Reuther. Über die polyphenol-oxidase der Trauben. Z. Lebensm. Untersuch. u. Forsch. **105**:77 (1957).

2. Casas Lucas, J. F. Evolution des Oxydases dans les mouts et les vins. Bul., O.I.V. **40**:1369 (1967).

3. Deibner, L. and H. Rifai. Die Polyphenoloxydase in Trau-bensaft. I. Über die Bestimmung der Activität der poly-phenoloxydase mit Purpurogallin. Rebe. Wein. **13**:56 (1963).

4. Harel, E. and A. M. Mayer. Partial purification and prop-erties of catechol oxidases in grape. Phytochemistry **10**:17 (1971).

5. Ivanov, T. and A. Ivanova. Sur l'oxydation du mout de raisin. III. Activité de la polyphenoloxydase et de la peroxydase au cours de la maturation du raisin des cépages 'Muscat rouge', 'Dimiat', 'Riesling' et 'Aligoté'. Ann. Technol. Agr. **17**:333 (1969).

6. Lerner, H. R., A. M. Mayer and E. Harel. Evidence for con-formational changes in grape catechol oxidase. Phyto-chemistry **11**:2415 (1972).

7. Mason, H. S. Comparative biochemistry of the phenolase complex. Adv. Enzymol. **16**:105 (1955).

8. Nelson, K. L., G. A. Baker, A. J. Winkler, M. A. Amerine, H. B. Richardson, and Frances R. Jones. Chemical and sensory variability in table grapes. Hilgardia **34**(1):1-42 (1963).

9. Pallavicini, C. Distribuzione degli enzimi fenolasi, fosfatasi, proteinasi e saccarasi nelle parti constituent gli acini di 4 uve e nei vini provenienti dalle uve stesse. Ind. Agr. **5**:603 (1967).

10. Poux, C. Polyphenoloxydase dans le raisin. An. Technol. Agr. **15**:149 (1966).

11. Radler, F. The prevention of browning during drying by the cold dipping treatment of Sultana grapes. J. Sci. Food Agr. **15**:864 (1964).

12. Rossi, J. A., Jr. and V. L. Singleton. Contributions of grape phenols to oxygen absorption and browning of wines. Amer. J. Enol. Viticult. **17**:231 (1966).

13. Singleton, V.L. and P. Esau. Phenolic substances in grapes and wine, and their significance. Adv. in Food Res. Suppl.

14. Singleton, V. L. and J. A. Rossi, Jr. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid re-agents. Amer. J. Enol. Viticult. **16**:144 (1965).

15. Singleton, V. L., C. S. Ough, and K. E. Nelson. Density separations of wine grape berries and ripeness distribution. Amer. J. Enol. Viticult. **17**:95 (1966).

16. Traverso-Rueda, S. Some studies on grape polyphenol-oxidase activity and their implications in winemaking. Re-search report. Library of the Dept. of Viticulture and Enology. University of California, Davis, California, (1971).

15 V. L. Singleton and F. H. Kratzer

PLANT PHENOLICS*

INTRODUCTION

The phenols and biochemically related substances of natural origin in plants can be divided roughly into two groups: the botanically widespread and structurally common ones, and those with exotic structures and limited specific occurrence. The first group consists of 25 or so substances such as *p*-coumaric, caffeic, ferulic, sinapic, and gallic acids or their derivatives. Included are the flavonoids with the common and analogous variations in the 5,7,3',4',5'-hydroxyl or 3',5'-methoxyl substituents in the anthocyanidin, catechin, flavone, flavonol, and leucoanthocyanidin series. Lignin, hydrolyzable and condensed tannins (p. 328), ellagic acid, and derivatives such as depsides and glycosides substantially complete the first group. Some of these substances almost invariably are contained in plant-derived foods, at least in traces and sometimes in fairly large amounts.

The second group is heterogeneous and includes most of the few dozen phenolic derivatives known to have high toxicity or potent physiological activity in animals.[1] These substances may also be present in very significant amounts in the particular plant tissues that contain them. A third group perhaps worthy of consideration would be the

* Literature reviewed to January 1971.

309
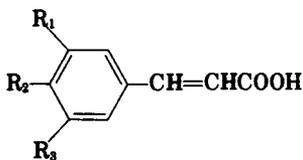
	R_1	R_2	R_3
p-Coumaric Acid	H	OH	H
Caffeic Acid	OH	OH	H
Ferulic Acid	OCH_3	OH	H
Sinapic Acid	OCH_3	OH	OCH_3
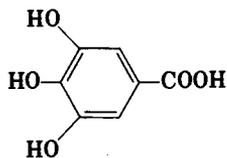
Gallic Acid
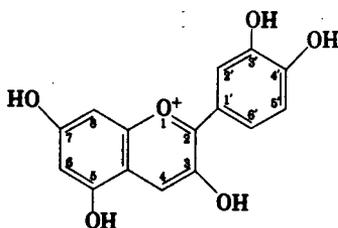
Cyanidin (an anthocyanidin)
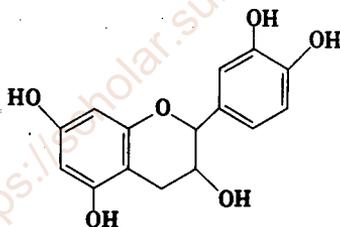
Catechin (a flavanol)
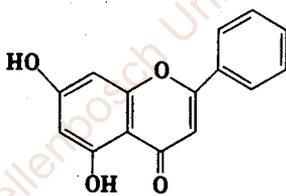
5,7-Dihydroxyflavone
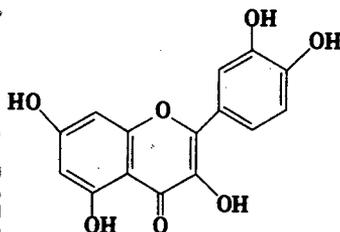
Quercetin (a flavonol)
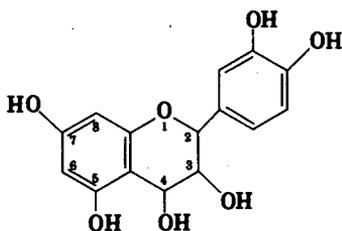
Leucoanthocyanidin (a flavan-3,4-diol)
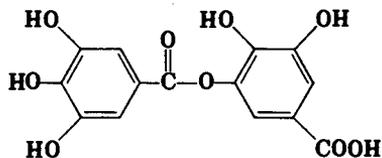
m-Digallic Acid (a depside)

smaller phenols produced by reactions such as pyrolysis. They could appear in foods as a result of smoking or other processing.

Owing, presumably, to long evolutionary adaptation, the common plant phenolics as usually consumed are readily detoxified. It appears[1] that carnivores tend to be more susceptible than herbivores to the acute toxicity of phenols, such as those from plants. Omnivores, for example, man and rats, appear to be intermediate. This relative sensitivity seems to be true parenterally as well as orally, so that it does not appear to be exclusively an effect of digestive-tract microflora. Considering that the diet of a herbivore may contain nearly 20% of its dry weight as lignin and other plant phenols, and that the diet of a carnivore has nearly none, an evolved difference in phenol tolerance seems reasonable. A case can be made for the concept that all phenols have some properties in common that render them potentially toxic to animals[1]; the fact that most plant phenols are harmless as usually encountered in the diet is largely the result of the animal's effective detoxification or nonabsorption of them. This leads to the corollary that if the animal's detoxification mechanisms can be overloaded by massive amounts of phenolic derivatives in the diet or circumvented by unusual structures of the phenols or unusual circumstances of ingestion, toxicity is likely to become manifest.

With a few exceptions,[1,2] it has been the fashion to consider effects of plant phenols according to botanical origin (mycotoxins, toxic plants, etc.), type of effect (antibiotics, antioxidants, carcinogens, estrogens, flavors, photosensitizers, etc.), or structures (cinnamates, coumarins, flavonoids, isoflavones, quinones, tannins, etc.). This practice tends to prevent recognition of general relationships in phenol toxicity, although to a degree it is continued in the present volume.

The reader is also referred to additional chapters in this volume in which other potential phenolic relationships are discussed: nitrogenous phenols such as tyrosine (Chapter 6) and mescaline (Chapter 8); phenolic vitamin E and quinoidal vitamin K (Chapter 11); phenolic enzyme inhibitors (Chapter 13); mycotoxins such as aflatoxin (Chapter 18); flavoring components such as myristicin and coumarin (Chapter 20); safrole (Chapter 23); dihydromethysticin and other kava components (Chapter 21); tremetol and related substances from white snakeroot in milk (Introduction); isoflavones and other phenols as estrogens (Chapter 24); and hypericin and psoralen relatives such as photosensitizers (Chapter 25).

Here some apparently general correlations of toxicity of nitrogen-free phenols from higher plants will be discussed, as well as a few exotic toxic phenols not covered in other chapters, notably gossypol. Some
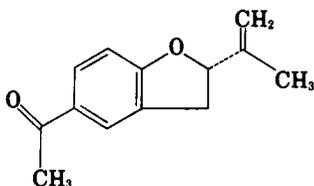
Tremetone

consideration will be given to the more widespread plant phenols, flavonoids, and tannins as related to food toxicity.

PLANT PHENOLS AS ACUTE TOXICANTS

The primary concern in this report is the significance of plant phenols as present or potential toxicants in human foods, but some useful perspective appears possible from brief consideraton of their participation in poisoning of animals or in "medicine cabinet" and "novices afield" accidents. Of about 300,000 species of plants, an estimated 700 have caused death or serious illness in this hemisphere, and doubtless many have yet to be recognized as poisonous.[3] Many instances go unreported, but thousands of persons receive medical treatment and hundreds of thousands of animals die annually from consuming poisonous plants.[3] Intoxications from plant ingestion are responsible for about 4% of all accidental poisonings in this country, and in most cases small children are involved.[4]

Implicated in 1,051 plant ingestions reported to poison control centers in the 50 states, Puerto Rico, and the Canal Zone during 1959–1960, plus several hundred reports from other years, were at least 175 plants in addition to mushrooms.[5] Of these, 11 plants, involving at least 123 patients and 5 fatalities, were those whose intoxicating properties are attributed at least in part to phenols. Other summaries[3-8] of plants with reported acute effects on humans attributable to phenols give a combined list including aloe, *Cassia, Rhamnus,* rhubarb, St. John's wort, and buckwheat containing anthraquinone derivatives; cashew, mango, poison ivy, and *Ginkgo biloba* containing vesicant phenols with long hydrocarbon side chains; and *Areca catechu,* aspidium, buckeye (*Aesculus*), daphne, *Derris,* hawthorn, marihuana, mayapple, nutmeg, and sassafras containing several different active phenolics. Examining these reports quickly leads to the conclusion that, although acute poisoning of people by plants with toxic phenols is real enough and some of the vectors are readily available, such poisoning is more often either a non-

fatal nuisance (as with poison ivy) or a relatively rare event in modern times.

Some of the listed plants may be toxic primarily because of compounds other than phenols. The anthraquinones of rhubarb are mainly in the root, and the cathartic drug is prepared from less edible varieties. Human poisonings occur mainly from eating rhubarb leaves (see Chapter 16), and the petioles, of course, are good food. The leaf poison is commonly thought to be oxalate, but other factors, possibly the quinones, may more likely be involved (see Chapter 16).[7] Daphne and *Aesculus* poisonings have been attributed to their content of the coumarin glycosides daphnin and esculin. There is not general agreement on this, however, and it appears that much of their acute toxicity should be attributed to an acid anhydride in daphne[4] and a saponin in buckeye or horse chestnut.[4,9,10] On the other hand, the toxicants in many plants have not been identified, and many toxicants are doubtless waiting in the wings to make an appearance. For example, 27 soldiers suddenly and permanently lost their sight after consuming the Australian finger cherry, *Rhodomyrtus macrocarpa*. The rhodomyrtoxin apparently is or contains completely substituted dibenzofurans derived from phloroglucinol.[11] The pure dibenzofuran was present in immature fruit at levels of at least 3,500 mg/kg fruit and was toxic to mice at oral doses of 30 mg/kg body weight.[11] Similar compounds had been found previously in lichens but not in higher plants.

A few of the plants listed as producing human toxicity are also of considerable practical importance in losses of farm animals, notably St. John's wort and white snakeroot. However, most of the economically significant phenolic plant poisons, such as dicumarol (the sweet clover anticoagulant), cocklebur, oak tannins, plant estrogens, and cottonseed gossypol, have evidently not directly caused human accidents. This is apparently because the plant materials are not likely to be consumed by humans and because, except for cocklebur, their most serious effects require repeated dosage. The apparent unlikelihood of human consumption is not always valid, however. Daphne, buckeye, and buckwheat toxins (possibly phenolics) are reportedly present in honey (see Chapter 22, p. 495) drawn from these plants.[5,8] Cashew oil, although removed and detoxified in preparing the nuts for sale, has been used as a coating for vanilla beans and thereby has caused irritation.

In terms of human lives lost from phenols originating in plants, the salicylate aspirin is probably the most dangerous. Aspirin is now obtained synthetically, but salicyl derivatives were first known from willow (*Salix*). Accidental and suicidal consumption of salicylates produces deaths of the order of four per million population per year.[12] Other

medicinal preparations such as aspidium and podophyllin, particularly in former times, offered considerable danger not only in the home but also to the gatherers and processors.

Only a few plant phenols are so highly toxic or so physiologically active that potentially lethal effects would result from a single dose that readily occurs in a natural product. Aspidium,[13] the anthelminthic drug (for elimination of tapeworms) prepared from male fern (*Dryopteris filix-mas*), contains at least 6.5% oleoresin, 24% or more of which is a mixture of substituted and oligomeric phloroglucinols. The oleoresin at a 1–5 g oral dose for humans produces headache, dizziness, nausea, and visual disturbances. At least one human fatality is known. An isolated constituent, desaspidin, had an acute oral LD_{50} of 595 mg/kg to mice, but its toxicity rose to 195–280 mg/kg when mixed with the nontoxic oils, etc., present in an ether extract of fern.[14] Filicin, another constituent, had an acute oral LD_{50} to rats of 1,076 mg/kg, but 90 mg/kg daily produced a number of toxic symptoms, including a gradual loss of the ability to generate sperm.[15] Cotoin[13,16] (2,6-dihydroxy-4-methoxy-benzophenone) from coto bark, formerly an antidiarrheal, is lethal at 8 mg/kg subcutaneously to frogs.
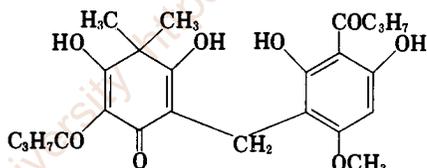
Desaspidin
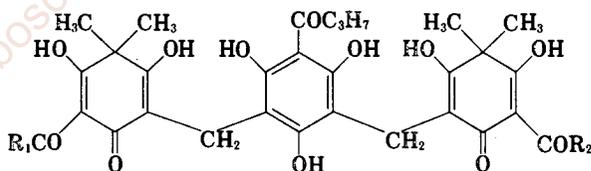
Filicin (a mixture of similar substances)
BBB, $R_1 = R_2 = C_3H_7$; PBB, $R_1 = C_2H_5$, $R_2 = C_3H_7$; PBP, $R_1 = R_2 = C_2H_5$
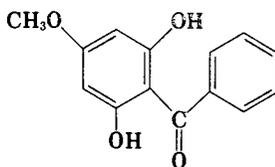
Cotoin

The mayapple, *Podophyllum peltatum*, contains in the dry roots about 8% of the resin podophyllin, of which about 20% consists of podophyllotoxin (a lignan) plus other related substances. The resin has irritant, cytotoxic, and cathartic effects, and a 130-mg medical dose has caused death.[16] The ripe fruits are often eaten with no effect or a mild catharsis, but green fruits are dangerous.[17] Podophyllotoxin has an oral LD_{50} to mice of 90 mg/kg.[13,16] Derris root powder has an oral LD_{100} to rats of about 400 mg/kg,[16] and the rotenone it contains has an LD_{50} of 133 mg/kg orally and 5 mg/kg intraperitoneally.[1,13] Of value as a natural fish poison and insecticide, rotenone (an isoflavone derivative) is ordinarily handled carefully but is considered relatively low in toxicity and in residual persistence compared to other insecticides. The lethal oral dose for humans has been estimated to be as low as 200 mg,[13] although this has been disputed.[18] The compound has cumulative toxicity on long exposure and is more toxic when inhaled.[13,18]

Most other phenols are less acutely toxic. Hydroquinone is the most toxic of the simple phenols at oral LD_{50} 320 mg/kg for the rat.[1,16] It is the toxicant in cocklebur seeds and sprouts that has caused much loss when animals consume the cotyledon stage of seedlings.[1,6] The seeds (not including the burrs) are toxic at about 3 g/kg of body weight, and apparently the hydroquinone is not present as a glycoside, in contrast to
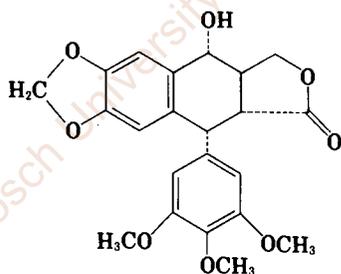
Podophyllotoxin
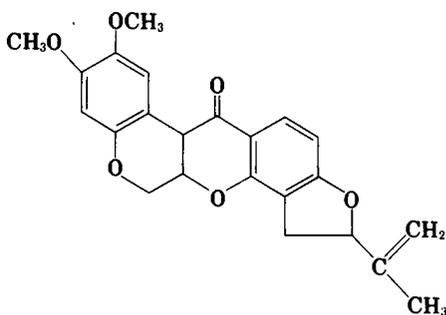
Rotenone

the usual situation.[6] The acute toxicities of the other phenols of the plant type are nearly all in the range considered only slight in oral toxicity, i.e., LD$_{50}$ 500–5,000 mg/kg, or even less toxic. Some representative acute oral toxicity values, not necessarily determined in a strictly comparable way,[1,13,16] are: vanillin 1,580, methyl salicylate 887, coumarin 680, thymol 980, safrole 1,950, menadione (a synthetic vitamin K) 1,000, dicumarol 542, and tannic acid 6,000 mg/kg.

Few values have been found for the lethal dose of common flavonoids or cinnamates, because they are essentially nontoxic in a single dose; however, they might be toxic if administered at high levels for a long time. Coumarin and safrole are no longer permitted additions to food, since, among other tests, long-term (nearly 1 yr to 6 yr) oral dosage of dogs at as little as 25 and 5 mg/kg, respectively, produced deleterious liver changes.[5] Higher levels, of course, gave more serious effects.

In general, flavonoids of the botanically usual types have negligible toxicity and show no harmful effects at oral doses of at least 500 mg/kg.[19] Rutin required 19.5 g/kg intraperitoneally to produce an LD$_{95}$ in rats, and similar levels given orally to guinea pigs were only marginally toxic.[20] Rutin is the 3-rhamnoglucoside of quercetin (see Chapter 25, p. 565). Daily feedings of 850 mg/kg for 3 months gave insignificant *gains* in growth rate of rats. In the same trials the sodium salt of the ethereal sulfate of 4-methylesculetin gave similar results (LD$_{95}$ intraperitoneally rat of 8.22 g/kg) and normal growth with 555 mg/kg daily for 3 months. A number of studies on naringin, hesperidin, rutin, quercetin, quercitrin (quercetin-3-rhamnoside), and dihydroquercetin have shown no acute toxicity, nor was toxicity noted when fed to rats at 1%
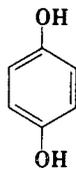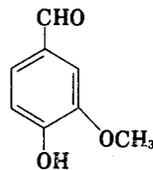
Hydroquinone   Vanillin
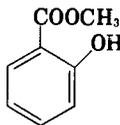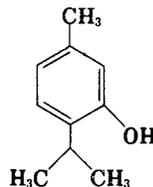
Methyl Salicylate   Thymol

of the diet for 200 days or longer.[21] The lethal dose of chlorogenic acid (3-caffeoylquinic acid) to mice intraperitoneally was 3.5 g/kg, but 4–5 g/kg orally or subcutaneously gave no toxicity.[22] Oral dosage of humans for 60 days with 1.5 g/day of 1,4-dicaffeoylquinic acid gave only favorable effects.[23] Parenteral toxicity of some of these substances is sometimes high, however, particularly that of the substances with high protein affinity of the tannin type. The IP LD_{50} of a leucocyanidin to rats was about 100 mg/kg[24] (although oral LD_{50} to mouse was 3 g/kg), and the tannic acid intravenous LD_{100} to mice was 80 mg/kg.[16]

Rhamnoglucoside—O

OH

Naringin

Rhamnoglucoside—O

OH

OCH_3

Hesperidin

HO COOH

HO—CH=CHCOO

OH

OH
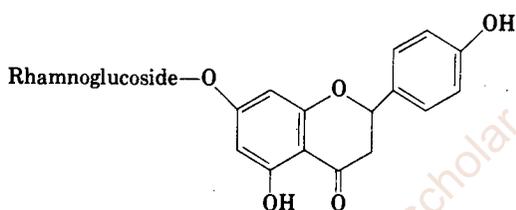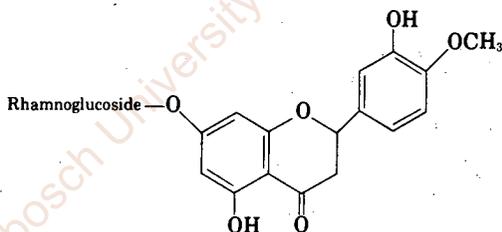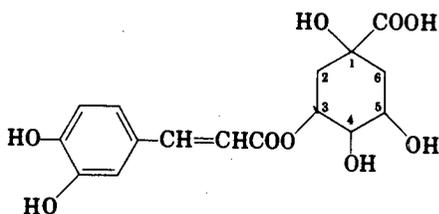
Chlorogenic Acid

SPECIFIC PLANT PHENOLIC TOXICANTS

It has been mentioned that several of the most important phenolic toxicants are being covered in other chapters. Several more have been discussed in the preceding sections, but, owing to their minimal significance in human foods, they need little elaboration here. A few others, however, deserve more consideration not only because of real or potential hazard to humans but also to illustrate some aspects of phenolic toxicity with, perhaps, general applicability.

Gossypol

A yellow phenol characteristic of the genus of cotton, *Gossypium*, gossypol would have probably remained insignificant as a natural hazard, but it has assumed importance because of the use of seed meal recovered from cotton production as a protein supplement for animal, and now human, diets. With major production in Brazil, Egypt, India, Mexico, and Pakistan, cottonseed is the protein-rich oilseed most widely available for supplementation of inadequate diets in tropical and subtropical countries.[25,26] For each 500-lb bale of cotton fiber, about 840 lb of seed is produced.[27] Total world production of cottonseed is as much as 25 million tons/yr, and about 3–6 million tons have been produced in the United States,[26] depending on fiber demand. Each ton of seed produces about 335 lb of oil and 945 lb of feed-grade meal.[27] If refined 60% protein cottonseed flour is prepared for human use, 300–400 lb/ton is produced; the remainder of the meal can be diverted to animal feed.[28] It is estimated that only one fourth of the cottonseed flour potentially available could satisfy the present serious deficiencies in protein in diet-deficient nations, and the protein is of high nutritional quality.[29]

There are no known instances of toxic effects of gossypol ingestion in humans, and there have been several reports on the absence of deleterious effects when cottonseed meal with low gossypol content has been consumed in moderate amounts, such as 60 g daily for 4.5 months of heated cottonseed cake originally containing 0.11–0.20% free gossypol.[30] A number of agencies have studied the use of cottonseed flour in foods, notably the Instituto de Nutricion de Centro America y Panama (INCAP), which developed Incaparina, a food containing 38% cottonseed flour.[31] Maximum gossypol in cottonseed preparations for human use has been set at 0.045% (450 mg/kg) free gossypol in the United States. Less than 0.06% free and 1.2% total is recommended by international groups.[25] Use of Incaparina by small children under close clini-

cal supervision for over 2 yr failed to cause any toxicity, and there was much improved protein status over an unsupplemented diet.[25] The product is already a commercial and nutritional success in some countries, notably Guatemala, and some families have used it for more than 4 yr without any indication of difficulty.[25,29,31]

Nevertheless, gossypol is a potential toxicant, and the fact that it is now possible to use processed cottonseed flour safely in the human diet depends on information developed in animal studies.

Gossypol is 1,1',6,6',7,7'-hexahydroxy-5,5'-diisopropyl-3,3'-dimethyl-[2,2'-binaphthalene]-8,8'-dicarboxaldehyde:
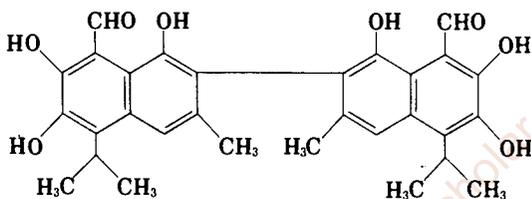
Gossypol

It occurs in the three equilibrium forms: the aldehyde, its enolic quinoid tautomer, and as the hemiacetal with the *peri*-hydroxyl.[1,25,32] It is strongly acidic for a phenol, is readily oxidizable, and acts as an antioxidant. It is soluble in most solvents of intermediate polarity but is not readily soluble in hexane or water. Along with it in cottonseed are found at least 15 related pigments in smaller amounts, including purple, orange, blue, and green substances. As far as they have been studied, they are derivatives of gossypol, or gossypol can be generated from them by treatments with acid, etc.[25] Biosynthesis is via the isoprenoid pathway, apparently by a specific cyclization of *cis-cis*-farnesyl pyrophosphate.[33] Gossypol pigments are confined in the cotton plant to "glands" (ovospheroidal bodies about 100–400 μm long) present in all but the woody tissues. Cotton without glands in the seed kernels (and therefore free of gossypol in the meal) has been bred and is beginning to be planted commercially. It remains to be seen if differing pest and disease resistance, fiber yield, quality, etc. will enable cotton with glandless seed to displace the glanded forms.[25,28,29,31]

The pigment glands represent about 2.4–4.8% of the kernel in varieties grown in the United States, and gossypol is about 20.6–39.0% of the weight of the glands; the other pigments contain about 2%.[25] In present commercial processes a small portion of the gossypol appears in the unrefined cottonseed oil, but it imparts color and is removed to a low level by refining and bleaching processes.[25,27] Heating in the pres-

ence of moisture disrupts the glands and causes the conversion of much
of the free gossypol to bound forms. The lack of toxicity of cottonseed,
even with intact glands, to ruminants is attributed to prolonged masti-
cation, water contact, and increased time in the rumen, which result in
binding of gossypol to protein.[34] Gossypol can be determined by spec-
trophotometry of the aniline derivative at 440 nm.[25] Total gossypol is
that recovered after hydrolysis with oxalic acid in methyl ethyl ketone
or after heating with 3-amino-1-propanol in *N,N*-dimethylformamide.
Free gossypol is that extracted at room temperature with aqueous ace-
tone or similar solvents; bound, of course, is total minus free.

Present commercial processes for cottonseed meal production all in-
volve the use of heat and other processes to remove, destroy, or bind as
much as possible (80–99%) of the gossypol.[25,28,31,35] The four types of
processes for oil removal are screw pressing, pressing then solvent ex-
traction, direct solvent extraction, and hydraulic pressing. The residual-
oil and free-gossypol contents of typical commercial meal products pre-
pared in these four ways would be about 2.5–5% and 0.02–0.05%, 0.4–1%
and 0.02–0.07%, 1% and 0.1–0.5%, and 4.5–7.5% and 0.04–0.10%, respec-
tively. The residual total gossypol is about the same for all and is in the
range of 0.5–1.2%. Hydraulic pressing has nearly disappeared. The trend
is toward solvent extraction, particularly prepress solvent extracting, at
the expense of screw pressing, although the latter still accounts for about
half of the production in the United States.

Bound gossypol is considered essentially nontoxic to animals. The
toxicity of cottonseed meal to nonruminants is usually attributed to
free gossypol, although there are complications. A simple analysis of
the free and total gossypol content does not truly measure the biological
activity under all circumstances. The activity is affected by the pH and
composition of the diet, the source and method of incorporation of the
gossypol, the test animal, etc.[25,36,37,38] It has been suggested that there
are several forms of both free and bound gossypol, each with different
activities,[37] or that toxicants in addition to gossypol are present in
cottonseed.[38] Cyclopropenoid fatty acids (p. 196) do occur in the
residual lipids of the meals, but, with a few exceptions such as pinking
of egg white from hens fed cottonseed meal, it has been difficult to
attribute the extra toxicity to them.[25,29]

Pigment glands can be isolated intact. They are quite variable in ac-
tion but may be as much as four times as toxic as pure gossypol in
equivalent amounts. The acute oral toxicity of gossypol is not high for
most animals. On a single-dose basis the oral LD_{50} to rats is about
2,400–3,340 mg/kg (averaging 2,630 mg/kg) when administered in
water; it is about 10% more toxic when administered in oil.[38] The

water-soluble equimolar glycine adduct did not reach LD_{50} values at 6,000 mg/kg. Intact glands have given acute oral LD_{50} values with rats of 925–2,170 mg/kg in water and were slightly less toxic in oil.[38] The three most toxic samples had the highest gossypol content (36.9–40.0%) and the least toxic the lowest (28.6%), but intermediate correlations were not proportionate. It appears that potentiation of absorption by oil and possibly by unidentified gland components, together with lesser inactivation in the gut or en route to a sensitive site (and possible synergism among the various forms of gossypol pigments) could account for the extra toxicity of glands and variable results with other preparations. So far at least, no different natural toxicant species has been identified, and gossypol pigments appear to be the significant toxicant of cottonseed meal.

In experimental animals, sensitivity to gossypol toxicity appears to decrease in the order guinea pig, rabbit, mouse, and rat.[38] Dogs appear quite sensitive; repeated oral dosage at 10–200 mg/kg/day is usually fatal in less than a month. Chickens are about as sensitive as rats, but pigs are more sensitive. Fed toxic levels of gossypol, pigs may appear normal for a few weeks to a year, then abruptly begin to gasp for breath (thump) and die in a few days with severe anemia and other complications.[1,6,32] Commercial meal with 0.04% or less free gossypol is believed to be safe as a supplement in balanced diets for chicks and pigs,[27] but 0.02% gossypol in the diet is the approximate borderline between toxic and nontoxic levels in the pig[39]; for safety, 0.01% or less free gossypol (or not over 9 percent cottonseed meal) in the diet is recommended.[40]

Common symptoms[6,25] with cumulative gossypol or cottonseed meal toxicity are loss of appetite; weight loss; hypoprothrombinemia[41]; diarrhea; hair discoloration; lowering of hemoglobin, red cell count, and serum protein; edematous fluid in body cavities, lungs, and heart; degenerative changes in liver and spleen; hemorrhages of liver, small intestine, and stomach; and yolk discoloration and decreased hatchability of eggs. Gossypol is more rapid but less effective than dicumarol in suppressing prothrombin formation, and the effect is antagonized by some forms of vitamin K; however, this effect is evidently contributory rather than primary in hemorrhage induction and the toxicity of gossypol.[41]

Gossypol forms stable, equimolar chelates of low water solubility with many metallic cations, apparently by linking through the carbonyl and *ortho*-hydroxyl groups.[42,43] The Schiff's base adducts formed between gossypol and amines (such as aniline) still chelate metals, though slightly less strongly. It appears that gossypol produces anemia by its binding of iron. Addition of iron salts to diets with free gossypol decreases their

toxicity, increases the proportion of the gossypol found in the feces, and at high levels can hasten the elimination of gossypol from the body and produce normal hematology.[44,45,46] Iron alone, however, does not prevent all toxic effects of gossypol, but the addition of 1% calcium hydroxide to the diet with iron did fully prevent gossypol toxicity at the levels involved.[44] This appears to be partly from rendering the iron complexes less soluble,[47] but could also be due to the fact that gossypol is unstable to alkali,[25] probably owing to catalysis of oxidation. Iron also appears to catalyze loss of the formyl group, and, although a major part of this action takes place in the digestive tract, it seems to be an important detoxification pathway for gossypol.[45] Little gossypol is eliminated via the urine, bile being the major excretory route.[45]

Combination, apparently of the Schiff's base type, between the carbonyls of gossypol and amino groups of amino acids and proteins is a major factor in the toxicity of gossypol and its cumulative effects. Part of the toxicity of gossypol can result from rendering amino acids unavailable by this reaction, particularly lysine via its terminal amino group, thus lowering the biological value of the diet.[48,50] A high intake of quality protein or supplementation of the diet with excess lysine lowers but does not abolish the toxicity of high gossypol levels.[39,50] The binding with gossypol lowers the digestibility of the protein and increases the proportion of the gossypol in the feces.[49,51,52] Bound gossypol suppresses liberation of some other amino acids by enzymatic digestion more than by the liberation of lysine.[53] Since both carbonyls of gossypol will link with amino groups, cross linking between protein chains could have a profound effect on enzymic hydrolysis.[49] The free carbonyls of gossypol could, of course, similarly link with enzyme protein to bind and inactivate it. Hill and Totsuka[56] showed that the addition of gossypol to a chick diet reduced the metabolizable energy of the diet. Rojas and Scott[57] observed increased metabolizable energy in chick diets in which the cottonseed meal was treated with phytase. Ferrous sulfate addition also improved the metabolizable energy value of diets containing glanded cottonseed meal but not glandless meal.

The development of an olive discoloration in egg yolk from hens fed cottonseed meal is due to the reaction between gossypol and iron in the yolk. Cyclopropenoid fatty acids, also from the cottonseed, enhance the discoloration by accelerating an increase in pH during storage, making the iron more readily available.[35] Removing the fat containing the cyclopropenoid fatty acids and reducing the amount of available gossypol are, therefore, both desirable objectives in processing cottonseed meal. The measurement of a gossypol-cephalin fraction in eggs from hens fed cottonseed meal has been used as an assay for the available gossypol in meals.[58]

Gossypol at superphysiological levels inhibits oxidative enzymes *in vitro*, but enzymes in liver mitochondria from animals showing gossypol toxicity were not inhibited when oxygen uptake was measured.[54] However, respiration involvement certainly seems indicated, and (by analogy to dicumarol and other phenols) uncoupling of oxidative phosphorylation, rather than inhibition of oxygen uptake, would be suspected as a gossypol effect.

The binding between gossypol and proteins no doubt also explains the high residual gossypol and cumulative effect of gossypol in the body. In the body, gossypol is especially high in the epithelial lining of the stomach and in the liver; considerable amounts are also bound in the kidney, spleen, blood, and muscle.[45,51,52] The liver may reach 900 mg of gossypol/kg in poisoned pigs; after cessation of intake, the level in the liver declined from 520 to 100 mg/kg total gossypol in 34 days.[46] The half-life of gossypol in the rat body, including the digestive tract, following a single small oral dose was about 48 h without—and 23 h with—added iron.[45] The ratio of extractable to residual gossypol in the tissues increased with time.[45] This indicates that gossypol was being eliminated by hydrolysis of the proteins to which it was bound and by excretion of the nontoxic[38] but soluble amino acid or peptide adduct. In trout,[55] evidence has been found of a slow buildup of gossypol bound in the tissues over an extended period of gossypol feeding. After a year or more of gossypol intake, shifting to a gossypol-free diet gave a decrease in free gossypol in body tissues, but bound gossypol decreased little—or even increased in certain tissues—over a 10-week period.

Tangeretin

The completely methylated flavonoids tangeretin, nobiletin, and the flavonol analog of nobiletin (3,3',4',5,6,7,8-heptamethoxyflavone) occur in citrus, the first two in tangerine, mandarin, and orange, and the latter in grapefruit.[59] They, of course, cannot occur as glycosides and are evidently confined to the oil receptacles such as those in the fruit peel (but not in the flesh). Juice pressed from orange peel contains about 20 mg/liter of nobiletin and perhaps 3 mg/liter of tangeretin. Along with other flavones and flavanones, tangeretin is nontoxic in various acute tests, including 500 mg/kg intraperitoneally to mice and 1,000 mg/kg orally to dogs (some diarrhea). However, tangeretin was second only to podophyllotoxin as a cytotoxin to zebra-fish embryos. It affected 50% of the embryos at 24 h at 0.2–0.4 mg/liter; nobiletin was less active but was not tested beyond 1 mg/liter.[60] With 10 mg/kg/day of tangeretin subcutaneously to rats during gestation, 83% of the offspring, although appearing normal, were born dead or died within 3 days.[61] The natural
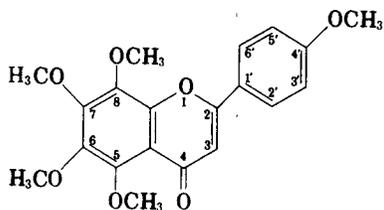
Tangeretin
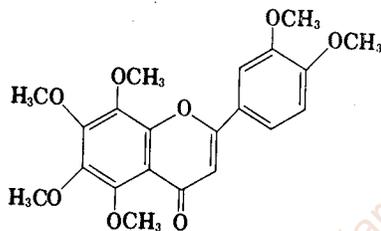
Nobiletin
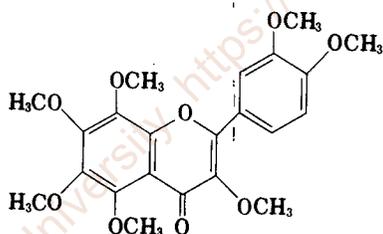
3,3',4',5,6,7,8-Heptamethoxyflavone

hazard of fruit to humans seems very small or absent. Pending further testing, regular consumption in copious amounts of tangerine peeling, and perhaps that of other citrus fruits, should be discouraged and is unlikely in any event. Its lipid solubility and completely substituted phloroglucinol ring would be suspected factors in tangeretin toxicity.

Phlorizin

The dihydrochalcones and their glycosides are of very limited and specific occurrence in plants.[59] Most of them have not been investigated toxicologically, even though some occur in toxic plants. Phloretin, 2',4',6'-trihydroxy-3-(p-hydroxyphenyl)propiophenone, occurs in apple as the 2'-glucoside (phlorizin) at levels of up to 1% in the fresh leaf, over 12% in the root bark (dry-weight basis), and about 300–400 mg/kg in
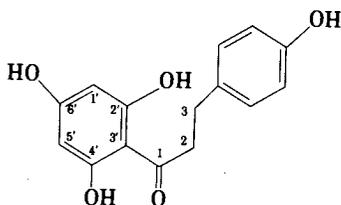
Phloretin

the fruit core and seeds.[62,63] Phlorizin produces the toxic effect of pronounced glucosuria in man and animals with oral doses of the order of 200–400 mg/kg of body weight.[64,65] The specific physiological effect is to block the active transport of glucose from kidney-tubule urine back into peritubular blood against the concentration and osmotic gradient by affecting the epithelial cells. Absorption of glucose in the small intestine is also inhibited.

The actual concentration of phlorizin at the active site in the kidney is very low, i.e., the substance is very potent. Phloretin is nearly inactive orally, and part of the activity loss en route to the kidney is probably due to hydrolysis of phlorizin.[64,65] Methylated phlorizin is inactive. Treatment of rats with 500 mg/kg subcutaneously of phlorizin for 6 days gave a slight weight loss, drowsiness, and—in spite of glucosuria— an increase in kidney glycogen.[66] This latter effect also occurs in liver and evidently results from inhibition of oxidation of carbohydrate. Phloretin (and to a lesser extent phlorizin) inhibits mitochondrial ATPase noncompetitively.[67] Phlorizin diminishes the efficiency of phosphorylation in mitochondria and causes them to swell. This swelling can be prevented by ATP addition. In plants, phlorizin and phloretin inhibit oxidative phosphorylation and have a distinct uncoupling effect,[68] phloretin being more active. Phloretin inhibits photophosphorylation by chloroplasts (phlorizin is about one third as effective) by affecting both transphosphorylation and electron transport during ATP synthesis.[69] Chloroplast ATPase is also inhibited. Studies with the absorbing cells of hamster intestinal and kidney epithelium have indicated that phlorizin is adsorbed instantaneously and strongly on a readily accessible receptor site.[70] There are a maximum of about 4×10^8 of these sites per absorbing cell membrane, and their binding would appear competitively to affect glucose uptake. However, the actual poison appears to be phloretin, which is liberated by hydrolase activity in the membrane; phlorizin serves only as a carrier of phloretin capable of penetrating some selective barrier in the membrane. Since glucose absorption is energy requiring, the phloretin would presumably act by interfering with

ATP production for this purpose, and the apparent conflict between the relative activity of phloretin and phlorizin in plants and animals would be reconciled.

Anthraquinone Cathartics

Vegetable purgatives of wide use (perhaps weekly by 30% of the elderly[71]) such as senna leaf and related products from *Cassia* spp., cascara and frangula barks from Rhamnaceae, aloe sap preparations, and rhubarb root (*Rheum* spp.) are active because of their content of anthraquinone derivatives. The species of rhubarb used are not the same as the garden food plant but are closely related.[72] Except for—or perhaps because of—the sometimes violent purging action, these compounds appear to be of low oral toxicity. A *Cassia* preparation of unknown content (but probably about 1% total anthraquinone derivatives) produced a single-dose oral LD_{50} to rats of 10 g/kg.[73] The total anthraquinone content of the natural dry drugs is usually 1–3% but may be up to 11% in fresh cascara bark. Typical laxative doses would contain 10–100 mg of anthraquinone derivatives,[13] but gross anthraquinone content is a poor measure of cathartic power.

The mixture of these substances and their chemistry is complex.[2,74,75,76] In the fresh plant the anthrone form is present, and apparently during drying and aging of the drug, dianthrones (usually two similar units but not always) and anthraquinones form or at least increase.[77] This is accompanied by a decrease in the violence and potency of the drug's action.[2] There appears to be a synergistic effect of the components. Both *C*- and *O*-glycosides are present, with the former considerably predominant. The anthraquinones present in these drugs include rhein (1,8-dihydroxyanthraquinone-3-carboxylic acid) and several others with more reduced substituents in position 3 or with additional hydroxyls. The particular group of substances present is, of course, variable by plant source. They may be biosynethsized by the acetate–malonate pathway as would be expected, or alternatively in higher plants such as *Cassia* and *Rheum* by a shikimic acid pathway as are cinnamates and flavonoids.[79]
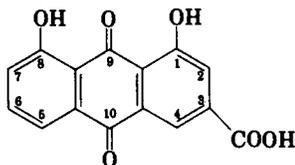
Rhein

High purgative activity is favored in these compounds by prevention of absorption en route to the site of action, the large intestine.[80] This is favored by high molecular weight and retention of glycosidation. Retention of dianthrone and reduced forms by prevention of oxidation increases activity; the more water-soluble aglycone is usually more active. The specific toxic action seems to be to initiate strong peristalsis in the large intestine; it may be related to 5-hydroxytryptamine metabolism since this substance appears to control peristalsis and it is more than just an "irritant" effect.[1] More serious and permanent effects appear related to destruction of nervous tissue in the intestinal wall, which seems to be a factor in the "cathartic colon" problem, particularly in the elderly after long dosage.[71] Rhein in dilute solution is an inhibitor of electron transport in mitochondrial NAD-linked oxidations. Only slight uncoupling of oxidative phosphorylation was observed and, unlike rotenone (p. 315), its effect was not reversed by synthetic vitamin K.[78,79] Rhein also tends to bind various proteins, but its effect is apparently not through HS-groups.[81,82,83]

Tannins

Any plant polyphenolic substance with a molecular weight greater than about 500 can be considered to be a tannin. The two distinctive groups are the hydrolyzable and the condensed tannins.[84] They have in common protein-binding and leather-forming activities, but they usually differ considerably in botanical distribution, other properties, and breakdown products. Tannic acid is typical of the hydrolyzable tannins. It is readily hydrolyzed enzymatically or hydrolyzes spontaneously to glucose and gallic acid (p. 310) with about 7 or less gallic acid units per glucose. Other tannins of this group may yield as hydrolysis products[84] ellagic acid, which replaced gallic acid, or quinic acid, which replaces glucose.

Tannic Acid
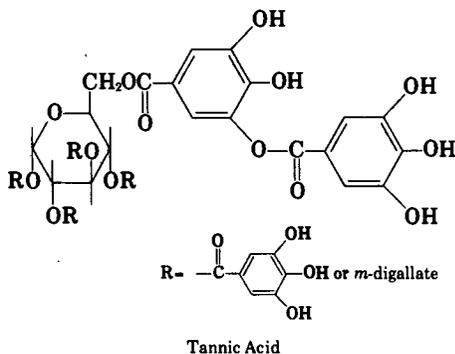
The condensed tannins (flavolans) are polymeric flavonoids composed predominantly of leucoanthocyanidin (p. 310) units linked carbon-to-carbon from the 4-position of one unit to the 6- or 8-position of the next.[84] They do not break down readily under physiological conditions; when treated drastically, they usually produce either less soluble polymeric "phlobaphenes" or flavonoid monomers, particularly catechins and anthocyanidins.[84]
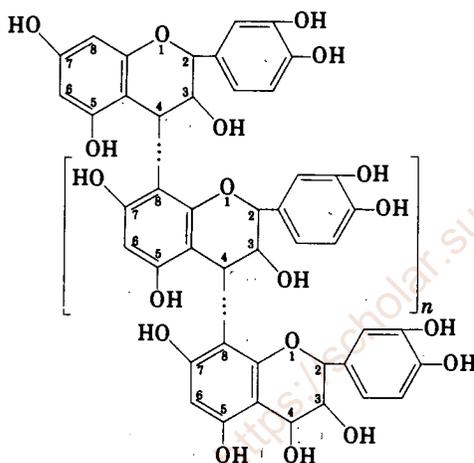
Condensed Tannin (flavolan)

Tannic acid has been the tannin most used in medical treatment; its single-dose oral acute toxicity is low, the LD_{50} to 160-g rats by gastric intubation being $2,260 \pm 83$ mg/kg.[85] One reason for the low oral toxicity is that tannic acid is hydrolyzed in passing through the normal gut, and only gallic acid or metabolic products thereof appear in blood or urine.[86] The absorption of an intact protein-binding, nondialyzable tannin macromolecule, whether hydrolyzable or condensed, seems quite unlikely, and the best evidence seems to be that it does not occur in the normally functioning alimentary tract of an animal.[1]

Liver and kidney toxicity and human fatalities, however, were produced when 3–5% tannic acid solution was used as a treatment on burned tissue. Absorption into the bloodstream must have occurred, since gallic acid does not produce the same effect.[87] Condensed tannins are somewhat less toxic in burn treatment. When injected, all tannins are rather toxic, as might be expected for powerful precipitants of proteins. A purified condensed tannin from hawthorn had LD_{50} values in mice of 300 mg/kg subcutaneously and 130 mg/kg intraperitoneally,[88] while another from spruce bark given intraperitoneally to rats had an LD_{50} of 100 mg/kg.[24] The LD_{100} value of tannic acid administered intravenously to mice was 80 mg/kg.[16]

Carcinogenicity seems to be a definite component in tannin toxicity.[87] The production of liver cancer in rats by repeated subcutaneous application of tannin has been demonstrated.[87,89] Habitual chewers of betel nut (*Areca catechu*) have a high incidence of buccal carcinoma that has been attributed to the 11–26% condensed tannins contained in the nut, although other components may be involved.[90,91] A series of epidemiological, demographic, and dietary correlations[92] present some evidence for human esophageal cancer related to food or beverage tannin. Perhaps the most nearly convincing case is the unusually high incidence of esophageal cancer in certain areas of the Transkei of South Africa when high-tannin sorghum is consumed. The cancer appears to result from chronic irritation of the mucous membranes of the throat caused by high consumption of the grain in Bantu beer and porridge; the incidence is particularly high in drought periods when high-tannin "bird-proof" grain sorghums are utilized to produce sufficient food.

Tannic acid has been used in barium enemas to improve definition of the colon wall in diagnostic x-rays. Severe acute liver damage, sometimes fatal, was produced in a small proportion of the patients.[1,93] The toxicity of tannic acid when administered rectally is about twice that of the substance when given orally.[85] The incidence of serious reactions after tannin enemas was greater when administered to juvenile patients, when the enemas were given repeatedly, when the patients had pre-existing ulcers or inflammation, when the enemas were retained in the bowel for increased periods of time, or when the tannin concentration was increased.[94,95,96] Liver injury from rectal tannic acid appears to result from damage to the intestinal wall that allows absorption of intact tannin. The damage is related to the tannin concentration; 0.25% or less in the enema preparation appears to be safe.[95] Tannic acid inhibits the absorption of glucose and methionine in the mouse intestine; this is believed to be the result of denaturation of the proteins of the protective outer cellular layer of the mucous membrane.[97] Localized destruction of epithelium of the gastrointestinal tract has been found to occur following oral administration of tannic acid and to be more severe in newborn than in older rats.[98]

A small injection of tannic acid gives recognizable cellular and subcellular changes within 1 h in kidney and liver and, if the animals recover, cells of these organs return to normal after about 7 days.[99,100,101] Effects include decreased liver glycogen, proteinuria, altered mitochondria, and fragmentation of polyribosomes with associated strong inhibition of protein and ribonucleic acid synthesis in the liver.

There are instances of significant farm-animal losses owing to high-tannin consumption in natural feed. Periodic losses in England from high consumption of oak acorns were attributed to the approximately 6%

tannin in the acorns, since no other toxic fraction was identified.[102] In the southwestern United States, annual losses of livestock may exceed $10 million from consuming shin oak (*Quercus havardi*) foliage at a time when other forage is lacking.[1,103] The toxicity results from a high level of hydrolyzable tannin, which produces liver necrosis and other effects similar to those described for other tannins. Some substances when added to the diet (e.g., iron salts, calcium salts, high protein levels) oxidize, precipitate, or bind the tannin and thus aid in avoiding this toxicity.[104]

Experimental feeding of diets with known or added tannins to chicks and to rats have shown growth depression with levels of the order of 1% of the diet, but effects are somewhat variable. Chicks are apparently more sensitive than rats. As much as 0.5% of various tannins in the drinking water of mice for 3 months gave no liver carcinoma and minimal other effects.[89] Condensed tannin, a pectinase inhibitor from sericea (*Lespedeza cuneata*) used in pickles, was fed to rats at 2% of the diet for as long as 150 days without effect on growth or other toxic effects.[105] Condensed tannin from grapes also produced no histopathological changes in rats fed 2% in their diet for 700 days.[105] Even intramuscular injection with 0.75 mg/kg of tannic acid in mice every other week for 18 months produced only a weak local reaction and no signs of cancer or other liver damage in one study.[106]

It appears that there are several causes of the growth-depressing and toxic effects of tannins, and the interplay between them and the experimental conditions account for variable observations. High tannin in the diet makes it astringent, and the animals must be starved to force them to eat it. Weight loss during this period is more serious for smaller, younger animals and seems to be at least one reason that larger animals are more tolerant of a high-tannin diet.[107] Paired feeding (i.e., the animal on the normal diet is limited to the intake of the animal on the high-tannin diet) shows that feed intake and presumably palatability are major but not the only factors in growth depression by high-tannin diets.[107,108,109]

The feeding of tannin also leads to lowered energy conversion from food and to the excretion of high levels of nitrogen in the feces.[109,110,111,112] The high nitrogen excretion results largely from the binding of dietary protein by tannin into an indigestible form. Any protein added in excess of the amount required to bind the tannin is utilized by the animal, resulting in greatly improved growth; the residual nitrogen in the feces remains about as it was before the addition.[112] Supplementation with 40% casein and 5% tannic acid gave growth equal to that of pair-fed rats without these supplements.[107] The protein–tannin

complex appears to be formed by multiple hydrogen bonding, as in leather tanning, because treatment of feces with urea liberates some of the tannin.[111] Similar nonspecific binding accounts for the highly inhibitory effect of free tannin on digestive enzymes[111] and, since the body responds to high-tannin diets by synthesizing severalfold as much proteolytic enzymes,[112] part of the high nitrogen excretion is endogenous enzyme protein. A net loss of nitrogen would be possible on protein-poor and high-tannin diets, and this would be expected to be most deleterious to young animals during the period of rapid muscular growth.

Depending upon the situation, supplementation with choline, methionine and ornithine, or arginine sometimes partially counteracts dietary tannic acid toxicity.[113,114] Since no arginine or ornithine conjugate was found in the excreta,[114] their effects would presumably reflect mere supplementation of a deficient diet. Free amino acids do not bind strongly with tannin. The methyl donors (choline and methionine), however, are necessary for detoxification of gallic acid to 4-*O*-methyl-gallic acid, a major excretory product when tannic acid is fed.[113,114] Methylated products are also important in the detoxification of flavonoids.[21] Thus a high absorption of tannin-breakdown products would deplete the supply of the essential amino acid methionine and place further stress on the animal unless methionine or other methyl donors are plentiful in the diet. Fatty livers, a symptom of methyl-donor deficiency, were produced by 5% gallic acid, but not with 5% tannic acid in the diet of rats.[107] Catechin is also growth depressing, although it and gallic acid are less toxic than tannic acid; they do not bind protein, and they appear to affect palatability less.[109,112,115] Additional load and nutrient loss may be occasioned by other similar mechanisms. For example, gallic acid is apparently partly detoxified, at least in the rabbit, by conjugation with glucuronic acid, since the urinary excretion of glucuronic acid is increased 10-fold following gallic-acid feeding, reaching values as high as 250 mg/100 ml of urine.[116] Two metabolic products from tannic acid, pyrogallol and pyrocatechol, are highly toxic to the chicken.[113,114]

It would appear from the data now available that tannins can be seriously toxic, perhaps carcinogenic, if they enter the bloodstream. However, it seems that unless high concentrations of soluble tannins *not*
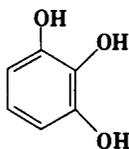
Pyrogallol
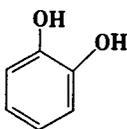
Pyrocatechol

complexed with protein or other binding agent contact mucosa in such a way as to damage it, or unless they contact burns or other unprotected areas, they are not absorbed. Unless absorbed intact, their effects seem related only to either the systemic effects of breakdown products known to be low in toxicity, or to "external" effects (particularly on palatability) and protein digestion. Since they tend to break down more, hydrolyzable tannins would be expected to be more toxic than condensed tannins in the systemic sense. The no-effect level for food-grade tannic acid in rats has been established to be 800 mg/kg body weight/day, and the total acceptable daily intake for a man is 560 mg.[96] This, however, is below the total intake of "tannin" by some persons, which may be of the order of 1,000 mg/day in coffee, tea, cocoa, etc. Mueller[117] has estimated that a child fed his entire milk intake as chocolate milk and eating additional candy might consume 160 mg/kg/day of cocoa tannin.

GENERAL CORRELATIONS AND PERSPECTIVE

Many other examples of reported plant phenol toxicity could be cited in addition to those noted above and elsewhere in this volume. However, it seems more useful to attempt to achieve some overall perspective on their various roles as a class of compounds including toxic members.[1] The phenolic substances that normally occur in foods are generally considered to be nontoxic[1,2,118,119] and certainly long history of apparently safe consumption in food and use in various experimental medical treatments[35,119–121] would bolster this view. In fact, many favorable effects have been claimed, although often later refuted or unsubstantiated, following administration of certain flavonoids and cinnamates.[35,120,121]

Some reports of toxicity of common phenols appear to result from the specific experimental conditions. Commercial rutin from eucalyptus produced cataracts after oral doses of about 25 mg to rats, but pure quercetin or rutin from other sources did not have this effect, which was evidently from contaminants.[122] In mice and rats with implanted tumors, the maximum tolerable (and tumor-inhibiting) oral dose of cyanidin-3,5-diglucoside was 350 mg/kg.[123] Purified mixtures of anthocyanidin glycosides from currants, blueberries, and elderberries produced no deaths in mice or rats with oral doses of 20 g/kg; the intravenous LD_{50} was 240 mg/kg in rats and 40 mg/kg in mice.[124] Oral doses of 6 g/day to rats for 3 months produced no gross or microscopic abnormalities. Anthocyanins and most other natural flavonoids are not very readily absorbed when fed, but rabbits fed 500 mg of grape anthocyanins ex-

creted 1–2% of the pigment in the urine, and dogs injected intravenously with the same pigment excreted portions of it within 20 min in both bile and urine.[125] Oral doses of 750 mg of anthocyanins to humans produced no toxic effects but produced in man and animals accelerated regeneration of visual purple and dark adaptation.[126,127] The flavonoids without glycosidation and with higher lipid solubility seem more toxic; flavone, for example, had an oral LD_{50} to mice of 400 mg/kg.[128] However, in the course of studies of the metabolism of flavonoids by humans, ingestion of 2-g samples of several common flavonoids produced no significant toxic effects.[21]

The common detoxification path of flavonoids in animals involves the destruction of the phloroglucinol "A" ring[129] and the excretion of the "B" ring products in forms like those predicted from cinnamic acid metabolism.[21] Of significance to our discussion, methylation of phenolic hydroxyls and conjugation with glucuronic acid or glycine are common reactions leading to the excreted metabolites. As already discussed with tannins, this metabolic loss of methionine, glycine, and metabolizable carbohydrate would be stress producing and in marginal situations could produce or aggravate toxicity. This would appear to be the toxic mechanism for the simpler and common plant phenols, very high dietary levels of which are ordinarily required to demonstrate toxic effects experimentally. In general, feeding at 1% of the diet to experimental animals for prolonged periods has produced little or no effect on growth or other significant toxicity for naringin, hesperidin, rutin, quercetin, dihydroquercetin, quercitrin, catechin, or gallic acid.[21,115] At 2% and more of the diet, gallic acid and catechin cause increasing growth depression[115] in the rat. Bioflavonoids from citrus at 2.5% in the diet did not depress chicken growth but 5% did.[130]

From these data, it would appear to be very unlikely that the plant phenols usually present in human diets would produce significant toxic effects. In some cases, however, the amounts of phenols consumed may reach high levels and may produce some negative effects, especially when the diet is suboptimal (as may be caused by famine or idiosyncrasy, for example). Unripe persimmons have a total phenol content of the order of 13 g/kg; although only about half this amount can be recovered from the ripe fruit, the remainder probably is present but insoluble.[131] Some bananas are nearly as high in total phenols as persimmon. If grapes are consumed, seeds and all, they could contribute about 5.6 g of total phenols/kg.[132] Nearly all other fruits and vegetables have caffeic and other cinnamic acid derivatives of the order of 100–300 mg/kg fresh weight and many have as much as 500 mg/kg of flavonoids in addition.[133] Coffee and tea may contribute 1 g of total phenols/day[96] in the

habitual diet in this country; in other countries consumption of an exhaustive decoction of 100 g tea/day, contributing up to 30 g of polyphenols to the diet, has led to toxic symptoms.[134] There appear to be significant positive (and some negative) correlations between cancer mortality of specific types and national per capita consumption of tea and coffee as well as cigarettes and other potential carcinogens such as solid fuels.[135]

A common observation in natural phenolic toxicity studies has been synergism, as reflected in the fact that the isolated components are not as toxic as they are when present in the natural mixture or added back together. Synergism of this general type is noted[1] with aspidium,[14] gossypol, anthraquinone cathartics, dihydromethysticin and kava components, myristicin and nutmeg components, and others. Perhaps the most striking example is the synergistic augmentation of rotenone's insecticidal activity by nontoxic phenols such as sesamin.[136] One component of such synergism appears to be the placing of extra demands on the animal's adaptive detoxification mechanisms when they are already having difficulty in coping with a toxicant. From this it would appear that normally innocuous plant phenols could increase the effect of toxic phenols. It is not uncommon to find a considerable percentage of usual, nontoxic phenols occurring in the same plant material with the unusual and toxic ones.
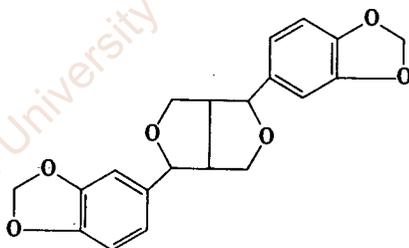
Sesamin

The toxicity of tangeretin would appear to be partly related to its lipid solubility and lack of glycosidation, but it also seems to depend on the animal's inability to metabolize its completely substituted phloroglucinol A-ring directly to carbon dioxide as is the case with the common flavonoids.[21,129] It seems highly significant that many of the most toxic or most physiologically potent phenolic derivatives are, or appear to be, derived from mevalonic acid–terpenoid biosynthesis: gossypol, tetrahydrocannabinol (from marihuana), and tremetone (from snakeroot), for example. Other toxic phenols appear to be derived primarily from the acetate pathway but seem to have been further modified so that they cannot be metabolized as simple phloroglucinol derivatives,

e.g., anthraquinones, hypericin, aspidium components, and rhodomyrtoxin. The shikimic acid biosynthetic pathway seems to give rise to relatively few toxic cinnamate or flavonoid components; when they are toxic they also are substituted in additional, unusual ways (e.g., tangeretin, isoflavones, rotenone, dicumarol, etc.). A common feature is the methylene dioxy grouping that is found in safrole and myristicin and is necessary in sesamin-type rotenone synergy.[1]

In addition to protecting the phenols from metabolism by usual animal detoxification mechanisms so that they can reach vulnerable sites and cause toxicity, substitutions of the types mentioned—methylation, methylene dioxy groups, polycyclization, alkyl substituents, etc.—would increase lipid solubility, and this tends to enhance toxicity. Administration of many phenolic toxicants in oil aids their absorption and increases the toxic effect; myristicin and gossypol afford two examples.[1] Related but opposite effects were noted with anthraquinone cathartics in that the glycosides (especially nonhydrolyzable *C*-glycosides) enable the medication to reach the lower bowel and act without absorption earlier. Phloretin to be active must reach the kidney as the glucoside phlorizin, even though the liberation of the aglycone in passing through the kidney cell membrane may be required for the actual toxic process.

The combination of lipid- and water-soluble properties of phenols appears important in their toxicity, membrane transfer, and penetration to active sites. Phenol itself is an extraordinary solvent for proteins and is used in the laboratory to extract proteins from nucleic acids, for example. A small amount of phenol spilled on appreciable skin area is rapidly absorbed and may easily be fatal. Penetration through the skin is a feature of some forms of natural phenol toxicity, notably poison ivy urushiol and related cashew and mango dermatitis and photosensitization (see Chapter 25) from topically applied psoralen.[1] Cumarin in ointment rubbed on the skin leads to the same urinary excretory products as does parenteral administration.[137] This ready penetration applies to internal tissues as well. Esters of salicylic acid applied to the skin in ointment rapidly penetrate into the muscles directly below but not lateral to the application.[138] Aspirin is rapidly absorbed through the stomach wall; since many phenols have the required properties of rather small molecular size, polarity, and nonionizability at stomach pH, they should be absorbed also.[139]

It appears to us that this solvent action and ready penetration by phenols may explain the observations that phenols can serve as promoters of carcinoma. Under certain conditions, repeated application of phenols following a single application of a carcinogen such as benzopyrene produced skin cancers (although a single application of a carcino-

gen to animal skin did not produce cancer and phenols alone did not);
similar promoting effects are found in inhalation-induced lung can-
cer.[140,141] Black tea contains phenol, cresols, and other small phenols.
An initial application to skin of 3,4-benzopyrene followed by frequent
applications of brewed tea produced epithelial carcinoma in 6 of 15
treated mice.[142] Pyrolysis produces smaller phenols such as guaiacol and
4-vinylguaiacol from lignin, ferulic acid, and other larger natural phenols;
however, carcinogenic hydrocarbons and phenols are also among the
products of pyrolysis of cellulose, glucose, fructose, sodium acetate,
proteins, amino acids, etc.[143]

$$OH$$

$$OH$$

$$n\text{-}C_{15}H_{25-31}$$

Urushiol
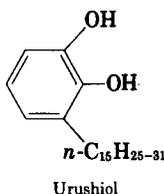
The analyzed content of phenols in the surface layer of smoked meat
is only of the order of 37 mg/kg, although it appears this is about half
the true value.[144] At this dilute level of phenol content, tumor promo-
tion by solubilization of carcinogenic hydrocarbons and by carrying
them along as the cells are penetrated would seem unlikely. Although
the possibility of tumor promotion by phenols seems to be real enough
and it should not be forgotten, as a food hazard it seems small, in view
of the long and apparently safe use of smoked foods without demon-
strated adverse effects.[144]

An action of phenols generally, including natural plant phenols, is
the uncoupling of oxidative phosphorylation in the mitochondria so
that respiration energy is not trapped in usable form as ATP but is
wasted. This effect can be predicted from physical and chemical data on
a series of substituted phenols, taking into account hydrophobic (lipo-
philic) character and tendency to ionize.[145] These data predict about
equally well the relative toxicity and uncoupling activities. Highly ion-
izable phenols tend to be more active in phosphorylation uncoupling,
and the nitrophenols are much more active than the natural phenols.
However, a major action involved in the toxicity of rotenone and di-
cumarol is the uncoupling of oxidative phosphorylation. The specific
site of action is apparently different, since they are affected differently
by antagonists such as vitamin K. Concentration of the phenol at or in
specific membranes seems to be involved (thus lipoidal solubility), and
inhibition of energy requiring membrane transport is a frequent effect,
as seems to be the case with phlorizin in glucosuria. The pain-relieving

effect of salicylates is believed to involve inhibition of oxidative phosphorylation at specific pain receptors.[12]

Common features of phenol toxicity are the cumulative effect and the relatively prolonged retention of the substance in the body. This is the case with gossypol, dicumarol, tetrahydrocannabinol, urushiol, hypericin, psoralen, and podophyllin.[1] These effects involve binding of the phenol to body constituents. In some cases, for example, with gossypol and perhaps tremetone, the binding is primarily via nonphenolic functionality. In other instances, it appears to involve hydrogen bonding and other linking dependent upon the phenolic character. Certainly the effect of tannin in tying up dietary protein is a related example. Urushiols from poison ivy are rapidly bound as the skin is penetrated.[1] Tetrahydrocannabinol binds to plasma lipoproteins.[146] Salicylates bind to albumin in blood and are no longer dialyzable.[1] Salicylates and phenylbutazone potentiate the effect of coumarin anticoagulants, such as dicumarol, apparently by displacing them from nonspecific binding sites in the liver and blood-plasma proteins.[147,148] The binding, however, need not be simple, readily displaced complexing. Covalent linking has been demonstrated between the catechol portion of urushiols and proteins.[149] The mechanism involves oxidation of the catechol to *ortho*-quinone and then linking of the protein groups (particularly sulfhydryl) to the activated, unsubstituted positions on the quinone ring. This strong binding mechanism is available to many plant phenols, including gossypol, which contains the common catechol type of substitution.

Another potential toxic effect involves metal chelation, as is evident in anemia related to gossypol and tannin toxicity and some other conditions such as prolonged overdosing with aspirin. Many phenols, including common ones with low toxicity, bind metal ions rather strongly through vicinal functional groups.

Since phenols are commonly detoxified by methylation, competitive inhibition of *O*-methyltransferase by plant phenols is apparently the reason they often prolong the action of adrenaline.[1,21] Interference with nerve-regulating animal phenols appears to be involved in the central-nervous-system effects reported for the active phenolic constituents of marihuana, nutmeg, sassafras, marking nut, and kava. Similar peripheral effects are indicated by trembling, analgesia, peristalsis, etc., as shown by salicylates, anthraquinone cathartics, tremetone, and hypericin.[1]

A mild goitrogenic action by anthocyanins from various sources, by catechin, quercitrin, and phloroglucinol, appears to result from the binding of iodine with these phenols instead of with tyrosine to form thyroxine.[150,151]

Irradiation of psoralen produces activation, which enables interstrand cross linking with DNA; this effect is related to photosensitizing activity in animals.[152] Light activation, production of free radicals, etc., are common properties of plant phenols, but these specific effects seem limited to substances that have a certain molecular structure. Similarly, the estrogenic activity of isoflavones and coumestrol appears to result from the positioning of two phenolic hydroxyls a certain distance apart in a relatively planar molecule.[1]

CONCLUDING REMARKS

Although some plant phenols are quite toxic, and a few phenols have caused occasional serious human poisonings or serious losses of animals, these are mostly caused by phenolic structures uncommon in human food. The common phenols are of low toxicity under most circumstances. However, this low toxicity appears to result from efficient barriers developed by animals through long contact with common plant phenols. Several toxic mechanisms appear to be potentially available for most plant phenols, but if natural defenses are overloaded (by large amounts) or evaded (by unusual forms of administration or unusual derivatives), toxicity may result. Barring accidental consumption of plant material or medicines not normally considered to be food for humans, the most likely toxicity to humans would appear to be marginal effects from unusually high consumption of a high-phenol beverage or food, coupled with a poor diet. Such effects appear rare and are difficult to demonstrate. They would be more likely in juvenile, senile, or debilitated individuals. Some suggestive relationships (such as those between mouth cancer and the chewing of high-tannin areca nut, or throat cancer and high-tannin grain sorghum consumption) need further investigation to be considered proved, but caution is certainly suggested.

REFERENCES

1. V. L. Singleton and F. H. Kratzer, Toxicity and related physiological activity of phenolic substances of plant origin. J. Agric. Food Chem. *17*:497 (1969).
2. J. W. Fairbairn (ed.), The pharmacology of plant phenolics. Academic Press, New York (1959).
3. J. M. Kingsbury. Deadly harvest, a guide to common poisonous plants. Holt, Rinehart and Winston, New York (1965).
4. K. F. Lampe and R. Fagerström, Plant toxicity and dermatitis, a manual for physicians. Williams & Wilkins, Baltimore (1968).
5. S. B. O'Leary, Poisoning in man from eating poisonous plants. Arch. Environ. Health *9*:216 (1964).

6. J. M. Kingsbury, Poisonous plants of the United States and Canada. Prentice-Hall, Englewood Cliffs, N.J. (1964).

7. J. W. Hardin and J. M. Arena, Human poisoning from native and cultivated plants. Duke University Press, Durham, N.C. (1969).

8. H. G. Scott, Poisonous plants and animals. In Food-borne infections and intoxications, H. Riemann (ed.). Academic Press, New York (1969).

9. F. Corcilius, Die Wirkstoffe der Rosskastanie und ihre Biochemischen Funktionen bei Erkrankungen des Venensystems. Q. J. Crude Drug Res. 7:1005 (1967).

10. G. Vogel, M. L. Marek, and R. Oertner, Untersuchungen zum Mechanismus der therapeutichen und toxischen Wirkung des Rosskastanien-Saponins Aescin. Arzneimittel-Forsch. 20:699 (1970).

11. N. H. Anderson, W. D. Ollis, J. G. Underwood, and R. M. Scrowston, Constitution of the dibenzofuran psi-rhodomyrtoxin, isolated from Rhodomyrtus macrocarpa Benth. J. Chem. Soc. C. 2403 (1969).

12. M. J. H. Smith and P. K. Smith, The salicylates: A critical bibliographic review. Wiley, New York (1966).

13. P. G. Stecher (ed.), The Merck index. 8th ed. Merck and Co., Rahway, N.J., p. 107 (1968).

14. M. J. Mattila and S. Takki, The effect of non-phloroglucinol residues of fern extracts on the toxicity and absorption of desaspidin. Ann. Med. Exp. Biol. Fenn. 45:357 (1967).

15. A. Georges, Y. Gerin, and J. Denef, Toxic effects of purified fern extracts. Proc. Eur. Soc. Stud. Drug Toxicity 10:218 (1969).

16. W. S. Spector, Handbook of toxicology, Vol. 1. W. B. Saunders, Philadelphia, (1956).

17. R. Chatterjee, Indian Podophyllum. Econ. Bot. 6:342 (1952).

18. A. Heyndrickx, Toxicology of insecticides, rodenticides, herbicides, and phytopharmaceutical compounds. Prog. Chem. Toxicol. 4:179 (1969).

19. K. Böhm, Die Flavonoide. Arzneimittel-Forsch. 9:539,647,778 (1959); 10: 54,139,188,468,547 (1960).

20. S. Radouco-Thomas, P. Grumback, G. Nosal, and F. Garcin, Etude toxicologique de quelques facteurs P. Therapie 20:879 (1965).

21. F. De Eds, Flavonoid metabolism, In Comprehensive biochemistry, M. Florkin and E. H. Stotz (eds.). 20:127–171. Elsevier, Amsterdam (1968).

22. F. Chassevent, L'Acide chlorogenique ses actions physiologiques et pharmacologiques. Ann. Nutr. Aliment. 23:1 (1969).

23. M. Mancini, P. Oriente, and L. D'Andrea. Hypocholesterolemic effects of quinic acid 1,4-dicaffeate in atheroscloerotic patients, p. 533–537. In S. Garattini and R. Paoletti (eds.) Drugs affecting lipid metabolism. Proceedings of a Symposium on Drugs Affecting Lipid Metabolism. Elsevier, Amsterdam (1961).

24. P. Claveau and M. Masquelier, Le metabolisme du leucocyanidol chez le rat. Can. J. Pharm. Sci. 1:74 (1966).

25. L. C. Berardi and L. A. Goldblatt. Gossypol, p. 211. In Toxic constituents of plant foodstuffs, I. E. Liener (ed.). Academic Press, New York (1969).

26. K. M. Decossas, L. J. Molaison, A. de B. Kleppinger, and V. L. Laporte, Cottonseed oil and meal utilization. J. Am. Oil. Chem. Soc. 45:52A (1968).

27. A. M. Altschul, C. M. Lyman, and F. H. Thurber. Cottonseed meal, p. 469. In Processed plant protein foodstuffs, A. M. Altschul (ed.). Academic Press, New York (1958).

28. U.S. Department of Agriculture, Proceedings of the 1967 Cottonseed Processing Clinic. ARS 72-61 Washington, D.C. (1967).

29. F. E. M. Gillham, Cotton in a hungry world. S. Afr. J. Sci. 65:173 (1969).

30. A. A. Adamova and M. A. Lebedeva, Khlopkovyi zhmykh kak dopolnitelnyi istochnik pitaniya. Gig. Sanit. 12:33 (1947).

31. G. A. Harper and K. J. Smith. Status of cottonseed protein. Econ. Bot. 22:63 (1968).

32. R. Adams, T. A. Geissman, and J. D. Edwards, Gossypol, a pigment of cottonseed. Chem. Rev. 60:555 (1960).

33. P. F. Heinstein, D. L. Herman, S. B. Tove, and F. H. Smith, Biosynthesis of gossypol. Incorporation of mevalonate-2-^{14}C and isoprenyl pyrophosphates. J. Biol. Chem. 245:4658 (1970).

34. R. Reiser and H. C. Fu, The mechanism of gossypol detoxification by ruminant animals. J. Nutr. 76:215 (1962).

35. R. A. Phelps, Cottonseed meal for poultry. World's Poult. Sci. J. 22:86 (1966).

36. R. Bressani, L. G. Elias, and A. Porras, Effect of pH on the free and total gossypol and nutritive value of cottonseed and protein concentrate. Arch. Latinoam. Nutr. 19:367 (1969).

37. L. C. Berardi and W. H. Martinez, Gossypol source versus biological activity. Proceedings of the Conference on Inactivation of Gossypol with Mineral Salts. National Cottonseed Products Association. Memphis (1966).

38. E. Eagle, A review of some physiological effects of gossypol and cottonseed pigment glands. J. Am. Oil Chem. Soc. 37:40 (1960).

39. A. J. Clawson, F. H. Smith, L. C. Osborne, and E. R. Barrick, Effect of protein source, autoclaving, and lysine supplementation on gossypol toxicity. J. Anim. Sci. 20:547 (1961).

40. F. Hale, C. M. Lyman, and H. A. Smith, Use of cottonseed meal in swine rations. Bull. 898 Tex. Agric. Exp. Stn. (1958).

41. W. S. Harms and K. T. Holly, Hypoprothrombinemia induced by gossypol. Proc. Soc. Exp. Biol. Med. 77:297 (1951).

42. H. N. Ramaswamy and R. T. O'Connor, Spectroscopic properties of some metal complexes of gossypol. Appl. Spectrosc. 24:50 (1970).

43. H. N. Ramaswamy and R. T. O'Connor, Metal complexes of gossypol. J. Agric. Food Chem. 17:1406 (1969).

44. J. E. Braham, R. Jarquin, R. Bressani, J. M. Gonzalez, and L. G. Elias, Effect of gossypol on the iron-binding capacity of serum in swine. J. Nutr. 93:241 (1967).

45. M. B. Abou-donia, C. M. Lyman, and J. W. Dieckert, Metabolic fate of gossypol: The metabolism of ^{14}C-gossypol in rats. Lipids 5:939 (1970).

46. J. A. Buitrago, A. J. Clawson, and F. H. Smith, Effects of dietary iron on gossypol accumulation in and elimination from porcine liver. J. Anim. Sci. 31:554 (1970).

47. T. R. Shieh, E. Mathews, R. J. Wodzinski, and J. H. Ware, Effect of calcium and phosphate ions on the formation of soluble iron-gossypol complex. J. Agric. Food Chem. 16:208 (1968).

48. E. J. Conkerton and V. L. Frampton, Reactions of gossypol with free epsilon-amino groups of lysine of proteins. Arch. Biochem. Biophys. 81:130 (1959).

49. C. M. Lyman, B. P. Baliga, and M. W. Slay, Reactions of proteins with gossypol. Arch. Biochem. Biophys. 84:486 (1959).

50. J. N. Tone and D. R. Jensen, Effect of ingested gossypol on the growth performance of rats. Experientia 26:970 (1970).

51. F. H. Smith and A. J. Clawson, Effects of dietary gossypol on animals. J. Am. Oil Chem. Soc. *47*:443 (1970).

52. C. M. Lyman, J. T. Cronin, M. M. Trant, and G. V. Odell, Metabolism of gossypol in the chick. J. Am. Oil Chem. Soc. *46*:100 (1969).

53. C. M. Cater and C. M. Lyman, Effect of bound gossypol in cottonseed meal on enzymic degradation. Lipids *5*:765 (1970).

54. L. A. Meksongee, A. J. Clawson, and F. H. Smith, The *in vivo* effect of gossypol on cytochrome oxidase, succinoxidase, and succinic dehydrogenase in animal tissues. J. Agric. Food Chem. *18*:917 (1970).

55. J. N. Roehm, D. J. Lee, and R. O. Sinnhuber, Accumulation and elimination of dietary gossypol in the organs of rainbow trout. J. Nutr. *92*:425 (1967).

56. F. W. Hill and K. Totsuka, Studies on the metabolizable energy of cottonseed meals for chicks, with particular reference to the effect of gossypol. Poult. Sci. *43*:362 (1964).

57. S. W. Rojas and M. L. Scott, Factors affecting the nutritive value of cottonseed meal as a protein source in chick diets. Poult. Sci. *48*:819 (1969).

58. C. R. Grau, E. Allen, M. Nagumo, C. L. Woronick, and P. A. Zweigart, A distinctive yolk component in the fresh eggs of hens fed gossypol. J. Agric. Food Chem. *2*:982 (1954).

59. J. E. Kefford and B. V. Chandler, The chemical constituents of citrus fruits. Advances Food Research, Suppl. 2. Academic Press, New York (1970).

60. R. W. Jones, M. G. Stout, H. Reich, and M. N. Huffman, Cytotoxic activities of certain flavonoids against zebra-fish embryos. Cancer Chemother. Rep. *34*:19 (1964).

61. M. G. Stout, H. Reich, and M. N. Huffman, Neonatal lethality of offspring of tangeretin-treated rats. Cancer Chemother. Rep. *36*:23 (1964).

62. A. H. Williams, Dihydrochalcones, p. 297. In Comparative phytochemistry, T. Swain (ed.). Academic Press, New York (1966).

63. A. B. Durkee and P. A. Poapst, Phenolic constituents in core tissues and ripe seed of McIntosh apples. J. Agric. Food Chem. *13*:137 (1965).

64. F. W. McKee and W. B. Hawkins, Phlorizin glucosuria. Physiol. Rev. *25*:255 (1945).

65. W. D. Lotspeich, Phlorizin and the cellular transport of glucose. Harvey Lect. *56*:63 (1960–61).

66. S. Babu, T. V. Madhavan, and K. R. Rao, Effect of phlorizin and related compounds on glucose excretion, kidney glycogen content and alkaline phosphatase activity of treated rats. Indian J. Med. Res. *55*:1226 (1967).

67. M. T. Tellez de Inon, Inhibition of mitochondrial ATPase by phlorizin. Acta Physiol. Lat. Am. *18*:268 (1968).

68. G. Stenlid, On the physiological effects of phlorizin, phloretin, and some related substances upon higher plants. Physiol. Plantarum *21*:882 (1968).

69. E. G. Uribe, Phloretin: An inhibitor of phosphate transfer and electron flow in spinach chloroplasts. Biochem. *9*:2100 (1970).

70. D. F. Diedrich, Is phloretin the sugar transport inhibitor in intestine? Arch. Biochem. Biophys. *127*:803 (1968).

71. Anonymous, Cathartic action. Br. Med. J. *4*(5633):723 (1968).

72. N. Taylor, Plant drugs that changed the world. Dodd, Mead, New York (1965).

73. D. G. Patel, S. S. Karbhari, O. D. Gulati, and S. D. Gokhale, Antipyretic and analgesic activities of *Aconitum spicatum* and *Cassia fistula*. Arch. Int. Pharmacodyn. *157*:22 (1965).

74. J. W. Fairbairn, The anthracene derivatives of medicinal plants. Lloydia *27*: 79 (1964).

75. A. Stoll and B. Becker, Sennosides A and B, the active principles of senna. Prog. Chem. Org. Nat. Prod. 7:248 (1950).

76. R. Kinget, Studies in the field of drugs containing anthracene derivatives. 17. The quantitative determination of anthracene derivatives of *Rhamnus purshiana* bark. Lloydia *31*:17 (1968).

77. R. P. Labadie, Anthracene derivatives in *Rhamnus frangula*. 1. The aglycones. Pharm. Weekbl. *105*:189 (1970).

78. T. J. McCarthy, Distribution of glycosyl compounds in South African aloe species. Planta Med. *17*:1 (1969).

79. E. Leistner and M. H. Zenk, Chrysophanol (1,8-dihydroxy-3-methylanthraquinone) biosynthesis in higher plants. Chem. Commun. *(5)*:210 (1969).

80. J. W. Fairbairn and M. J. R. Moss, Relative purgative activities of 1,8-dihydroxyanthracene derivatives. J. Pharm. Pharmacol. *22*:584 (1970).

81. E. A. Kean, Rhein: An inhibitor of mitochondrial oxidations. Arch. Biochem. Biophys. *127*:528 (1968).

82. E. A. Kean, Inhibitory action of rhein on the reduced nicotinamide adenine dinucleotide-dehydrogenase complex of mitochondrial particles and on other dehydrogenases. Biochem. Pharmacol. *19*:2201 (1970).

83. E. A. Kean, M. Gutman, and T. P. Singer, Rhein, a selective inhibitor of the DPNH-flavine step in mitochondrial electron transport. Biochem. Biophys. Res. Commun. *40*:1507 (1970).

84. E. Haslam, Chemistry of vegetable tannins. Academic Press, New York (1966).

85. E. M. Boyd, K. Bereczky, and I. Godi, The acute toxicity of tannic acid administered intragastrically. Can. Med. Assoc. J. *92*:1292 (1965).

86. F. W. Blumenberg and F.-J. Kessler, Untersuchungen über die Resorbierbarkeit oral zugeführten Tannins. Arzneimittel-Forsch. *10*:742 (1960).

87. B. Korpássy, Tannins as hepatic carcinogens. Prog. Exp. Tumor Res. *2*:245 (1961).

88. W. Rewerski and S. Lewak, Einige pharmakologische Eigenschaften der aus Weissdorn (*Crataegus oxyacantha*) isolierten Flavan-Polymeren. Arzneimittel-Forsch. *17*:490 (1967).

89. K. S. Kirby, Induction of tumours by tannin extracts. Br. J. Cancer *14*:147 (1960).

90. V. Raghava and H. K. Baruah, Arecanut: India's popular masticatory—History, chemistry, and utilization. Econ. Bot. *12*:315 (1958).

91. D. Schmahl, Krebserzeugung durch Naturstoffe. Dtsch. Med. Wochenschr. *89*:575 (1964).

92. J. F. Morton, Tentative correlations of plant usage and esophageal cancer zones. Econ. Bot. *24*:217 (1970).

93. M. L. Janower, L. L. Robbins, and D. E. Wenlund, A review of the use of tannic acid in patients with ulcerative colitis. Radiology *89*:42 (1967).

94. F. F. Zboralske, P. A. Harris, S. Riegelman, O. N. Rambo, and A. R. Margulis, Toxicity studies on tannic acid administered by enema. 3. Studies on the retention of enemas in humans. 4. Review and conclusions. Am. J. Roentgen. *96*:505 (1966).

95. Anonymous, A reprieve for tannic acid on the way? Food Cosmetol. Toxicol. *5*:244 (1967).

96. Anonymous, Tannic acid again. Food Cosmetol. Toxicol. 7:364 (1969).

97. S. Mitjavila, G. De Saint-Blanquat, and R. Derache, Effet de l'acide tannique sur l'absorption intestinale chez le souris. Food Cosmetol. Toxicol. *8*:27 (1970).

98. M. S. Weinberg, R. E. Goldhamer, and S. Carson, Acute oral toxicity of various drugs in newborn rats after treatment of the dam during gestation. Toxicol. Appl. Pharmacol. *9*:234 (1966).

99. R. K. Boler, J. S. Broom, and R. B. Arhelger. Ultrastructural renal alterations following tannic acid administration to rabbits. Am. J. Pathol. *49*:15 (1966).

100. A. A-B. Badawy, A. E. White, and G. H. Lathe, The effect of tannic acid on the synthesis of protein and nucleic acid by rat liver. Biochem. J. *113*:307 (1969).

101. J. K. Reddy, M. Chiga, C. C. Harris, and D. J. Svoboda, Polyribosome disaggregation in rat liver following administration of tannic acid. Cancer Res. *30*:58 (1970).

102. E. G. C. Clark and E. Cotchin, A note on the toxicity of the acorn. Br. Vet. J. *112*:135 (1956).

103. B. J. Camp, E. Steel, and J. W. Dollahite, Certain biochemical changes in blood and livers of rabbits fed oak tannin. Am. J. Vet. Res. *28*:290 (1967).

104. G. V. N. Rayudu, R. Kadirvel, P. Vohra, and F. H. Kratzer, Effect of various agents in alleviating the toxicity of tannic acid for chickens. Poult. Sci. *49*:1323 (1970).

105. A. N. Booth and T. A. Bell, Physiological effects of sericea tannin in rats. Proc. Soc. Exp. Biol. Med. *128*:800 (1968).

106. J. Bichel and A. Bach, Investigation on the toxicity of small chronic doses of tannic acid with special reference to carcinogenicity. Acta Pharmacol. Toxicol. *26*:41 (1968).

107. Z. Glick and M. A. Joslyn, Food intake depression and other metabolic effects of tannic acid in the rat. J. Nutr. *100*:509 (1970).

108. P. Handler and R. D. Baker, The toxicity of orally administered tannic acid. Science *99*:393 (1944).

109. P. Vohra, F. H. Kratzer, and M. A. Joslyn, The growth depressing and toxic effects of tannins to chicks. Poult. Sci. *45*:135 (1966).

110. J. K. Conner, I. S. Hurwood, H. W. Burton, and D. E. Fuelling, Some nutritional aspects of feeding sorghum grain of high tannin content to growing chickens. Aust. J. Exp. Agric. *9*:497 (1969).

111. M. Tamir and A. Alumot, Carob tannins—Growth depression and levels of insoluble nitrogen in the digestive tract of rats. J. Nutr. *100*:573 (1970).

112. Z. Glick and M. A. Joslyn, Effect of tannic acid and related compounds on the absorption and utilization of proteins in the rat. J. Nutr. *100*:516 (1970).

113. G. V. N. Rayudu, R. Kadirvel, P. Vohra, and F. H. Kratzer, Toxicity of tannic acid and its metabolites for chickens, Poult. Sci. *49*:957 (1970).

114. D. K. Potter and H. L. Fuller, Metabolic fate of dietary tannins in chickens. J. Nutr. *96*:187 (1968).

115. M. A. Joslyn and Z. Glick, Comparative effects of gallotannic acid and related phenolics on the growth of rats. J. Nutr. *98*:119 (1969).

116. F.-W. Blumenberg and R. Dohrmann, Über den Ausscheidungsmechanismus oral zugeführter Gallussäure. Arzneimittel-Forsch. *10*:109 (1960).

117. W. S. Mueller, The significance of tannic substances and theobromine in chocolate milk. J. Dairy Sci. *25*:221 (1942).

118. E. C. Bate-Smith, Flavonoid compounds in foods. Adv. Food Res. *5*:261 (1954).

119. W. Pearson, Flavonoids in human nutrition and medicine. J. Am. Med. Assoc. *164*:1675 (1957).

120. J. J. Willaman, Some biological effects of the flavonoids. J. Am. Pharm. Assoc. Sci. Educ. *44*:404 (1955).

121. Anonymous, Today's drugs—Vitamin P. Br. Med. J. No. 5368:235 (1969).

122. Y. Nakagawa, M. R. Shetlar, and S. H. Wender, Physiological effects of commercial rutin samples on rats. Life Sci. *4*:753 (1965).

123. O. K. Kabiev and S. M. Vermenichev, O protivoopukholevykh svoistvakh 3,5-diglikozid-tsianidina. Vopr. Onkol. *16*(8):60 (1970).

124. H. Pourrat, P. Bastide, P. Dorier, A. Pourrat, and P. Tronche, Preparation and therapeutic activity of some anthocyanin glycosides. Chem. Ther. *2*:33 (1967).

125. M. K. Horwitt, Observations on behavior of the anthocyan pigment from Concord grapes in the animal body. Proc. Soc. Exp. Biol. Med. *30*:949 (1933).

126. R. Alfieri and P. Sole, Influence des anthocyanosides administrés par voie oro-perlinguale sur l'adapto-électrorétinogramme (AERG) en lumière rouge chez l'homme. C. R. Soc. Biol. *160*:1590 (1966).

127. R. Wegmann, K. Maeda, P. Tronche, and P. Bastide, Effets des anthocyanosides sur les photorecepteurs. Aspects cytoenzymologiques. Ann. Histochim. *14*:237 (1969).

128. V. Demole, Toxicité, résorption, élimination de la flavone synthétique. Helv. Physiol. Acta *20*:93 (1962).

129. N. P. Das and L. A. Griffiths, Studies on flavonoid metabolism. Metabolism of (+)-[^{14}C]-catechin in the rat and guinea pig. Biochem. J. *115*:831 (1969).

130. C. W. Deyoe, L. E. Deacon, and J. R. Couch, Citrus bioflavonoids in broiler diets. Poult. Sci. *41*:1088 (1962).

131. T. O. M. Nakayama and C. O. Chichester, Astringency of persimmons. Nature *199*:72 (1963).

132. V. L. Singleton and P. Esau, Phenolic substances in grapes and wine and their significance, Adv. Food Res., Suppl. 1, p. 282. Academic Press, New York (1969).

133. K. Heintze, Untersuchungen über die Vitamin P-wirksam Flavonoidverbindungen und ihre Bedeutung in Pflanzlichen Lebensmitteln. Dtsch. Lebensm.-Rundsch. *61*:309 (1965).

134. A. Porot, Les toxicomanies, étude medico-sociale. Libraire Ferraris, Algiers. (1945).

135. P. Stocks, Cancer mortality in relation to national consumption of cigarettes, solid fuel, tea, and coffee. Br. J. Cancer *24*:215 (1970).

136. P. Budowski and K. S. Markley, The chemical and physiological properties of sesame oil. Chem. Rev. *48*:125 (1951).

137. I. Pekker and E.-A. Schäfer, Vergleich von enteraler und perkutaner Resorption von Cumarin. Arzneimittel-Forsch. *19*:1744 (1969).

138. J. Pütter, Resorptionsuntersuchung an Salicylaten. 2. Die percutane Resorption von Salicylsäure-2'-hydroxyläthylester bei äusserlicher Anwendung. Arzneimittel-Forsch. *20*:1721 (1970).

139. A. R. Cooke, Aspirin, ethanol and the stomach. Australas. Ann. Med. *19*:269 (1970).

140. R. K. Boutwell, Phenolic compounds as tumor-promoting agents, p. 120. In Phenolic compounds and metabolic regulation, B. J. Finkle and V. C. Runeckles (eds.). Appleton-Century-Crofts, New York (1967).

141. B. L. Van Duuren, A. Sivak, L. Langseth, B. M. Goldschmidt, and A. Segal, Initiators and promoters in tobacco carcinogenesis. Nat. Cancer Inst. Monogr. *28*:173 (1968).

142. H. E. Kaiser, Cancer-promoting effects of phenols in tea. Cancer *20*:614 (1967).

143. E. B. Higman, I. Schmeltz, and W. S. Schlotzhauer, Products from the thermal degradation of some naturally occurring materials. J. Agric. Food Chem. *18*: 636 (1970).

144. P. Issenberg, M. R. Kornreich, and A. O. Lustre, Recovery of phenolic wood smoke components from smoked foods and model systems. J. Food Sci. *36*: 107 (1971).

145. T. Fujita and M. Nakajima, The correlation between physiological activity and physicochemical property of the substituted phenols. Residue Rev. *25*:319 (1969).

146. M. Wahlqvist, I. M. Nilsson, F. Sandberg, S. Agurell, and B. Granstrand, Metabolism of cannabis. 4. Binding of Δ^1-tetrahydrocannabinol to human plasma proteins. Biochem. Pharmacol. *19*:2579 (1970).

147. A. I. Sandler, Interactions of oral coumarin anticoagulants with other drugs. Drug Intel. Clin. Pharm. *4*:146 (1970).

148. R. A. O'Reilly and G. Levy, Pharmacokinetic analysis of potentiating effect of phenylbutazone on the anticoagulant action of warfarin in man. J. Pharm. Sci. *59*:1258 (1970).

149. J. C. Byck and C. R. Dawson, Assay of protein-quinone coupling involving compounds structurally related to the active principle of poison ivy. Anal. Biochem. *25*:123 (1968).

150. N. R. Moudgal, E. Raghupathy, and P. S. Sarma, Studies on goitrogenic agents in foods. J. Nutr. *66*:291 (1958).

151. E. Jeney, Review and new data on the goitrogenic properties of flavonoids, Kiserl. Orvostud. *20*:514 (1968) (Trans.)

152. R. S. Cole, Light-induced cross-linking of DNA in the presence of a furocoumarin (psoralen). Studies with phage *Lambda, Escherichia coli,* and mouse leukemia cells. Biochim. Biophys. Acta *217*:30 (1970).

THE PRODUCTION OF ALDEHYDES AS A RESULT OF OXIDATION OF POLYPHENOLIC COMPOUNDS AND ITS RELATION TO WINE AGING

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ABSTRACT

Evidence is presented showing that oxidation of ethanol to acetaldehyde by direct chemical reaction with air occurs at an appreciable rate in wine only by a coupled autoxidation of certain phenolic substances occurring in the wine. The oxidation of vicinal di- and tri-hydroxyphenols like caffeic acid, catechin, or myricetin in 12% ethanol model wine produced acetaldehyde. Similar reaction in aqueous 1-propanol produced propanal showing that the source of the volatile aldehyde is the wine's alcohol.

A mechanism is postulated which appears to be general for autoxidation of phenols, ascorbic acid, melanoidins, reductones, enediols, and related compounds whereby the oxidation of the phenol to a quinone (or ascorbic to dehydroascorbic, etc.) co-produces a strong oxidant, probably hydrogen peroxide, which then can oxidize other substances in the wine such as ethanol. Relevance of these findings to wine storage and aging reactions is discussed.

The volatile aldehyde of wines generally increases during aging. This has been noted with many different types of wine in many places and is a fact generally accepted in enological texts (1). During oxidative aging or baking of sherries and other maderized wines not only does volatile aldehyde content almost invariably increase, but this change is considered an essential part of the maderization process (1, 2, 3). Of course, in some instances high aldehyde production in wine arises from microbial action; aerobic yeast growth in flor sherry production, for example. We are not concerned here with these microbiological reactions. In other instances, however, microbial action is very unlikely and a chemical reaction mechanism for the production of volatile aldehyde must be sought. Examples of this include maderization of wines such as oloroso sherries and tawny ports containing sufficient alcohol to suppress growth of microorganisms, oxida-

tion of pasteurized wines, and the temporary "bottle sickness" attributed to the production of a small amount of acetaldehyde by oxygen contact at bottling of microbiologically stable wines.

In one of the more detailed of the early studies of acetaldehyde's role in the aging of red wines (4) it is noted that although aldehyde eventually accumulates during autoxidation of red wines it is not known whether it is a result of direct oxidation of alcohol or is formed by an induced oxidation. Later work has not made that statement obsolete.

It seems highly significant that ethanol is relatively stable on the chemist's shelf, with little or no aldehyde produced after long exposure to air. It therefore appears that other constituents of wine have an important role in promoting the reaction in wine.

Phenolic substances have a primary role as oxidation substrates in wine. In their review on the

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119

significance of polyphenols of wine Singleton and Esau (5) summarized many of the pertinent observations and inferences drawn from them. They postulated that the reaction with oxygen of phenols in wine not only produced oxidized quinoidal products from suitable phenols but also produced, coincidentally, a strong oxidant, presumed to be hydrogen peroxide, which in turn oxidized additional wine constituents, including ethanol to acetaldehyde.

This hypothesis was highly speculative because it was based upon little actual data and involved extrapolation from media and conditions very different from those in wine storage. As a consequence, this research was begun with the objective of clarifying the oxidation of ethanol in wine and, if possible, verifying or refuting the hypothetical role of phenols as participants in aldehyde production during wine oxidation. If successful, considerable practical importance of the research was seen in relation to wine aging and oxidative spoilage reactions.

MATERIALS AND METHODS

Wines and model solutions: The majority of the work was done on a model solution resembling wine (called "model wine" for convenience) composed of potassium bitartrate at 1.11 g/l plus tartaric acid at 0.7 g/l (pH 3.7) in 12% (v/v) aqueous ethanol. The ethanol (and in other experiments, methanol, l-propanol, carbon tetrachloride, etc.) was freed of possible carbonyl contaminants prior to use by refluxing 3 hours with 2,4-dinitrophenylhydrazine at 1.0 g/l and 2.0 ml of concentrated sulfuric acid followed by distillation.

Phenolic compounds were added as indicated to the model wine to make final concentrations of 1.586×10^{-3}M. This level, equivalent to 200 ppm of pyrogallol, was chosen to approximate the usual total phenol content for white table wines without being unrealistically high for a single phenolic component in comparison with wines in general.

Oxidation test conditions: Model wine or other sample solutions were placed, 100 ml, in 16-fluid-ounce screw-capped prescription bottles (Owens Duraglas Ovals, Owens-Illinois Glass Co.). The headspace of the bottle was sparged for 30 sec with a 5-lb/in² stream of oxygen gas from commercial cylinders before capping. The bottles were stored during oxidation on their flat sides to allow maximum wine-O_2 surface of about 113 cm². Dark chambers maintained at controlled temperatures, usually 50°C (122°F), were used for bottled sample storage.

To compensate for possible individual variation, a weekly sampling consisted of 5 replicates of these bottled samples.

Aldehyde analysis: Quantitative analysis of aldehyde content was by two methods: the titrimetric direct bisulfite method (6,7), and colorimetry of the alkali-treated 2,4-dinitrophenylhydrazones. The latter method was modified from that of Johnson and Scholes (8) by improvements suggested by Basson (9) and, as a result of our studies, a lowering of the 2,4-dinitrophenylhydrazine used (to save reagent and reduce contamination of the hydrazones with hydrazine), a combination of the ethanol and KOH solutions (to save time), and measurement at 430 nm as maximum absorbance rather than the 420 nm used previously. The direct bisulfite method was used on distillates prepared from the sample to be analyzed and produced most of the data reported. The colorimetric method was used on either distillates or carbon tetrachloride extracts, but was considered less desirable. Carbon tetrachloride extracts the hydrazone derivatives but little of the hydrazine hydrochloride reagent.

The aldehydes produced were isolated from distillates by production of their 2,4-dinitrophenylhydrazones in sufficient amount to crystallize directly from the solution or after extraction with CCl_4 and evaporation of the extract to dryness. The weight of hydrazone recovered was also a good approximation of the amount of aldehyde in the distillate. For identification, the 2,4-dinitrophenylhydrazones were recrystallized and compared with similar preparations from known samples of different aldehydes. Criteria used included melting points, infrared spectra (Beckman IR8) in Nujol mull, and paper chromatography as described by Ronkainen (10).

RESULTS AND DISCUSSION

Ethanol autoxidation: The first consideration was whether ethanol could be autoxidized to aldehydic products in the wine-like model solution without any additions. Starting with the carbonyl-free model wine held at 50°C with oxygen, the apparent volatile aldehyde calculated as acetaldehyde varied between 0.9 and 0.6 mg/l over the period of 7 to 35 days. This is equivalent to zero volatile aldehyde production since it represents the minimum of iodine titrant to change the indicator color in the bisulfite analytical method. Raising the temperature to 75°C apparently produced a slight autoxidation of model wine components to volatile aldehyde, resulting in values of 6.5 mg/l in 30 days and 5.7 mg/l at 44 days. Therefore, the temperature was held to 50°C or lower in further tests to avoid possible contribution of direct autoxidation. It is thus concluded that ethanol or tartaric acid is not capable of reacting with oxygen to produce significant volatile aldehyde under conditions that obtain in wine storage or sherry baking. Of course, no browning occurred in these solutions either.

In contrast to the lack of aldehyde production from direct autoxidation of a simple model wine, wines do autoxidize to produce volatile aldehyde. Both our own studies and the literature show this. Heitz et al (11) showed that volatile aldehyde cal-

culated as acetaldehyde developed during sherry baking at 49 ± 1.1°C from an original level of 10 mg/l to 24.4 at 30 days, 44.8 at 60 days, and 36.5 at 90 days without aeration, and to 59.8, 83.3 and 77.4, respectively, with aeration. They considered that the aldehyde arose from direct oxidation of ethanol, noting that the ethanol decreased slightly even in unaerated samples, where evaporation could not account for the losses. Joslyn and Comar (4) reported that a young red wine increased in aldehyde from 22 to 38 mg/l during bottling and one year of storage at room temperature. A second barrel-aged wine similarly treated decreased after bottling from 75 mg/l to 29 mg/l in total volatile aldehyde calculated as acetaldehyde.

It thus appears to be an understatement in the literature (4) that aldehyde during oxidation is formed faster and in larger amounts in wine than in alcoholic solution. According to our results, volatile aldehyde is not formed, or, at most, is formed in traces, in aqueous alcohol model solution under conditions normal to wine storage or processing.

Phenol requirement: Since a solution of ethanol, tartaric acid, potassium bitartrate, and water did not generate volatile aldehyde when autoxidized whereas white wines do, what is the oxidizable component of wine which is necessary to produce this reaction? Various reasons implicate phenols (5), primarily the fact that they constitute the only naturally and universally present group of substances capable of significant direct oxygen uptake under wine conditions. Addition of 200 ppm of pyrogallol (0.001586 M) to the model wine solution (pH 3.7) gave the results shown in Table 1. Note that volatile aldehyde was rapidly produced, reaching an apparent maximum at 3 weeks with a level (73 ppm as acetaldehyde) similar to that which is produced in a white wine that would have a total phenol content similar to the level of pyrogallol used (5). Note also that aldehyde production reached a maximum near 1 mole per mole of phenol, and browning paralleled aldehyde production initially but continued to increase after aldehyde content had begun to decrease.

Table 1

Production of Volatile Aldehyde and Browning During Oxidation of Pyrogallol in Model Wine at 50°C

Days	Vol. aldehyde (mg/l AcH)	Moles AcH per mole phenol	Absorbance (420 nm, 1 cm)
0	0.0	0.0	0.0
7	33.7	0.48	0.51
14	50.3	0.72	0.69
21	72.9	1.04	0.82
28	63.0	0.90	0.87
35	65.8	0.94	0.89

Figure 2. The production of acetaldehyde by oxidation of model wines containing 1.586 x 10^{-3} M caffeic acid (Cf), 4-methyl catechol (M), quercitrin (Q), (+)−catechin (Ct), dihydroquercetin (D), or protocatechuic acid (P).

Figure 1. The production of acetaldehyde by oxidation of model wines containing 1.586 x 10^{-3} M pyrogallol (Py), 2,3,4-trihydroxybenzoic acid (T), gallic acid (G), myricetin (M), methyl gallate (MG), phloroglucinol (Ph), or quinic acid (Q).

Pyrogallol is not a natural wine component, although a number of wine constituents such as gallic acid and delphinidin glucosides are pyrogallol derivatives. Therefore, a number of additional substances were tested for volatile aldehyde production when added to model wine. The results are in figures 1 and 2, showing the moles of volatile aldehyde produced (calculated as acetaldehyde) per mole of phenol contained in the solution. This puts different phenols on a directly comparable basis. Since the same molar concentration (1.586 x 10^{-3}M) of all

phenols was used, the plots are also directly proportional to the actual concentration of volatile aldehyde produced. Curve A in figure 1 is from data in table 1. All compounds listed gave appreciable volatile aldehyde production except quinic acid, phloroglucinol, and protocatechuic acid. Quinic acid, although a polyhydroxy compound, is not a phenol, and its lack of reaction further indicates that aldehyde production during oxidation is a concomitant of phenol content. On the other hand, poorly oxidizable phenols such as monophenols and phloroglucinol do not produce appreciable volatile aldehyde either. It appears that from the viewpoint of natural wine constituents we can expect vicinal di- and trihydroxyphenols to produce volatile aldehyde when oxidized under wine conditions.

The rate of volatile aldehyde production is evidently related to the oxidizability of the phenol. Instances of oxidation-reduction potentials readily available from the literature (12) are paralleled by volatile aldehyde production. For example, pyrogallol (E_0 = .713V) is much more active (Figure 1, 2) in volatile aldehyde production than gallic acid (E_0 = 0.799V), and protocatechuic acid (E_0 = 0.883V) gives very little volatile aldehyde. Caffeic acid, a major component of the nonflavonoids of wine (5), is very active in volatile aldehyde production, and flavonoid components such as (+)-catechin and quercetin are also capable of causing the production of volatile aldehyde. In so far as these data are definitive, it appears that electron-withdrawing substituents, like carboxyl or carboxyester groups, decrease the substance's ability to generate volatile aldehyde in wine just as they decrease the phenol's ease of oxidation. Electron-supplying substituents (methyl or hydroxyl substituents, for example) would generally increase the oxidizability and the volatile-aldehyde-generating effect. These results are consistent with predictions from benzenoid chemistry.

Aldehyde source and identity: Having shown that volatile aldehyde in wine does not arise from simple
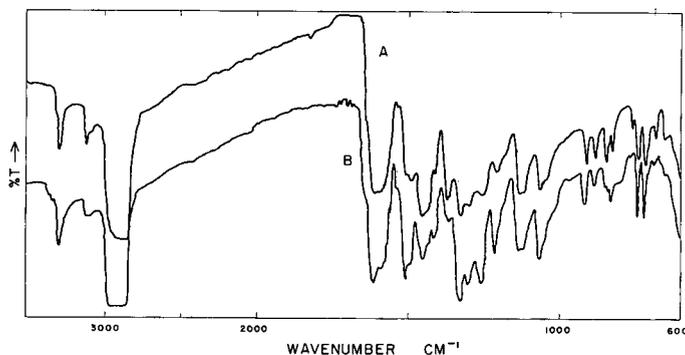
Figure 3. The infrared spectra of acetaldehyde-2,4-dinitrophenyl-hydrazone (A) and of the 2-4-dinitrophenylhydrazone produced from the aldehyde resulting from oxidation of pyrogallol in model wine solution (pH 2.7) for 28 days (B).

TABLE 2

Production of Volatile Aldehyde and Browning During Oxidation of Pyrogallol in Various Solutions at 50°C

Days	Vol. aldehyde (mg/l AcH)	Moles AcH per mole phenol	Absorbance (420 nm, 1 cm)
0% ethanol			
0	0.0	0.0	0.0
25	1.2	0.02	0.68
8.3% methanol			
0	0.0	0.0	0.0
22	6.1	0.09	0.81
15.3% propanol			
0	0.0	0.0	0.0
5	26.4	0.38	0.74
12	23.4	0.34	0.85
19	20.4	0.31	0.88
26	40.0	0.57	0.92
32	58.4	0.84	0.94

autoxidation of ethanol and that readily oxidizable phenols will cause the formation of volatile aldehyde, what is the source of the aldehyde? The volatile aldehyde produced from autoxidation of pyrogallol in model wine was isolated as a crystalline 2,4-dinitrophenylhydrazone. Acetaldehyde-2,4-dinitrophenylhydrazone crystallizes in two forms. One form melts at 156-157°C, the other at 169°C, and a mixture of the two at 146-148°C. We obtained the last melting point after recovery from carbon tetrachloride of extracts of the hydrazone of the aldehyde produced in experimental pyrogallol oxidation, and a mixed melting point with an authentic sample prepared from acetaldehyde-2,4-dinitrophenylhydrazone together with their infrared spectra (Figure 3) convinced us that the volatile aldehyde being produced in the experiment was acetaldehyde. The high yield of the acetaldehyde derivative in relation to the analytical volatile aldehyde content and the relatively high initial purity of the hydrazone derivative indicated that the volatile aldehyde was mostly, if not exclusively, acetaldehyde. The volatile aldehyde from oxidation in model wine of pyrogallol (at both pH 2.7 and 3.7), myricetin, and 4-methylcatechol was shown by melting point and infrared spectral studies of their 2,4-dinitrophenylhydrazones to be the same, acetaldehyde.

It appeared desirable to prove that the volatile aldehyde arose from a coupled oxidation of the ethanol and not from some other reaction. Table 2 shows that when pyrogallol was oxidized in aqueous tartrate solution without ethanol, no volatile alde-

hyde was produced although browning (and there-fore oxidation) occurred. When methanol was sub-stituted on an equimolar basis for the 12% ethanol, some volatile aldehyde production was detected, and the yield was about the same with 1-propanol as with ethanol. The properties of the derivative of the volatile aldehyde produced in the presence of 1-pro-panol were consistent with those of authentic pro-panal-2,4-dinitrophenylhydrazone; the infrared spec-tra are shown in figure 4.
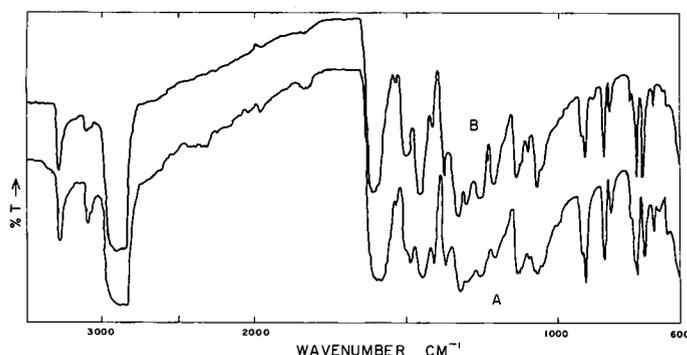
Figure 4. The infrared spectra of propanal-2,4-dinitrophenylhy-drazone (A) and of the 2,4-dinitrophenylhydrazone (B) produced from the aldehyde from the oxidation of pyrogallol in model wine solution containing 15.3% propanol rather than ethanol.
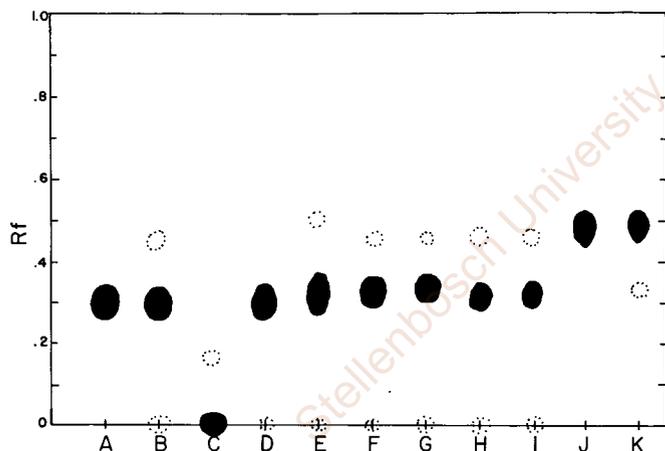
Figure 5. Paper chromatograms of the reagent (C), authentic 2,4-dinitrophenyldrazones of ethanal (A) and propanal (J), the oxidation of pyrogallol in aqueous propanol (K), and the oxida-tion in model wine of pyrogallol (B), ascorbic acid (D), pyrogallol with CaSO$_4$ (E), gallic acid (F), myricetin (G), (+)-catechin (H), and caffeic acid (I).

Paper chromatography of the 2,4-dinitrophenylhy-drazones also agreed with those findings and showed that propanal was the greatly predominant aldehyde produced when propanol was the alcohol in the wine-like model solution, whereas in every instance and regardless of conditions the volatile aldehyde pro-duced from ethanolic model wine was acetaldehyde (Figure 5). Figure 5 was redrawn from several different chromatograms, resulting in an apparent slight variation in R$_f$ values. In fact, on each chrom-atogram the indicated authentic sample cochromato-

graphed indistinguishably from the indicated ex-perimental samples with propanal derived from 1-propanol and acetaldehyde from ethanol. These data seem to us convincing proof that the volatile alde-hyde arising in wine from chemical reactions comes from conversion of ethanol to acetaldehyde by a coupled autoxidation of polyphenols.

Conditions of reaction: Some study was made of the effect of various reaction conditions. Oxidation of pyrogallol at pH 2.7, 3.7, and 4.7, the extremes of the wine range, indicated (Figure 6) that the more
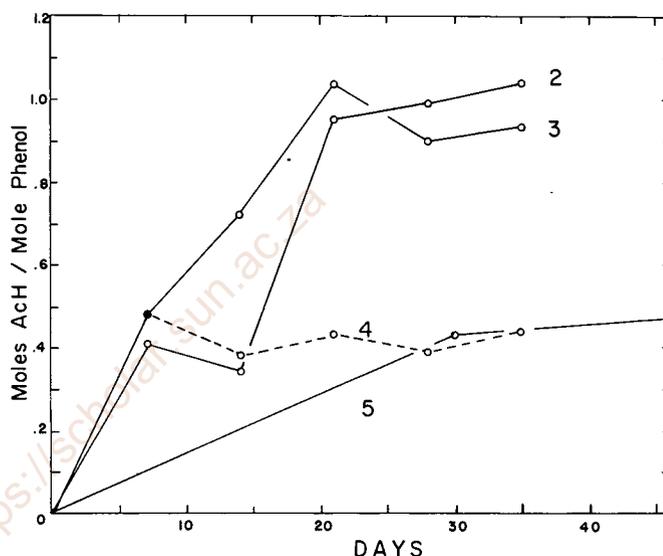
Figure 6. The production of acetaldehyde by oxidation of 1.586 x 10^{-3} M pyrogallol in model wine at pH 2.7 (2), pH 3.7 (3), pH 4.7 (4), all at 50°C, and at pH 3.7 at 25°C (5).

acidic conditions slightly inhibited the oxidation, as would be predicted. (The pK$_a$ of most phenols is 8-10, and the phenolate ion oxidizes much faster than the phenol.) The pH 4.7 sample produced acetaldehyde rapidly at first, but ceased production at a relatively low mole ratio of about 0.4, compared with about 1.0 for the other two pH's. This is attributed to com-petitive reactions at the higher pH, which decrease the production or retention of free acetaldehyde. The reaction of aldehydes with phenols without a phloroglucinol or resorcinol configuration is known to be faster at higher pH and completely prevented in strongly acid conditions (13). It seems signifi-cant that the browning reaction was much faster in the early stages and reached a higher color intensity at pH 4.7 than at pH 3.7 or 2.7. Addition of 86 ppm of acetaldehyde prior to autoxidation of pyrogallol in model wine at pH 3.7 gave rapid browning with-out further increase in volatile aldehyde content. Joslyn and Comar (4) showed that the interpreta-tion of gain or loss of acetaldehyde in wine was com-plicated by reactions which consumed it. In our tests also, as in those of Heitz et al. (13), volatile aldehyde content generally decreased slightly after reaching a maximum. All these observations indi-cate that acetaldehyde production ceases after a time and that further reactions may reduce the

amount formed or consume it after formation.

The effect of temperature was not studied extensively. Acetaldehyde was produced amounting to 30.4 mg/l in 30 days at 25°C with the pyrogallol model wine system, whereas at 50°C about twice that concentration was produced in a little less time (Figure 6, Table 1). Assuming linear production in either case, the apparent acetaldehyde production rate was 3.47 mg/l/day at 50°C and 1.01 mg/l/day at 25°C. This would indicate a Q_{10} of about 1.4, a not unreasonable figure for a complex chemical reaction, and clearly suggests appreciable contribution of this reaction even at cool wine cellar temperatures (10°C) given sufficient time (as in the months and years of wine aging). For example, pyrogallol gave maximum aldehyde production in 21 days at 50°C and at 10°C 81 days should be required. Similar estimations predict maximum aldehyde production at periods of about 1/3 to 1¼ years with other phenols at 1.586 x 10^{-3} M and 10°C.

Water content also seems to be an important variable, because acetaldehyde was found to be produced from pyrogallol oxidation in model wine with 20% ethanol, but not in 99% ethanol or absolute ethanol. Furthermore, acetaldehyde was not recovered from pyrogallol autoxidations in solutions with increasing dimethylformamide and decreasing water content, starting with 12% ethanol, 44% dimethylformamide, and 44% water.

Addition of 5% fructose or 5% glucose had nearly identical slightly depressing effects on acetaldehyde generation by coupled pyrogallol oxidation in model wine. The fructose data are shown in figure 7. No interference from the formation of furfural derivative
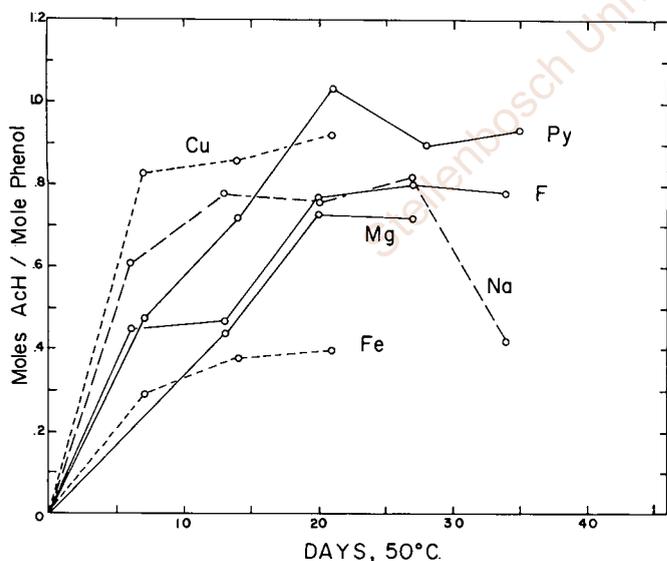
of sweet wines than of dry wines. Addition of 5% sodium chloride (Figure 7) stimulated the early production of acetaldehyde, but the production ceased at a lower concentration and decreased as a brown precipitate formed. Calcium chloride or magnesium sulfate addition at 1.586 x 10^{-3} M had nearly identical inhibitory effects on the reaction. The MgSO$_4$ data are shown in figure 7. Addition of cuprous chloride, cupric sulfate, ferrous sulfate, or ferric chloride at 1.586 x 10^{-3} M affected the acetaldehyde production. The copper salts stimulated the reaction and the iron salts inhibited it. Since the effect of each pair was nearly identical, only the data for the two sulfates are shown in figure 7. Together, these data indicated that other components of wines may influence the production of acetaldehyde by coupled phenol oxidation, but they do not prevent it and appear to play rather subordinate roles.

The fact that ascorbic acid can behave as a promoter of oxidation rather than the expected antioxidant effect has been known for some time (14). Ascorbic acid added to the model wine produces acetaldehyde (Figure 5, 8) even more rapidly than pyrogallol. Addition of 177 ppm of sulfur dioxide (2.8 x 10^{-3} M) greatly reduced but did not eliminate the production of acetaldehyde during oxidation of either ascorbic acid or pyrogallol (Figure 8). After sufficient reaction (about 3 weeks) and presumably consumption of the SO$_2$, the production of acetaldehyde increased although browning was still prevented. Note that the effect of SO$_2$ was not simply to bind acetaldehyde, because both free and sulfite-bound acetaldehyde are determined by the method used.

Figure 7. The production of acetaldehyde by oxidation of 1.586 x 10^{-3} M pyrogallol in model wine at pH 3.7 (Py) with the addition of 5% fructose (F), 5% NaCl (Na), or 1.586 x 10^{-3} M CuSO$_4$ (Cu), FeSO$_4$ (Fe), or MgSO$_4$ (Mg).
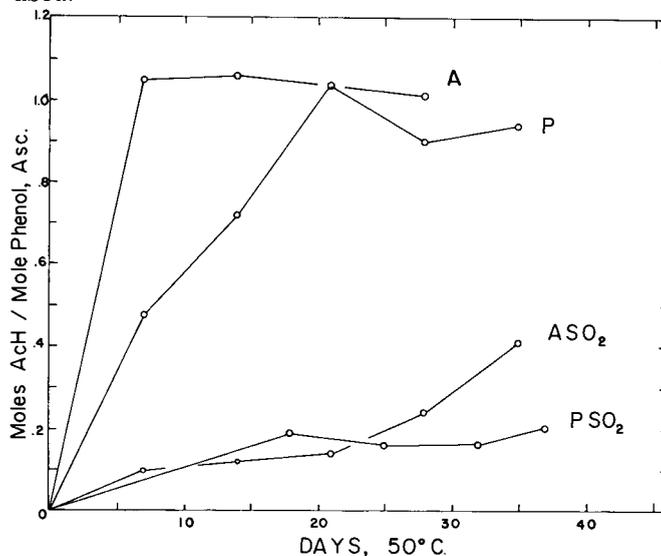
Figure 8. The production of acetaldehyde by oxidation of 1.586 x 10^{-3} M ascorbic acid (A) or pyrogallol (P) in model wine and similarly after addition to each of 177 ppm of SO$_2$ (ASO$_2$, PSO$_2$).

CONCLUSIONS AND INTERPRETATIONS

Oxidation of vicinal di- and trihydroxyphenols, including the types naturally occurring in wines, has been shown to cause the production of acetaldehyde

tive was indicated. The depressing effect might be related to binding of the water and does seem related to the generally greater resistance to oxidation

in model wine solutions. We believe that this is an important reaction in wine contacting oxygen. Oxygen, ethanol (the source of the acetaldehyde), and a readily oxidizable phenol are required. Since reactions involving three molecules simultaneously are very unlikely, and since certain other aspects of the reaction such as brown color development can proceed without involvement of ethanol, a coupled reaction is indicated. The phenol reacts with oxygen, and one of the products is a strong oxidant which then proceeds to oxidize to acetaldehyde the most available substrate in wine, ethanol. Similar oxidation-promoting action by ascorbic acid had already been shown (14), and while this research was in progress Hashimoto (15) reported the oxidation of alcohols in beer by a similar reaction with melanoidins or reductones produced during wort boiling. Our data extend the finding to wine phenols, and we therefore propose that this is a general reaction whereby substances which readily react with O_2 from the air (such as vicinal diphenols, enediols, and reductones, but not sulfur dioxide) all produce coupled oxidations by forming a strong oxidant as they are themselves oxidized. This strong oxidant is capable of rapidly converting alcohols to aldehydes.

In a beverage such as wine the high concentration of ethanol ensures that the first oxidizable molecule encountered by the strong oxidant is likely to be ethanol. It seems highly significant that the most readily and rapidly reacting substances in these tests (pyrogallol, caffeic acid, and ascorbic acid) approached as an apparent limit a maximum of 1 mole of acetaldehyde produced per mole of oxidizable substance present. All other substances tested either had not reached a maximum or had leveled off below 1 mole of aldehyde per mole of phenol. There was no evidence of continued oxidation to acetaldehyde (or, for that matter, brown products) once one molar equivalent of oxidizable phenol was accounted for. It thus appears that a limit of one mole of acetaldehyde per mole of autoxidation substrate is produced unless either competition for the strong oxidant or loss of acetaldehyde interferes.

It is believed that the reaction involves oxidation of the vicinal diphenol with O_2 to produce an ortho-quinone. Preliminary experiments indicate that 1,4-hydroquinones can produce the same reaction, but they are not generally present in beverages or foods. The O_2 is postulated as accepting the two hydrogen atoms, and since water appears to be necessary for the reaction a two-step transfer is hypothesized, resulting in the production of hydrogen peroxide as the strong oxidant. Organic hydroperoxides seem an unlikely alternative since they are very unstable in aqueous solution at elevated temperature. They should result in products differing between phenols and ascorbic acid, and their reaction is not simply related to the oxidation-reduction potential of the parent compound. Analytical methods capable of detecting very small levels failed to detect hydrogen

peroxide as an intermediate, presumably owing to the rapid consumption of the hydrogen peroxide in further reactions. In one experiment, catalase, whose only known substrate is hydrogen peroxide, changed the amount of concomitant browning during pyrogallol autoxidation in model wine whereas heat-inactivated catalase in the same amount had no effect. Further experiments are planned, but hydrogen peroxide appears very likely as the strong oxidant. Hydrogen peroxide is known to be able to oxidize ethanol to acetaldehyde. The postulated reactions then are:

$$R\text{-(ring)-}OH,OH + O_2 \longrightarrow R\text{-(ring)-}O,O + H_2O + (O)$$

$$(O) + H_2O \longrightarrow H_2O_2$$

$$H_2O_2 + CH_3CH_2OH \longrightarrow CH_3CHO + 2 H_2O$$
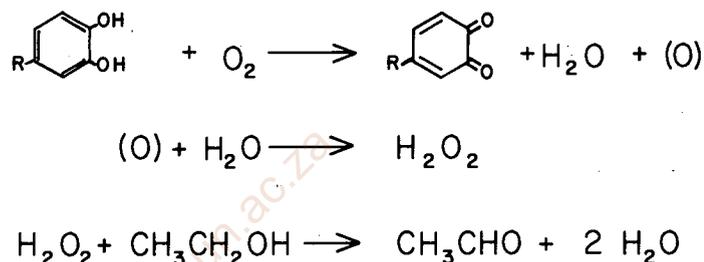
The proposed reactions not only account for the products and molar ratios, but also for the participation of water as a promoter of the reaction and for some of the inhibitory effects. Sulfur dioxide could inhibit the aldehyde-forming reaction by competing for the hydrogen peroxide. Hydrogen peroxide very rapidly oxidizes sulfite to sulfate. The fact that acetaldehyde production was *not completely suppressed* (and yet browning was) is attributed again to the relative concentration ratio of high ethanol to low SO_2. The increase in acetaldehyde production as SO_2 was consumed would agree (Figure 8). Thus, even in the presence of SO_2 and no evident browning, some acetaldehyde can be formed.

These findings appear to have great practical application in explaining the aldehyde production during barrel aging, "bottle sickness," etc. Even more important, we believe, they point to the need to reconsider some beliefs about aging reactions. Air contact with wine produces a strong oxidant, probably hydrogen peroxide, as shown by these results. Therefore, the old idea seems doubtful that rapid oxidation and "excessive" exposure to air gives different products and less desirable aging than slow diffusion and limited air exposure. There is, of course, no question that excessive oxidation does change most wines for the worse. On the other hand, since air contact leads immediately to the production of a strong oxidant, the effects of "strong" oxidation should be the same as limited oxidation provided the wine was kept well mixed and the same final amount of oxidation occurred. Thus, quick aging by metered amounts of air in stirred stainless-steel tanks appears more feasible than has been generally believed. If a time lag is required, it

must be related more to the further reactions of acetaldehyde and other products of the reaction than to the oxidation itself.

LITERATURE CITED

1. Amerine, M. A., H. W. Berg, and W. V. Cruess. The technology of wine making. 3rd ed., 802 p. Avi Publishing Co., Westport, Conn. (1972).

2. Joslyn, M. A., and M. A. Amerine. Dessert, appetizer, and related flavored wine; the technology of their production. 483 p. Divn. of Agricultural Sciences, University of California, Berkeley, California (1964).

3. Singleton, V. L. Aging of wines and other spiritous products; acceleration by physical treatments. Hilgardia 32:319-91 (1962).

4. Joslyn, M. A., and C. L. Comar. Role of acetaldehyde in red wines. Ind. Eng. Chem. 33:919-28 (1941).

5. Singleton, V. L., and P. Esau. Phenolic substances in grapes and wine, and their significance. Adv. Food Research, Suppl. 1, 282 p. Academic Press, N.Y. (1969).

6. Guymon, J. F., and E. A. Crowell, determination of aldehydes in wines and spirits by the direct bisulfite method. J. Am. Offic. Agr. Chem. 46:276-84 (1963).

7. Guymon, J. F., and D. L Wright. Collaborative study of the direct bisulfite method for the determination of aldehydes in brandy and wine spirits. J. Am. Offic. Agr. Chem. 55:566-9 (1972).

8. Johnson, G. R. A., and G. Scholes. Micro-determination of acetaldehyde as its 2,4-dinitrophenylhydrazone. Analyst 75:217-19 (1954).

9. Basson, R. A. Some sources of error in the spectrophotometric determination of acetaldehyde as its 2,4-dinitrophenylhydrazone. Anal. Chem. 38:637-8 (1966).

10. Ronkainen, P., and S. Brummer. Chromatographic identification of carbonyl compounds. I. Isolation of carbonyl compounds from solutions as their 2,4-dinitrophenylhydrazones. J. Chromatography 27:374-9 (1967).

11. Heitz, J. E., E. B. Roessler, M. A. Amerine, and G. A. Baker. A study of certain factors influencing the composition of California-type sherry during baking. Food Research 16:192-200 (1951).

12. Clark, W. M. Oxidation-reduction potential of organic systems. Williams & Wilkins Co., Baltimore. 584 p. (1960).

13. Kramling, T. E., and V. L. Singleton An estimate of the non-flavonoid phenols in wines. Am. J. Enol. Viticult. 20:86-92 (1969)

14. Chapon, L., and E. Urion. Ascorbic acid and beer. Wallerstein Comm. 23:38-44 (1960).

15. Hashimoto, N. Oxidation of higher alcohols by melanoidins in beer. J. Inst. Brewing. 78:43-51 (1972).

Analytical Fractionation of the Phenolic Substances of Grapes and Wine and Some Practical Uses of Such Analyses

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9

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The molar color yield under recommended conditions with a molybdotungstophosphoric heteropoly anion (Folin-Ciocalteu) reagent was determined for 150 phenol derivatives and eight potentially interfering substances. Determination of total phenol content was shown to be reliable, consistent, and stoichiometrically predictable from the reactions of known phenols. The expression of results as mg gallic acid equivalent per kg (grapes) or per liter (wine) is recommended. Potentially interfering substances are either very low in wines or grapes, or they can be corrected for by separate analysis. Among grape and wine constituents, only flavonoids readily precipitated when allowed to react with acidic formaldehyde. Total phenol analysis before and after such precipitation estimates flavonoid and nonflavonoid content. Practical analyses of wines, effects of barrel aging, and browning tendency are described.

The importance of phenolic substances in wines cannot be over-emphasized (1). Although generally present in smaller amounts than alcohols, acids, and carbohydrates, they are among the next most abundant substances with a typical range of a few tenths of a gram to a few grams per liter of wine and roughly 1 to 7 grams/kg for whole fresh grape berries (1). Wines would be much less interesting beverages without phenols. They form the red, golden, and brown pigments of most wines. Astringent and bitter flavors in wines involve phenols. The major reservoir of readily autoxidizable substances in a wine is its phenols,

and they are crucial in aging changes, browning reactions, and oxidation changes. The phenolic substances of grapes appear in wine made by various methods of vinification to varying degrees. In wine, the grape's phenols are modified by fermentation and storage, and additional phenols may be contributed to wine from storage in wooden containers.

Because they are important, the phenolics and their roles in wine have been studied considerably (1), but more needs to be known. Since different classes of phenols behave quite differently in the various roles as pigments, oxidation substrates, flavors, etc., separate consideration and analysis is essential to completely understand them. Every individual phenol should be separately determined precisely and quantitatively. Chromatography may solve these problems, but for now, chromatography and other detailed separatory methods have drawbacks, particularly for economical application to many individual wine or grape samples.

Methods of analysis are needed to determine total phenolic content and the relative content of phenolic fractions by means of their different characteristics. Many analytical methods used for phenols have been empirical and not easily reproduced or rationalized (1). Procedures that are based on sound chemical principles and that are sufficiently verified deserve wider application. We are concerned here with recent work on such analyses for phenols in wines. Application of these results may help solve a major problem in phenol research—the many different, too empirical, unrelatable values (ml $KMnO_4$, vanillin-to-leucoantho-cyanin ratio, etc.) obtained in different ways by different researchers. Uniform use of verified methods and uniform standards and methods of expressing results will aid in developing an understanding in this field.

Total Phenol Content of Wine

Singleton and Esau (1) reviewed the methods for phenol analysis of wine. They pointed out that study would be greatly advanced if one could determine the total content of phenolic substances and express it in such a way that analysis of subclasses of phenols could be related to the original total and a balance sheet could be obtained. One could then say, for example, "this wine has a total phenolic content of 1200 mg/liter calculated as gallic acid, and of that total, cinnamic acid derivatives account for 200 mg/liter, anthocyanins for 300 mg/liter, other small flavonoids for 200 mg/liter, and condensed tannins complete the total with 500 mg/liter of gallic acid equivalent." To accomplish this, the total phenol analysis not only must meet ordinary criteria of reproducibility and precision, but it also must be based on chemical relationships such that fractions determined separately can be converted to units of the total. Of course when clearcut fractionation can be accomplished by

other means, analysis for total phenols before and after treatment would then yield the desired information.

The colorimetric method based on the reagents of Folin and Denis or of Folin and Ciocalteu has been generally preferred over other methods to determine total phenols in complex natural materials such as wines and fruits (1, 2, 3, 4, 5). This method is relatively simple, convenient, reliable, generally applicable, and it is accepted as an official analysis in several countries for total phenols in wines and a number of other products. Although it is a preferred method, it can be even better than is commonly recognized.

Swain and Goldstein (6, 7) noted a rather large difference in the molar color yield from different phenols with the Folin-Denis reagent. They attributed this to differences in relative oxidation–reduction potentials of the different phenols, but under their conditions pyrogallol gave about half the color of catechol and more than resorcinol. However, they also reported that the molar absorptivity produced by a flavonoid was approximately equal to the sum of the values for the separate phenolic moieties which it contained.

The Folin-Ciocalteu Assay for Total Phenols

The Folin-Ciocalteu (F-C) reagent is superior to the Folin-Denis formulation (8, 9), and improved conditions for the assay have been developed (9). With the improved F-C procedure (compared with Folin-Denis analysis), the molar color yield was higher and more consistent, particularly with less reactive phenols. The improved method gave less deviation among replicate analyses, better recovery of added gallic acid, and was affected less by nonphenolic reductants. Based on limited numbers of known phenol structures, it appeared that with this procedure (9) monophenols reacted similarly and polyphenols gave additional reductive color generation depending on their quinoidal possibilities, e.g., phloroglucinol reacted as a monophenol and pyrogallol as a diphenol (1). Further studies have now been made with a wider group of phenols.

Method. The method of Singleton and Rossi (9) was used. Commercial samples of the compounds to be tested, generally without further purification, were accurately weighed (about 100–200 mg unless the supply was very limited), dissolved in ethanol, and diluted with water so that the final solution was 10 vol % ethanol and had a known concentration of phenol which yielded an absorbance of about 0.3 in the analysis. For incompletely soluble substances the suspension was kept dispersed, fine, and homogeneous. The analysis was essentially as published (9): 1.00 ml sample, gallic acid standard, or blank solution was mixed with 10.00 ml distilled water, 1.00 ml F-C reagent, 3.0 ml 2% Na_2CO_3 solution, and 5.00 ml distilled water. The absorbance was determined in a

Zeiss PMQII spectrophotometer, 1-cm cells at 765 nm after either 5 min at 50°C in a water bath or 2 hrs at room temperature (24°C).

The Beer-Lambert plots for this method are linear, and it is sensitive —approximately $5 \times 10^{-5}M$ monophenol in the final assay solution (*e.g.*, 7 mg/liter 4-hydroxybenzoic acid) gives about 0.6 absorbance at 765 nm in 1-cm cells. Five min at 50°C gave maximal absorbance at 765 nm with gallic acid. The absorbance produced was nearly the same as that obtained with 2 hrs at room temperature whereas more rapid fading tended to produce lower maximum absorbance at 60°C. For 150 different phenols, the absorbance produced by 2 hrs at room tempreature averaged 100.6% of the absorbance produced in 5 min at 50°C with a standard deviation of ±0.5%. In the few instances that the hotter incubation gave appreciably higher readings than did the room temperature one, the phenols were suppressed in activity and produced lower than usual molar absorptivities (3,5-diisopropylcatechol, ellagic acid, ethyl *p*-hydroxyben-zoate, *p*-hydroxybenzaldehyde, *p*-hydroxyacetophenone, and pentachloro-phenol).

The gallic acid mean molar absorptivity and standard deviation of 21 replicates which were weighed separately, diluted, and allowed to react with the assay reagents for 5 min at 50°C was 24967 ± 847. The standard deviation thus was 3.4% for replicate assays and would be expected to be about 5% for a single analysis compared with a single standard and about 7% for analysis by difference between two such assays. The reproducibility of a given value would be better if more standard levels, duplicate unknown samples, etc., were used. Nearly identical mean molar absorptivity and standard deviation were obtained for the samples held 2 hrs at room tempreature and, since the warmer temperature had advantages including slightly greater reaction with difficult-to-oxidize phenols, only the 5 min at 50°C values will be reported further.

Monophenol Derivatives. The molar absorptivities at 765 nm, produced in the F-C assay (9) with 150 phenols or phenolic derivatives and eight other substances after color development for 5 min at 50°C, are listed in Tables I through IX. The values shown are mostly from a single assay, but any result which seemed questionable was repeated one or more times with the same chemical from another source if possible. Table I lists 28 monophenols and one biphenol. The maximum molar absorptivity for a monophenol is about 16,000 in this assay. The typical value is *ca.* 12,500–13,000 for phenol itself and electronically equivalent structures. Phenols producing extinction coefficients higher than 13,000 generally have electron-repelling substituent groups, and those producing lower than this have electron-attracting substituents. However, substitu-ents which strongly suppress proton loss to form the phenolate ion (*e.g.*,

Table I.   Molar Absorptivity in the Total Phenol Assay and
Precipitability with Acidic Formaldehyde: Monophenols

Substance	ε_{765} ($\div 1000$)	% Pptd by HCHO
2-Hydroxycinnamic acid	15.9	0
4-Hydroxystilbene	15.8	insol.
Tyrosine	15.7	0
3-Hydroxycinnamic acid	15.6	0
4-Hydroxycinnamic acid	15.6	0
4-Hydroxybenzoic acid	15.6	0
3,5-Dimethylphenol	13.6	83
Phenol	12.7	0
4-Chlorophenol	12.1	0
2,4,6-Trichlorophenol	11.4	0
3,4-Dimethylphenol	11.1	40
2,4-Dichlorophenol	10.6	0
Salicylamide	10.2	0
4-tert-Butylphenol	10.2	0
2,2'-Biphenol	($20.2 \div 2$) 10.1	0
Salicylic acid	8.0	0
2,6-Dimethylphenol	8.0	0
2,6-Di-tert-butylphenol	7.2	insol.
2,4,5-Trichlorophenol	4.6	0
Phenyl salicylate	3.4	insol.
Methyl 4-hydroxybenzoate	2.4	0
4-Hydroxyacetophenone	1.9	0
Ethyl 4-hydroxybenzoate	1.7	0
4-Hydroxybenzaldehyde	0.6	0
Pentachlorophenol	0.4	insol.
4-Chloro-2-nitrophenol	0.2	0
4-Nitrophenol	0.1	0
2,4-Dinitrophenol	0.1	0
Picric acid	0	0

2,6-dimethylphenol) tend to lower the reducing power toward the F-C
reagent. Conversely, substituents which make the phenol very acidic but
suppress the oxidative removal of electrons (*e.g.*, 2,4,5-trichlorophenol)
suppress or prevent oxidation of the phenol by the F-C reagent. The
only phenols almost completely unreactive in this assay are the highly
acidic nitrophenols (Table I). Aldehyde, ketone, and ester groups sup-
pressed the phenol's oxidizability and therefore the color yield as might
be expected. However, in the alkaline assay solution (about pH 8.8),
carboxyl ions enhanced rather than suppressed the removal of electrons
by the heteropoly anions of the F-C reagent (*cf.* 4-hydroxybenzoic acid
with its esters, Table I).

Catechol and Guaiacol Derivatives.   The only diphenol in Table I
behaved as two separate monophenols. Catechols also generally react as
diphenols, *i.e.*, they produce twice the 765 nm absorbing color with F-C
reagent as does a typical monophenol. Table II shows molar extinc-

tions from 14 catechol and eight guaiacol derivatives. The catechols show much the same effects as, and perhaps more clearly than, the monophenols. Molecular configuration conveying ready ionization and also ready oxidative removal of the electron promotes reduction of the F-C reagent to produce the blue pigment. A few highly inhibited catechols behaved as monophenols, or at least their color yield was less than the maximum expected from a monophenol.

Table II. Molar Absorptivity in the Total Phenol Assay and Precipitability with Acidic Formaldehyde: Catechol and Guaiacol Derivatives

Substance	ε_{765} ($\div 1000$)	Phenolic Hydroxyls A Free	Phenolic Hydroxyls B Reactive	ε/B	% Pptd by HCHO
Pyrocatechol violet	52.7	3	3	17.6	0
Chlorogenic acid	28.9	2	2	14.4	0
3,4-Dihydroxyphenylalanine	24.6	2	2	12.3	0
Caffeic acid	22.9	2	2	11.4	0
3-Methoxycatechol	22.7	2	2	11.4	7
Catechol	22.5	2	2	11.2	0
4-Methylcatechol	21.6	2	2	10.8	0
3-Isopropylcatechol	20.9	2	2	10.4	7
3-Methylcatechol	20.4	2	2	10.2	0
2,3-Dihydroxybenzoic acid	17.8	2	2	8.9	0
3,4-Dihydroxybenzoic acid	17.3	2	2	8.6	0
3,5-Diisopropylcatechol	12.5	2	1	12.5	7
4-*tert*-Butylcatechol	11.1	2	1	11.1	0
Tetrabromocatechol	9.9	2	1	9.9	insol.
3-Hydroxy-4-methoxy-cinnamic acid	19.2	1	1 (2)	19.2 (9.6)	0
Ferulic acid	19.2	1	1 (2)	19.2 (9.6)	0
Vanillic acid	18.5	1	1 (2)	18.5 (9.2)	0
3-Ethoxy-2-hydroxy-benzaldehyde	16.3	1	1	16.3	0
Vanillin	14.9	1	1	14.9	0
Zingerone	14.3	1	1	14.3	0
Acetovanillone	12.8	1	1	12.8	0
Ethyl vanillate	10.1	1	1	10.1	0

Guaiacol derivatives (Table II) mostly react as monophenols as expected. The three most reactive guaiacol analogs gave more than the maximum color expected from monophenols, and the rest tended to be slightly higher than might be predicted from Table I. There may be some tendency for phenol regeneration from the methoxy groups. Veratric acid

did not give a small color yield from F-C assay but 2,4-dimethoxycinnamic acid did (Table IX).

Pyrogallol Derivatives. Table III lists molar absorptivities for 11 pyrogallol derivatives. The result with sinapic acid was uniform with samples from three different commercial lots, and it is interpreted as clear evidence for conversion of methoxyl to hydroxyl by the assay conditions. Furthermore, recrystallization of one sample twice from 95% ethanol did not change its melting point (193–195°C, dec, uncorrected) or color yield. The demethylation does not appear to be the result of excessive acid contact during assay, because the color yield from sinapic acid did not increase as the time between F-C reagent and sodium carbonate addition was increased from 2 to 16 min. Such demethylation has been reported from other studies [sinapyl alcohol readily loses a methyl group during storage in solution (10)] to be relatively facile, but apparently it occurred only to a smaller degree with the carbonyl analogs and not with syringic acid. Otherwise, the data in Table III agree well with predictions which could be made from Tables I and II.

Table III.   Molar Absorptivity in the Total Phenol Assay and Precipitability with Acidic Formaldehyde: Pyrogallol Derivatives

Substance	ε_{765} ($\div 1000$)	Phenolic Hydroxyls		ε/B	% Pptd by HCHO
		A Free	B Reactive		
Bromopyrogallol red	47.4	5	3	15.8	0
Purpurogallin	33.7	4	3	11.2	84
Methyl gallate	30.0	3	2	15.0	0
Gallic acid	25.0	3	2	12.5	0
Pyrogallol	24.8	3	2	12.4	20
Ellagic acid	23.5	4	2	11.8	insol.
2,3,4-Trihydroxybenzoic acid	18.3	3	2	9.1	0
Syringic acid	11.9	1	1	11.9	0
Syringaldehyde	15.9	1	1	15.9	0
3,5-Dimethoxy-4-hydroxyacetophenone	19.6	1	2	9.8	—
Sinapic acid	33.3	1	2	16.6	0

Namely, these series show that monophenols react according to their oxidizability, catechols react similarly except twice as much (presumably *via* o-quinone production), and pyrogallol derivatives generally react as catechols if vicinal hydroxyls are free and as monophenols if not. Ellagic acid appears perhaps anomalous, but it is known that two of the four hydroxyls are considerably more acidic than the other two (11).

Table IV. Molar Absorptivity in the Total Phenol Assay and Precipitability with Acidic Formaldehyde: *m*-Polyphenols

Substance	ε_{765} ($\div 1000$)	Phenolic Hydroxyls A Free	Phenolic Hydroxyls B Reactive	ε/B	% Pptd by HCHO Alone	% Pptd by HCHO With Phloroglucinol
Phloroglucinol Derivatives						
Rottlerin	24.4	5	2	12.2	97	—
2,4,6-Trihydroxybenzoic acid	13.6	3	1	13.6	95	—
Phloroglucinol	13.3	3	1	13.3	99	—
2,4,6-Trihydroxyacetophenone	12.7	3	1	12.7	97	—
Resorcinol Derivatives						
2,2′,4,4′-Tetrahydroxybiphenyl	26.5	4	2	13.2	91	—
2,2′,4,4′-Tetrahydroxybenzophenone	22.0	4	2	11.0	31	52
Resorcinol	19.8	2	2	9.9	98	—
Resorcinol monoacetate	10.2	1	1	10.2	84	—
3,5-Dihydroxybenzoic acid	16.2	2	1	16.2	0	13
Orcinol	15.4	2	1	15.4	97	—
2,4-Dihydroxybenzoic acid	14.6	2	1	14.6	0	17
2,6-Dihydroxybenzoic acid·	14.6	2	1	14.6	94	—
Sesamol	11.7	1	1	11.7	23	77

Phloroglucinol and Resorcinol Derivatives. Table IV lists absorptivities for four phloroglucinol and nine resorcinol derivatives. Each phloroglucinol ring reacts as a monophenol. However, resorcinol and certain of its analogs react as diphenols. That this is not merely a difference in oxidizability or oxidation–reduction potential is shown by the fact that resorcinol monoacetate gives almost exactly half the molar yield of blue pigment as does resorcinol, yet the dipole moments of the hydroxyl and acetate groups are nearly identical. Further, this result indicates that under the conditions of the assay the acetate group was not appreciably hydrolyzed. Other resorcinol derivatives, depending on their substitution, apparently react as either monophenols (especially with electron-repelling substituents) or diphenols (especially with electron-attracting substituents). Reaction of sesamol as a monophenol shows that the methylene dioxy group is not destroyed in the conditions of the assay.

Hydroquinone Derivatives. The nine hydroquinone derivatives listed in Table V all appear to act as monophenols. It appears from the quin-

Table V.   Molar Absorptivities in the Total Phenol Assay and
Precipitability with Acidic Formaldehyde:
Hydroquinone Derivatives

| Substance | ϵ_{765} ($\div 1000$) | Phenolic Hydroxyls | | ϵ/B | % Pptd by HCHO |
		A Free	B Reactive		
2,5-Dihydroxybenzoic acid	16.1	2	1	16.1	0
Tetrahydroxy-1,4-benzoquinone	14.7	4	1	14.7	0
Quinhydrone	14.7	2	1	14.7	0
Hydroquinone	12.8	2	1	12.8	0
4-*n*-Butoxyphenol	12.1	1	1	12.1	0
4-Methoxyphenol	11.6	1	1	11.6	0
2-*tert*-Butylhydroquinone	10.0	2	1	10.0	0
2,5-Di-*tert*-butyl-hydroquinone	8.7	2	1	8.7	insol.
2,5-Dihydroxy-1,4-benzoquinone	8.4	2	1	8.4	87

hydrone complex that phenols oxidized to the quinone form are not
further oxidizable by the F-C reagent as would be expected. However,
oxidations expected to give 1,4-benzoquinones give half the molar color
yield with F-C reagent than when 1,2-benzoquinones are expected. This
is noteworthy especially since the oxidation–reduction potential is about
0.8 for *o*-quinone and 0.7 for *p*-quinone (*i.e.*, hydroquinone is a stronger
reductant than catechol). Furthermore, it appears that the second
phenol equivalent of a catechol can be produced by the F-C reagent
acting on a hydroxyl substituent on a 1,4-benzoquinone (Table V).
Since phenol oxidation by the F-C reagent is demonstrated to be very
reproducible and quantitative, a study of the nature of the oxidation
products with different classes of phenol would be worthwhile.

Naphthalene and Anthracene Derivatives. In Table VI the molar
color yield in the F-C assay with 11 naphthalene and 6 anthracene-
derived phenols is given. In general, the results seem consistent with
what one would predict from the preceding tests and general chemical
knowldge. Anthraquinone (Table IX) does not give appreciable color
formation and, presumably owing to strong hydrogen bonding of the
peri OH with the quinone carbonyl, the 1- and 8-hydroxyanthraquinones
are strongly suppressed in reducing power. Apparently analogous to
1,4-benzoquinone, 1,4,9,10-tetrahydroxyanthracene, and 1,4-dihydroxy-
naphthalene react as monophenol equivalents. Further hydroxyls in the
anthraquinone series apparently contribute up to two more monophenol
equivalents depending on their position in the different rings. The meta
substitution in 1,3-dihydroxynaphthalene parallels to reactivity of orcinol

(Table IV) and the other naphthols react according to the number (one or two) of their hydroxyls.

Flavonoid and Coumarin Derivatives. Table VII lists the results with 17 aglycones and nine glycosides in the flavonoid and chalcone series and five coumarins including one glycoside. In general the results agree excellently with predictions from preceding tables. The phloroglucinol (or resorcinol) ring reacts as a monophenol, and the other ring reacts according to its substitution. Malvin, like sinapic acid, behaves as a diphenolic B-ring through loss of a methyl substituent to regenerate a catechol structure. Similarly, the color yield from biochanin A, hesperetin, and neohesperidin dihydrochalcone is higher than would be predicted from the free phenolic groups only, and conversion from the 4'-methoxy to the relatively acidic 4'-hydroxyl is indicated.

Table VI.   Molar Absorptivity in the Total Phenol Assay and Precipitability with Acidic Formaldehyde: Naphthalene and Anthracene Derivatives

Substance	ε_{765} ($\div 1000$)	A Free	B Reactive	ε/B	% Pptd by HCHO
Naphthalenes					
2,6-Dihydroxynaphthalene	27.8	2	2	13.9	98
2,3-Dihydroxynaphthalene	26.0	2	2	13.0	92
2,7-Dihydroxynaphthalene	24.0	2	2	12.0	94
1,7-Dihydroxynaphthalene	21.8	2	2	10.9	99
1,5-Dihydroxynaphthalene	18.5	2	2	9.2	96
1,3-Dihydroxynaphthalene	15.9	2	1	15.9	99
1,4-Dihydroxynaphthalene	5.6	2	1	5.6	81
1-Hydroxy-2-carboxy-naphthalene	12.9	1	1	12.9	81
1-Naphthol	12.4	1	1	12.4	96
2-Naphthol	8.8	1	1	8.8	84
Naphthochrome green	2.4	1	1	2.4	6
Anthracenes					
1,2-Dihydroxy-anthraquinone	31.8	2	2	15.9	96
1,2,5,8-Tetrahydroxy-anthraquinone	24.9	4	2	12.4	94
Emodin (1,3-8-trihydroxy-6-methyl-anthraquinone)	15.8	3	1	15.8	89
1,4,9,10-Tetrahydroxy-anthracene	15.6	4	1	15.6	85
1,8-Dihydroxy-anthraquinone	2.6	2	1	2.6	70
1-Hydroxyanthraquinone	0.7	1	0	—	42

Table VII. Molar Absorptivity in the Total Phenol Assay and Precipitability with Acidic Formaldehyde: Flavonoids and Coumarins

Substance	ε_{765} ($\div 1000$)	Phenolic Hydroxyls A Free	B Reactive	ε/B	% Pptd by HCHO Alone	With Phloroglucinol
Aglycones						
Myricetin	48.5	5	3	16.1	35	80
Dihydroquercetin	49.1	4	3	16.4	90	—
Quercetin	48.3	4	3	16.1	92	—
Morin	42.5	4	3	14.2	96	—
l-Epicatechin	43.9	4	3	14.6	91	—
d-Catechin	38.7	4	3	12.9	95	—
Cyanidin	34.3	4	3	11.5	90	—
Fisetin	42.7	3	3	14.2	0	0
Hesperetin	34.9	3	2 (3)[a]	17.6 (11.6)	85	99
Naringenin	32.2	3	2	16.1	79	97
Kaempferol	29.6	3	2	14.8	91	—
Apigenin	28.6	3	2	14.3	97	—
Biochanin A	23.8	2	1 (2)[a]	23.8 (11.9)	95	—
Chrysin	10.8	2	1	10.8	96	—
Acacetin	3.0	2	1	3.0	85	—
Tectochrysin	1.8	1	1	1.8	51	—
2-Hydroxychalcone	7.0	1	1	7.0	insol.	—
Glycosides						
Quercitrin	44.8	4	3	14.9	14	38
Rutin	43.4	4	3	14.5	12	24
Neohesperidin di-hydrochalcone	36.4	3	2 (3)	18.2 (12.1)	34	47
Phlorizin	25.6	3	2	12.8	43	55
Naringin di-hydrochalcone	21.2	3	2	10.6	13	29
Malvin	40.5	2	2 (3)	20.2 (13.5)	28	65
Pelargonin	24.9	2	2	12.4	20	51
Robinin	20.0	2	2	10.0	17	46
Naringin	18.9	2	2	9.4	0	16
Coumarins						
4-Methylesculetin	31.1	2	2	15.6	0	11
4-Methyldaphnetin	28.0	2	2	14.0	0	—
Esculin	16.8	1	1	16.8	1	4
7-Hydroxycoumarin	10.0	1	1	10.0	0	21
4-Methyl-7-hydroxycoumarin	7.6	1	1	7.6	4	25

[a] The higher value assumes conversion of the 4'-methoxyl to a hydroxyl.

The color yield of acacetin is low in spite of a 4′-methoxyl group, and this is attributed to suppression of the A-ring activity owing to 5-hydroxyl to 4-keto hydrogen bonding, carbonyl attraction of electrons, and perhaps low solubility.

Chrysin shows a similar effect which is much enhanced in tecto-chrysin, owing presumably to methylation of the 7-hydroxyl in techto-chrysin leaving only the keto-associated 5-hydroxyl free in that flavone. Opening the flavone heterocyclic ring to produce 2-hydroxychalcone leaves monophenol activity still partly suppressed because of the remaining carbonyl effect on oxidizability and H-bonding. Since the structure is relatively free to rotate about the ring-to-carbonyl bond, the suppression is less than in tectochrysin.

The coumarins (Table VII) react as expected with some suppression of reactivity of the 7-hydroxyl which is attributable to the alkyl–ester substitution. Note that the ester function is not appreciably hydrolyzed (Tables VII, IX) to liberate the 2-hydroxyl under the conditions of the assay.

Table VIII.   Molar Absorptivity in the Total Phenol Assay and Precipitability with Acidic Formaldehydes: Amines

| | | Amine + Hydroxyls | | | % Pptd |
| | ε_{765} | A | B | | by |
Substance	($\div 1000$)	Free	Reactive	ε/B	HCHO
3-Aminophenol	24.1	2	2	12.0	46
2-Aminophenol	21.8	2	2	10.9	36[a]
1,2-Diaminobenzene	21.4	2	2	10.7	95[a]
3-Diethylaminophenol	19.1	2	2	9.6	1[a]
N-(4-Hydroxyphenyl) glycine	19.9	2	2	10.0	0
4-Aminophenol	12.5	2	1	12.5	0
p-Toluidine	11.6	1	1	11.6	0
p-Anilinophenol	17.1	2	2	8.6	46[a]
2-Amino-4-nitrophenol	10.7	2	1	10.7	0

[a] Not precipitated but lost to the assay presumably by carbonyl amine reaction.

Amine and Aminophenol Derivatives. Amines and aminophenols (Table VIII) react with the F-C reagent about as predicted considering the aromatic amino groups equivalent to phenolic hydroxyls. This would be an important interference with total phenol assay in samples with appreciable aromatic amine content. Fortunately, for this and other reasons as well, the major wine grapes and most other fruit and vegetable products are free of significant concentrations of aromatic amines which would interfere. Correction might be made for methyl anthranilate

present at low levels in foxy grapes. Formaldehyde can tie up and render primary amine groups inactive in this assay (Table VII). This might be developed as a means of differentiation between the two functions in a mixture.

Table IX.   Molar Absorptivity in the Total Phenol Assay and
Precipitability with Acidic Formaldehyde:
Nonphenolic Substances

Substance	ε_{765} ($\div 1000$)	% Pptd by HCHO
Ferrous sulfate	3.4	0
Sodium sulfite	17.1	40[a]
D-Fructose	0	0
D-Glucose	0	0
Ascorbic acid	17.5	0
Mandelic acid	0.04	0
Veratic acid	0	0
2,4-Dimethoxycinnamic	0.1	yes
Acetylsalicylic acid	0.2	0
Chalcone	0.08	0
Flavone	0.1	insol.
Flavanone	1.9	0
3-Hydroxyflavone	3.5	insol.
Rotenone	1.8	insol.
Coumarin	0.1	0
4-Hydroxycoumarin	0.09	0
Anthraquinone	0.3	12

[a] Prevented from reaction with F-C reagent, but not precipitated.

Other Substances. Table IX gives the reaction in the F-C assay of several other substances which might interfere and a number of nonphenolic derivatives of phenols. Ascorbic acid (and no doubt other reductones and ene-diols) could be an important source of interference, but apparently reducing sugars and α-hydroxy acids are not. Readily reduced inorganic substances such as ferrous ion and bisulfite ion may interfere, but bound bisulfite is less reactive (Table IX). The presence of these substances is low in grapes or wine, and it is routinely determined. Their effect could be subtracted from a total phenol analysis when necessary, but it is seldom necessary since they ordinarily make a very small contribution. The amount of ascorbic acid can be appreciable in fresh grapes, but neither it nor significant amounts of any other substance reductive enough to react rapidly with sodium 2,6-dichloroindophenol is present in ordinary wine unless it is added. Good success has been seen in separately determining the ascorbic acid content with Tillman's reagent, calculating its contribution to the F-C analysis, and subtracting to adjust the total phenol value.

Reference has already been made to the effect in F-C determinations of a number of the phenolic derivatives no longer containing free

phenolic hydroxyls (Table IX). The near absence of reaction with acetylsalicylic acid reinforces the result (Table IV) with resorcinol monoacetate. These results together with those for the coumarins lacking free phenolic groups show that esters of phenolic hydroxyls generally do not hydrolyze sufficiently in the course of the assay, as used here, to cause interference.

Methoxyl groups can convert to or behave as phenolic hydroxyls in assay under some conditions of activation. However, in most cases, particularly when all phenolic hydroxyls are methylated, they are unreactive. Quinones do not react unless further hydroxylated (or reduced to hydroquinones). There is some reaction by 3-hydroxyflavone that is attributed to enol reaction involving the substitution pattern of carbons 2, 3, and 4 in the flavonol series. This may explain the general tendency of flavonols to give higher color yield, based simply on the phenolic hydroxyls, than do flavanols (*e.g.*, quercetin *vs.* catechin, Table VII). Flavanone gives (Table IX) small but measurable color formation in the F-C assay, and this is attributed to partial formation of the chalcone, a known equilibrium reaction in solution, thus producing a new free phenolic hydroxyl. This effect is used to justify the slightly high color yield from naringenin and dihydroquercetin (Table VII). If one assumes no conversion of the 2-hydroxychalcone (Table VII) to the flavanone, then 27% flavanone is converted to the chalcone (Table IX) under the assay conditions.

Phenols Alone and in Mixtures. The phenolic substances of grapes and wine have been well classified. They are predominantly common types with the expected substitution patterns and they are well covered by the model substances studied here (*1*). The phenolics are known not to contain significant amounts of polycyclic phenols like naphthols or anthraquinones; the flavonoids seem to be the common types (*e.g.*, phloroglucinol rather than resorcinol derivatives) as do the cinnamates. The content of potentially interfering nonphenols is generally small and subject to correction by separate determination. From the data presented, a good estimate of the molar absorptivity to be expected in the F-C determination for any one of the phenols known in grapes can be made by inspecting the structure and comparing it with compounds already tested. Several levels of sophistication are possible. Suppose an assay has been made and the total phenol content has been determined as so many gallic acid quivalents (*e.g.*, 340 mg/liter GAE) in a wine. To estimate the equivalent concentration of, say, *d*-catechin, the GAE value could be multiplied by the ratio of the two phenolic equivalents in the assay for gallic acid and the three equivalents for catechin, and then by the molecular weight ratio of the two substances. The result (340 × 2/3 × 290/170) indicates that 387 mg/liter of catechin would be equiva-

Table X.   Behavior of Phenols Alone and in Mixtures Toward Total

Assay	Caffeic Acid, 44.8[a] 40.9[b]	Catechin, 47.2[a] 42.5[b]	Vanillic Acid, 74.8[a] 56.0[b]
Assay Without Formaldehyde			
Mg/liter GAE by assay (A)	43.4	42.0	55.8
% A/C	106.3	98.9	99.8
Mixture 1, mg/liter GAE	17.4	8.4	11.2
Mixture 2, mg/liter GAE	8.7	16.8	11.2
Mixture 3, mg/liter GAE	8.7	8.4	22.3
Mixture 4, mg/liter GAE	8.7	8.4	11.2
Assay With Formaldehyde			
Mg/liter GAE by assay (B)	40.0	5.8	62.4
% B/C	97.9	13.5	111.5
Apparent % pptd by HCHO (100-100 B/A)	7.8	86.3	0
Mixture 1, mg/liter GAE	16.0	5.8	12.5
Mixture 2, mg/liter GAE	8.0	5.8	12.5
Mixture 3, mg/liter GAE	8.0	5.8	25.0
Mixture 4, mg/liter GAE	8.0	5.8	12.5

[a] Mg/liter added.

lent to 340 mg/liter of gallic acid in the F-C assay. In many instances of adding phenols to wine, such estimations have proved accurate enough. By applying actual molar absorptivity ratios or other improvements culminating in direct co-analysis of the components involved, improved estimates can be made. The above figure becomes 340 mg of gallic acid which is equivalent to 374 mg of catechin by our best estimate.

To demonstrate that such calculations are valid and that the different phenols act independently when a mixture is analyzed, solutions of four phenols chosen to represent different types important in wine were analyzed alone and as mixtures (Table X). Separate assays of solutions of the four phenols gave gallic acid equivalent concentrations 98.9–106.3% of that calculated from the known concentration of each phenol corrected for the molecular weight and average molar absorptivity compared with gallic acid. A second assay (Table X) of the same solutions after treatment with acidic formaldehyde gave similar results except for slightly larger deviations and nearly quantitative precipitation of catechin. Analysis of four mixtures, each with all four phenols but with one in turn higher in concentration, showed very good agreement in both assays with the value calculated from the assays of the individual substances. Therefore, the phenols reacted independently with the F-C reagent and did not affect each other's assay. A similar conclusion can be

Phenol Assay Before and After Formaldehyde Treatment

Syringic Acid, 99.9[a] 40.8[b]	Total Phenol in Mixture, GAE mg/liter		
	By Sum (E)	By Assay (D)	Recovery % E/D
40.8			
100.0			
8.2	45.1	43.5	96.5
8.2	44.8	45.8	102.2
8.2	47.6	46.2	97.0
16.3	44.6	43.4	97.3
49.8			
122.1			
0			
10.0	44.2	45.4	102.8
10.0	36.2	38.9	107.6
10.0	48.7	47.1	96.8
19.9	46.2	44.2	95.7

[b] Mg/liter GAE by calculation: gallic acid equivalent (GAE) calculated from the differences in molecular weight and molar absorptivity in the assay (C).

reached from quantitative recovery of gallic acid added to wine (9) and other similar tests.

The exact chemical nature of the yellow heteropoly molybdo- and tungstophosphoric anions and their blue reduction products is still uncertain. Several types and intergraded forms appear to exist, but detailed discussion would be out of place here. Many of the studies on these substances have been related to their use in phosphate determination which is carried out in acidic solution. Nevertheless, a few observations from recent reports seem significant in relation to the F-C assay for total phenols. The F-C reagent is prepared as a mixture of molybdo- and tungstophosphates. The tungsten ions can substitute freely for the molybdenum ions in the complex yellow heteropoly acid formed in making the active reagent (9).

The heteropoly molybdophosphates are much more reducible than the corresponding tungstates, but the latter exhibit one-electron transfer steps at high pH whereas the molybdophosphates did not (12). Otherwise, the electronic structures and behavior of the two are very similar (13), and perhaps the F-C reagent operates well, partly because one-electron transfers are possible, yet the reagent is a good oxidant.

Alkali slowly decomposes the yellow heteropoly anions, but the reduced blue forms are more stable. Presumably alkaline conditions are

required to convert the phenols to phenolate ions and this fact would explain poor reactions of cryptophenols. The reduction involves conversion of some of the molybdenum (or tungsten) ions in the complex from valence six to five without the complex structure of the yellow anion being broken down in converting to the blue form (*13, 14*). Therefore, any two substances, say phenols, capable of reducing the complex to the blue form behave similarly and without interference as long as excess oxidized heteropoly anion is present. Furthermore, the phenol oxidized by the F-C reagent apparently acts as an electron-contributing reductant and does not become a part of the blue pigment. The blue pigment formed seems to be the same regardless of the nature of the reductant.

The amount of blue pigment formed by reduction of a given portion of yellow heteropolyanion is relatively constant, but the rate of reduction increases as the acidity decreases (*15*), becoming pH independent with molybdophosphate blue at pH 4 for the transfer of the first two electrons and at pH 8 for the second pair (*12*). The molar absorptivity of the blue pigment is proportional to the number of electrons accepted at about ε 6000 for each electron transferred in either the molybdenum or tungsten series (*14, 15, 16*). Thus molar absorptivity in the F-C analysis of 12,000 as for phenol itself would indicate the transfer of a pair of electrons and gallic acid at ε 25,000 two pair. Blue pigments formed from increasing pairs of electrons transferred to the same pigment molecule have maxima shifted toward shorter wavelengths within 850–660 nm, with the 4-electron molybdophosphate blue at about 760 nm (*16*).

In summary, the F-C assay for total phenols (*9*) is stoichiometrically reproducible for a given phenol within a modest standard deviation. The color formed is reasonably predictable from the number of monophenol equivalents to be expected in a given phenolic molecule, and predictions can be improved if they are based on the activation and deactivation effects of substituents. The latter effects can best be interpreted from properly chosen model substances but they generally conform to predictions from well known chemical principles. The quantitative assay of total phenols by the F-C reagent is thus highly suitable for comparing the relative contributions of different classes of natural phenols in products like wine. The potentially interfering substances in wine, grapes, and most other foods are limited or analyses can be easily corrected for their presence.

Carbonyl Reactions as a Flavonoid Assay

The substitution of aldehydes as electrophiles into activated nucleophilic positions of aromatic rings, such as the ortho or para positions in a phenol, is well known from the commercial importance of phenol–formal-

dehyde resins. The reaction can be catalyzed by heat and alkali, in which case many phenols react and oxidation is likely. The reaction can also be catalyzed by strongly acidic conditions, but the more strongly nucleophilic centers produced in *m*-dihydric phenols then may be required for reaction unless the mixture is heated. The products of reaction between aromatic aldehydes, particularly vanillin, and phenols have been analyzed by colorimetry. In the strongly acidic solution used, molar equivalents of vanillin and phloroglucinol rings, such as the A-rings of flavonoids and certain resorcinol derivatives, react to produce color at 500–520 nm maximum (6, 7). The molar extinction coefficients indicate stoichiometric conditions ranging from 28,000 to 45,000 with resorcinol and phloroglucinol derivatives (6, 7). Chlorogenic acid did not react. Although reaction with vanillin has been used to determine catechins and other flavonoids in wine (1, 17), it has serious drawbacks. The strong mineral acid used converts some of the anthocyanogens to anthocyanidins which absorb at the same wavelength (1, 18) while use in red wine is virtually impossible. Furthermore, phloracetophenone and flavonoids with a 4-keto group do not react nor do phlobaphenes from acid-catalyzed polymerization of catechin (1, 5, 6). Polymeric condensed tannins react, but the reaction is decreased, apparently by loss of reactive sites to the polymer linkages and by steric coverage (6). Therefore, the vanillin/HCl reaction gives values which are very difficult to interpret (1).

Formaldehyde in the Stiasny test (with HCl) has long been used to distinguish (by their precipitation) condensed from hydrolyzable tannins, and high formaldehyde uptake is a characteristic of vegetable tannin preparations that have high flavonoid *vs.* gallic acid derivatives. Hillis and Urbach (19) showed that the reaction of formaldehyde at room temperature was confined to the phloroglucinol A-ring, and at pH 1 this remained so even if the solution was heated. Formaldehyde is both more reactive and smaller than vanillin so that A-ring sites inaccessible to vanillin are substituted by formaldehyde (20). Hillis and Urbach (19) tested formaldehyde/HCl precipitation as a gravimetric determination but found the resin too difficult to prepare and dry reproducibly. The combination of this reaction with the reliable F-C total phenol assay before and after precipitation by formaldehyde seemed to be a good prospect (1) for a meaningful flavonoid (precipitable) *vs.* nonflavonoid (nonprecipitable) determination in products such as wine.

Formaldehyde Precipitation of Phenols

Kramling and Singleton (21) developed an analytical procedure which gave reproducible values on a series of wines with a variability of about 2.5%. They found that the formaldehyde did not significantly

affect the F-C total phenol assay. Provided the pH was below 0.8, catechin, phloroglucinol, and wine flavonoids were nearly quantitatively precipitated by excess formaldehyde in 24 hrs at room temperature, but pyrocatechol was essentially unaffected. Formaldehyde should be present in excess (about 10-fold molar excess was used (21)), but at very high levels the precipitate was solubilized, and it could be dissolved in ethanol (22).

The factors affecting the completeness of the precipitation of phenols reactive to formaldehyde under the assay conditions have been studied, and it is planned to report improvements in the method later (22). For example, a nomograph to correct for the moderate but real effects of temperature, ethanol content of wine samples, and sugar content of juice samples is under consideration. In this report the general applicability and specificity of the reaction is considered further. The products of the reaction of formaldehyde with flavonoids would be first a methylol substitution followed by crosslinking *via* a methylene bridge to a second flavonoid (or phloroglucinol) unit. The natural flavonoids of grapes, wine, and most foods are phloroglucinol rather than resorcinol derivatives and have two free nucleophilic centers at positions 6 and 8. Thus the reaction can continue to produce a polymer. The insolubilization of the flavonoid–formaldehyde polymer depends on the effects of the increased size, decreased polarity, and decreased hydration of the polymer. An additional factor may be the tendency of the relatively dense structure to roll up and self associate by hydrogen bonding (23). Also, in the presence of ethanol, methylol groups can convert to ethers, and with HCl they can convert to chloromethyl derivatives (24) which would reduce solubility. Since initial formaldehyde reaction with the phenol would still be required to produce precipitation, these side reactions would only improve the method. The phenols already discussed were also tested to determine their precipitability by reaction with formaldehyde in acid solution.

Method. Essentially the method of Kramling and Singleton (21) was used. The wine or phenol solution, 10.00 ml, was mixed with 5.00 ml HCl solution (100 ml concentrated HCl diluted to 250 ml with distilled water) and 5.0 ml aqueous formaldehyde (13 ml of 37% HCHO diluted to 100 ml with distilled water). The air in the test tube was displaced with nitrogen and the stoppered tube was left at room temperature for 24 hrs. Any precipitate was centrifuged, and the supernatant liquid was filtered through a 0.45μ membrane filter. The filtered solution was assayed in the usual (9) manner for total phenols with the F-C reagent using 5 min at 50°C and correcting for the additional dilution. When phloroglucinol was added, it was added at 4–5-fold molar equivalents of the co-precipitating phenol in such a manner that the final solution volume remained the same.

The results with different substances are shown in Tables I–IX. Among the monophenols (Table I) appreciable precipitation occurred only with 3,4- and 3,5-dimethylphenols. Since the methyl group has an electron-repelling effect similar to, but smaller than, that of a hydroxyl group, it is to be expected that these could readily react and precipitate with formaldehyde. However, the other monophenols did not react, and this type of phenol should not occur among the substances present in grapes, wines, or most foods (1). None or insignificant precipitation occurred with acid formaldehyde among the catechol and quaiacol derivatives (Table II).

Most pyrogallol derivatives did not precipitate either (Table III). A small precipitation occurred with pyrogallol (which may be considered as a hydroxyresorcinol) and it reacts with vanillin/HCl also (7). However, any additional substitution eliminates this reactivity, e.g., gallic acid (Table IV). Purpurogallin did precipitate with formaldehyde. It is not very soluble in the reaction mixture, and it is a tropolone. Structures of this type might interfere, but in tea and probably in wine the known tropolone derivatives are flavonoids (1, 25).

The resorcinol and phloroglucinol derivatives (Table IV) all precipitated rather completely as expected except for 3,5- and 2,4-dihydroxybenzoic acids. Their precipitation was enhanced if phloroglucinol was added; this indicates that formaldehyde substitution occurred but the products were too soluble and too polar to precipitate until crosslinked with phloroglucinol. It had been shown previously that phenols which did not react with formaldehyde were not appreciably entrained in the precipitate formed with those which did (21) (see Table X). Hydroquinone derivatives, except for one which is also a resorcinol derivative, did not precipitate with formaldehyde (Table V).

Naphthalene- and anthracene-derived phenols did, however, almost uniformly precipitate (Table VI). In natural materials (not grapes or wines) which contain them they would be included in the formaldehyde precipitable group. Several primary amines capable of Schiff's base formation reacted with formaldehyde to lose their F-C oxidizability, but only the resorcinol analog, 3-aminophenol, precipitated (Table VIII). Sulfite also reacted but did not precipitate with formaldehyde, and the F-C oxidizability was suppressed (Table IX). The resorcinol derivative, 2,4-dimethoxycinnamic acid, formed a precipitate with formaldehyde, but it did not react appreciably in the F-C assay.

The most important group of compounds for our purposes, the flavonoids, are shown with coumarins in Table VII. The flavonoid aglycones precipitated nearly quantitatively except for fisetin, myricetin, and tectochrysin. Tectochrysin, 5-hydroxy-7-methoxyflavone, still preceipitated to 51% without phloroglucinol even though two of the three

potential hydroxyl groups of the molecule are converted to ethers with lesser dipoles and the third is strongly hydrogen-bonded to the 4-keto group and, therefore, decreased in acidity. Myricetin apparently is too polar for complete precipitation alone, but it readily precipitates when crosslinked with phloroglucinol. Fisetin is the only flavonoid in the series tested lacking the 5-hydroxyl, a flavonoid structure uncommon among food plants. Its failure to precipitate with formaldehyde even with phloroglucinol agrees with the failure of such compounds to react with vanillin (26). Since it represents a type of flavonoid restricted to wattle and a few other natural sources, its failure to react does not present a problem in flavonoid estimation in wine and similar materials.

The coumarins did not precipitate (Table VII) without phloro-glucinol and not very well with it. This also indicates the importance of the phloroglucinol substitution pattern as opposed to the resorcinol. These data show that the coumarins reacted weakly at a single center and therefore could not propagate polymers, although some linking and co-precipitation with polymers of other phenols can occur. Esculin, the 6-glucoside of esculetin, has only the 8 position available for formalde-hyde substitution and, by analogy with fisetin, its apparent lack of reac-tion seems logical.

The glycosides of normal flavonoids (Table VII), except naringin, showed some precipitation alone, and without exception, precipita-tion increased considerably when they co-reacted with a few moles of phloroglucinol. The chalcones tended to precipitate more completely than the true flavonoids, probably because of the more free and reactive phloroglucinol moiety.

It is difficult for the formaldehyde product to drag the flavonoid polymer from aqueous solution if it is highly solubilized by attached sugars, as evident from these data. That the presence in the same re-action mixture of sufficient phloroglucinol will crosslink and over-whelm the solubilizing effect and precipitate the glycosides is also seen in the data. This is not as great a problem for successful flavonoid estimation in wine and grape extracts as it may first appear because these products contain a relatively large fraction of their flavonoids in non-glycoside forms, especially catechins and oligomeric condensed anthocyanogenic tannins (1). The resultant mixed precipitate renders the glycosides insoluble also.

The glycosidic anthocyanins are almost completely precipitated with red wines of a few months age and normal tannin content. However, with young wines made from the same grapes, a rosé wine with only 713 mg GAE/liter total phenol gave 311 mg GAE/liter as not-precipitated nonflavonoid, and with increasing levels of total phenol in red wines to

a high of 1421, the apparent nonflavonoid content decreased until a constant level of 250 mg GAE/liter was reached (*21*). Although with grape wine the method has been quite informative, further improvement through routine addition of a glycoside-precipitating co-reactant such as phloroglucinol is under study.

Table X illustrates the successful application of formaldehyde precipitation as a means of estimating the flavonoid and nonflavonoid contents in a mixture. The mixture consisted of catechin as the flavonoid and caffeic, vanillic, and syringic acids as the nonflavonoids. The catechin was 86% precipitated (lower than usual because of the low level), but the other substances were not significantly precipitated. The slight apparent loss of caffeic acid is attributable to experimental variation since in many other experiments the lack of reaction and precipitation or co-precipitation of caffeic acid or chlorgenic acid has been demonstrated. Allowing for the same slight solubility of the catechin-formaldehyde product in the mixtures as in the single component solution, the analysis of the mixtures gave 95.7–107.6% of the calculated value. This indicates no significant co-precipitation or entrainment of the nonflavonoids as the flavonoid was removed. This result has been verified a number of times with different substances added to model solutions and wines (*21, 22*).

Viewing the data (Table X) as if it had been the usual assay of unknowns and subtracting the assay values after formaldehyde treatment from those before, the mixtures 1, 3, and 4 would apparently contain no flavonoid when in fact they contained 8.4 mg/liter GAE by separate assay. On the other hand, mixture 2 with 16.8 mg/liter GAE of flavonoid by separate assay gave 6.9 mg/liter by formaldehyde precipitation. If correction was made for 5.8 mg/liter GAE residual solubility of the catechin–formaldehyde product then mixures 1–4 would be indicated to have, respectively, 3.9, 12.7, 4.9, and 5.0 flavonoid and 39.6, 33.1, 41.3, and 38.4 mg/liter GAE nonflavonoid. These values are considered very close to the true content considering the results are based on differences between two assays with the attendant increase in variability.

In summary, formaldehyde precipitates only phloroglucinol derivatives (including flavonoids), some resorcinol analogs, and polycyclic phenols of naphthalene or higher ring systems under the conditions tested. Total phenol analysis before and after such precipitation gives a useful estimate of nonflavonoids (remaining in solution) and flavonoids (precipitating) provided that, as in grape wine, conditions are such or can be arranged so that flavonoid glycosides precipitate, and potentially interfering phenols such as 5-deoxyflavonoids, certain coumarins, and polycyclic phenols are absent or in low concentration.

Examples of Practical Application of These and Similar Analyses to Wine

Kramling and Singleton (21), in addition to the original development of the flavonoid–nonflavonoid analysis just described, showed that white and red table and dessert wines had very similar nonflavonoid phenol content. In the wines studied, the total phenol content ranged from 205 to 1421 mg/liter GAE, but the nonflavonoid phenols were relatively constant at 190–343 mg/liter GAE. Nearly all of the variation was in the formaldehyde-precipitable flavonoid content which ranged from 10 to 1169 mg/liter GAE. The nonflavonoids of grapes, largely caffeic acid derivatives like chlorogenic acid (1), were shown to be essentially confined to the vacuolar fluids of the grape which are easily expressed as juice. The flavonoids are very low in juice, but very high in grape solids, skins, and seeds. As a result, white wine phenols are almost exclusively nonflavonoid unless appreciable pomace extraction had been made, and red wines had increased flavonoid in proportion to pomace extraction. Although this has been inferred from other largely qualitative studies (1), it is believed that this study (21) represented the first direct quantitative evidence for it.

Grape varieties vary slightly in nonflavonoid content, but they vary considerably in flavonoid contribution to wines prepared in similar ways (21, 27); large differences in the flavonoid content caused by vinification and processing often obscure varietal differences. Wines made (21) from the same grapes had a flavonoid content of 132 mg/liter for the lightest rosé and 1169 mg/liter GAE for the darkest red, but the nonflavonoid content assayed 201 and 252 mg/liter, respectively, and averaged 262 mg/liter GAE for a series of five wines.

Table XI. Average Analysis of Nonflavonoid Phenol Content of Typical Woods Which Contact Wine (27)

Wood	Solids Extd, grams/ 100 grams Dry Wood	Total Phenols, mg GAE/gram Extd Solids	Non-flavonoid Phenols, mg GAE/gram Extd Solids	Non-flavonoids, % of Phenols
American oak	6.45	365.1	320.2	87.7
European oak	10.39	560.7	505.3	87.7
Redwood	15.94	607.5	590.8	97.2
Cork	2.44	141.4	137.9	97.5

Formaldehyde analysis has been used to detect and measure oak extract in wines aged in wood cooperage and to correlate the amount of extract with the aging effect (27). Tannins and phenols of oak (and redwood and cork) are predominantly nonflavonoid–hydrolyzable tannins (Table XI), and they add to the otherwise relatively low and

Table XII. Selected Analyses of Wines with Known Treatments with Oak (21)

Wine	Wood Treatment[a]	Total Phenol, mg/liter GAE	Non-flavonoid Phenol, mg/liter GAE	Estd[b] Non-flavonoid from Oak, mg/liter GAE
Carignane	None	950	164	0
Carignane	AN 2.5 grams/liter, 4 days	1000	203	39
Carignane	AN 5 grams/liter, 4 days	1034	256	92
Carignane	AN 10 grams/liter, 4 days	1040	318	154
Carignane	AN 20 grams/liter, 4 days	1204	477	313
Carignane	AN 50 grams/liter, 4 days	1504	884	720
Cabernet Sauvignon	none	1744	353	0
Cabernet Sauvignon	EU 60 gal., 3 mo.	1798	352	0
Cabernet Sauvignon	EU 60 gal., 1 yr	1670	414	61
Cabernet Sauvignon	EU 60 gal., 3 yr	1374	458	105
Chardonnay	none	—	200	0
Chardonnay	EN 60 gal., 6 mo.	404	314	114
Chardonnay	EN 60 gal., 1 yr	518	478	278
Chardonnay	EN ———, .5 mo.	374	320	120
Chenin blanc	none	291	233	0
Chenin blanc	EU 340 gal., 2 mo.	326	251	18
Chenin blanc	EU 340 gal., 6 mo.	311	248	15
Flor sherry	AU 50 gal., 8-9 yr	324	300	100

[a] A = American oak, E = European oak; N = new, U = previously used; chips grams/liter, barrels capacity; time of contact.
[b] Nonflavonoid content of treated wine minus that of the untreated wine (or for Chardonnay and sherry values on the high end of the typical range).

constant flavonoid content of wine during aging (Table XII). Thus, increased nonflavonoid in wine is proportional to the newness of the barrel, time, and contact of wood surface per unit contents (28), and it can be followed even if total phenol varies considerably because of various reactions during aging. Results with wines having known barrel aging treatments suggest that this technique is capable of reliable detection and estimation of treatment by aging in wooden cooperage of purchased bottled wines whose history is unknown. This is feasible because of the relatively constant and low content of nonflavonoid phenols in

wines from a single variety and in wines in general so that a relatively small increase in nonflavonoid phenol from the barrel or other source becomes significant (*21, 27, 28*).

The flavonoid fraction, especially catechin, from grapes has been reported to be the primary source of yellow and brown colors in white wines (*29*). In work not yet reported in detail (*30, 31, 32*) the flavonoid content was shown to determine and control the potential of a white table wine to brown. Wines were prepared from several white grape varieties with increasing periods of contact between the juice and the pomace, allowing various extractions of flavonoid compounds. When exposed to oxygen under standardized conditions, each wine's increase in absorbance at 420 nm correlated almost perfectly with the assayed flavonoid content. Experimental data on two of the varieties, Clairette blanche (with the greatest tendency to brown) and Colombard (with the least tendency) are shown in Figure 1 (*32*). These results explain the greater stability and resistance to browning of wines or beers deliberately treated with formaldehyde (*33, 34*).
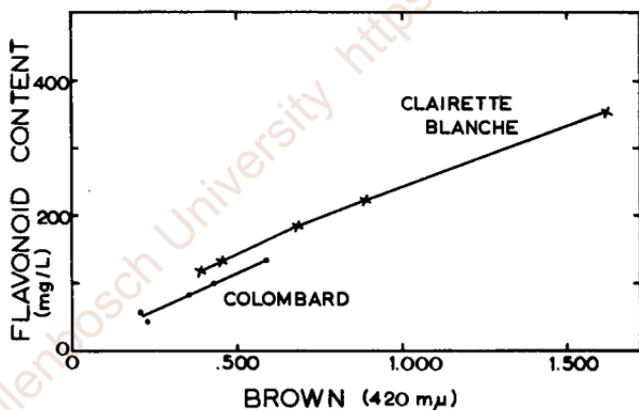
Figure 1. Flavonoid content mg/liter GAE, in wines made from the same grapes, as correlated with browning of each wine upon exposure to oxygen under standardized conditions

Peri and Pompei (*35, 36*) have helped verify the methods elaborated here (*9, 21*), and they have extended them by adapting cinchonine precipitation to separate tannins from nontannins. Thus, in a mixture of the two tannins precipitated by cinchonine they were able to redissolve them and quantitate tannic acid separately from grape seed tannin because of tannic acid's failure to precipitate with formaldehyde and by appropriate application of the F-C analysis. They applied these techniques to analysis of

a rather tannic white wine containing a total of 813 mg/liter GAE phenols, 33% (267 mg/liter GAE) of which was nonflavonoids, 25% (205 mg/liter GAE) of which was nontannin flavonoids, and 42% (347 mg/liter GAE) of which was tannins. Thin layer chromatograms illustrated the validity of the separations.

Table XIII.   Total Phenol, Tannin, and Nontannin Content of Wines From the Data of Mitjavila et al. (37)

Wine	Total Phenol by F-C Assay, mg/liter GAE	Nontannin, mg/liter GAE	Tannin (Pptd), mg/liter GAE	Sum, % of Total
			PVP/Cl₃CCOOH Pptn	
Fronton red	1328	846	418	95
Rhone red	1701	1019	568	93
Burgundy red	1664	1019	627	99
Bordeaux red	2019	1210	882	104
Rioja red	1842	1064	782	100
Toulouse red	1595	1364	200	98
Piedmont red	2092	1455	746	105
Corbières rose	455	364	91	100
Sherry white	437	313	84	91
Juracon white	318	293	20	98

Other separations can easily take advantage of the F-C total phenol method in the same way that has been indicated for formaldehyde and cinchonine precipitation. In one recent example (37), tannins were determined by complexing with polyvinylpyrrolidinone and precipitation of the complex with trichloroacetic acid. The precipitate was redissolved and assayed by the improved F-C method. They showed quantitative absence of tannic acid and enotannin in the supernatant liquid and a similar absence of gallic acid and catechin from the precipitate by this method. The only component tested which divided into both fractions was leucocyanidin. Monomeric flavan-3,4-diols are very low if not absent in wines (1, 38). In a series of wines, the sum of the precipitated tannins and the nonprecipitated phenols by this method (38) was identical (within experimental variation) with a separate assay of the total phenol content (Table XIII). Direct comparability with values obtained by others is an advantage of this method. Their data (37) in Table XIII are recalculated to gallic acid equivalents, since *d*-catechin had been used as the standard, by multiplying by the conversion factor derived from the best data in this paper, 0.9094. It appears to us most convenient to express such results in terms of mg of gallic acid equivalent per liter of beverage or per kg of fruit (1), and it is hoped others will adopt this terminology for even easier comparisons.

Literature Cited

1. Singleton, V. L., Esau, P., "Phenolic Substances in Grapes and Wine, and Their Significance," 282 p., *Advan. Food Res.* Suppl. 1, Academic, New York, 1969.
2. Pro, M. J., *J. Ass. Off. Agr. Chem.* (1952) **35**, 255; (1954) **37**, 665; (1955) **38**, 757.
3. Ribéreau-Gayon, P., Satore, F., *Chim. Anal.* (Paris) (1970) **52**, 627.
4. Swain, T., in "Chemistry and Biochemistry of Plant Pigments," T. W. Goodwin, ed., p. 545, Academic, New York, 1965.
5. Swain, T., Hillis, W. E., *J. Sci. Food Agr.* (1959) **10**, 63.
6. Goldstein, J. L., Swain, T., *Phytochemistry* (1963) **2**, 371.
7. Swain, T., Goldstein, J. L., in "Methods in Polyphenol Chemistry," J. B. Pridham, ed., p. 131, Macmillan, New York, 1964.
8. Joslyn, M. A., Morris, M., Hugenberg, G., *Mitt. Rebe Wein Obstbau Fruchteverwert. (Klosterneuburg)* (1968) **18**, 17.
9. Singleton, V. L., Rossi, J. A., Jr., *Amer. J. Enol. Viticult.* (1965) **16**, 144.
10. Schroeder, H. A., *J. Chromatogr.* (1967) **30**, 537.
11. Singleton, V. L., Marsh, G. L., Coven, M., *J. Agr. Food Chem.* (1966) **14**, 5.
12. Papaconstantinou, E., Pope, M. T., *Inorg. Chem.* (1967) **6**, 1152.
13. Varga, G. M., Jr., Papaconstantinou, E., Pope, M. T., *Inorg. Chem.* (1970) **9**, 662.
14. Reznik, B. E., Tsyganok, L. P., *Zh. Neorg. Khim.* (trans.) (1968) **13**, 548.
15. Crouch, S. R., Malmstadt, H. V., *Anal. Chem.* (1967) **39**, 1084.
16. Papaconstantinou, E., Pope, M. T., *Inorg. Chem.* (1970) **9**, 667.
17. Pompei, C., Peri, C., *Vitis* (1971) **9**, 312.
18. King, H. G. C., White, T., in "The Chemistry of Vegetable Tannins," p. 51, Society of Leather Trades' Chemists, Croydon, 1956.
19. Hillis, W. E., Urbach, G., *J. Appl. Chem. London* (1959) **9**, 474.
20. Hillis, W. E., Urbach, G., *Nature* (1958) **182**, 657.
21. Kramling, T. E., Singleton, V. L., *Amer. J. Enol. Viticult.* (1969) **20**, 86.
22. Singleton, V. L., Kramling, T. E., Sullivan, A. R., private communication.
23. Imoto, M., Ijichi, I., Tanaka, C., Kinoshita, M., *Makromol. Chem.* (1968) **113**, 117.
24. Mironov, G. S., Farberov, M. I., Shem, V. D., Vetrova, V. V., *Kinet. Katal.* (trans.) (1968) **9**, 612.
25. Singleton, V. L., in "The Chemistry of Plant Pigments," C. O. Chichester, ed., p. 143, *Advan. Food Res.*, Suppl. 3, Academic, New York, 1972.
26. Hillis, W. E., Urbach, G., *J. Appl. Chem.* (London) (1959) **9**, 665.
27. Singleton, V. L., Sullivan, A. R., Kramer, C., *Amer. J. Enol. Viticult.* (1971) **22**, 161.
28. Singleton, V. L., ADVAN. CHEM. SER. (1974) **137**, 254.
29. Rossi, J. A., Jr., Singleton, V. L., *Amer. J. Enol. Viticult.* (1966) **17**, 231.
30. Kramling, T. E., M.S. Thesis, University Calif., Davis, 1969.
31. Singleton, V. L., *Die Wynboer* (1969) No. 455, 13.
32. Singleton, V. L., DeWet, P., Kramling, T. E., Van Wyk, C. J., private communication.

33. Cantarelli, C., Pompei, C., Peri, C., Montedoro, G., *Amer. J. Enol. Viticult.* (1971) **22,** 59.
34. Macey, A., Stowell, K. C., White, H. B., *J. Inst. Brewing* (1965) **72,** 29.
35. Peri, C., Pompei, C., *Amer. J. Enol. Viticult.* (1971) **22,** 55.
36. Peri, C., Pompei, C., *Phytochemistry* (1971) **10,** 2187.
37. Mitjavila, S., Schiavon, M., Derache, R., *Ann. Technol. Agr.* (1971) **20,** 335.
38. Weinges, K., Piretti, M. V., *Justus Liebigs Ann. Chem.* (1971) **748,** 218.

COMPOSITION AND SENSORY QUALITIES OF WINES PREPARED FROM WHITE GRAPES BY FERMENTATION WITH AND WITHOUT GRAPE SOLIDS

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ABSTRACT

Wines were prepared from seven harvests representing six white grape varieties. The must of each harvest was divided into 3 or 5 equivalent lots. Lots were fermented dry at 16 C from juice clarified by settling, from turbid juice with double the normal amount of suspended solids, and from the entire destemmed, crushed grape mass inoculated with yeast and maintained in contact with the pomace for 24, 48, or 120 hours.

The expert panel of tasters judged wines prepared from clarified juice to be much higher in quality and desirable aroma than any of the other wines. Chemical analyses were essentially identical for the wines prepared from clarified juice or turbid juice, but the wines from turbid juice were much lower in quality and considered harsher (higher ratings for astringency and bitterness). The major quality difference between wines from these two treatments was due to the fresh, clean, delicate, fruity, characteristic odor called fermentation bouquet in wines from the clarified samples and its decrease or absence, plus off odors (particularly hydrogen sulfide) in wines from the turbid samples. The wines with increasing pomace contact exhibited increasing total phenol content coupled with increasing astringency rating. Pomace contact sufficient to give additional total phenol (as gallic acid) of about 100 mg/L from grape solids of seeded white grapes gave just recognizably increased astringency in white table wines. The wines with up to 5 days of pomace contact during fermentation at low temperature did not resemble red wines in flavor or odor, but remained similar to normal dry white wines, although with generally lower quality. Bitterness ratings did not parallel astringency or phenol content, and there appeared to be higher bitterness in intermediate levels of pomace contact and phenol content.

Many different methods are in use to make white wines in various parts of the world. The style of wine produced or the sensory features of white table wines that are considered desirable or inevitable in one locality may be unknown or considered undesirable in another. Even in wineries with up-to-date technology and awareness of practices in other countries, there may be disagreement as to which procedures produce the "best" wine. In the final analysis, at least from the viewpoint of financial success, the definition of "best" wine is controlled by the consumers of the wine.

Some of this sort of individuality and disagreement as to the style of wine that one should produce and which technology should be used to make white table wine (or other wines for that matter) can be highly desirable. It has led to standardization upon a specific style of wine eventually lending reputation and economic success to a winery or a winemaking district without at the same time stifling

Am. J. Enol. Viticult., Vol. 26, No. 2, 1975

62

the successful development and earning of a reputation for a different style by another winery or another region. A great deal of the appeal of wines rests on their diversity in contrast to the sameness and mass production of most other products today.

Among dry white table wines there are many gradations between such extremes as the very light, fresh, fruity, unaged style versus the robust, full-flavored, complex, and often well-aged wines. In making the fresh, young style of wine, rapid separation of the juice from the pomace is usually recommended, and clarification of the juice by sedimentation before fermentation is the usual practice in South Africa. The full-flavored style of dry white wine has been popular in many other parts of the world, and its production often involves more contact between the pomace and the juice as well as more aging of the wine.

From a subjective consideration it often seems the fresh young style of wine could benefit from more grapey, varietal character, more depth and fullness in its flavor, while the robust style is often lacking in lively fruitiness. The experiments presented here were designed to estimate some of the sensory and compositional effects of winemaking practices ranging from rapid separation and clarification of the must to fermentations with the pomace similar to red wine production. It was hoped that these extreme measures would yield a greater understanding of the possible range of white table wines and a more scientific basis for further refinement of winemaking practices to emphasize different features and styles of wine.

MATERIALS AND METHODS

The grapes were the same lots characterized in population studies by Singleton et al (5). The cultivars, vineyard source, and 1969 date of harvest were: 'Steen' ('Chenin blanc') (I), Nietvoorbij, 25 February; 'Riesling,' Nietvoorbij, 10 March; 'Green' ('Semillon'), Elsenburg, 18 March; Steen (II), Elsenburg, 19 March; 'White French' ('Palomino'), Elsenburg, 24 March; 'Colombar' ('French Colombard'), Elsenburg, 26 March; 'Clairette blanche,' Elsenburg, 31 March. The South African varietal designation is retained in the later discussion because of possible clonal differences, etc. The grapes were usually picked in the afternoon, stored overnight in a 15 C cold room, and processed early the next morning. In a few instances picking was early in the morning and sampling followed as soon as possible. Harvests were intended to be about 225 kg, but that quantity was not always available because of the small experimental plots and some damaged clusters.

Clusters with appreciable mold or other damage were discarded during berry sampling for the population characterization (5). The remaining healthy clusters were crushed and destemmed in the R.I.O.V.

experimental winery at Nietvoorbij, near Stellenbosch. Sufficient potassium metabisulfite was added to the grapes as they were crushed to furnish 75–100 ppm of SO_2. The last three harvests, 'White French,' 'Colombar,' and 'Clairette blanche,' were low in sugar, and sufficient sucrose was added at the crusher to raise the average must to about 20 Brix so as to ensure a normal fermentation. That procedure is not permitted for commercial wine in South Africa or California but was considered necessary for experimental validity in the low-Brix musts.

The crushed grapes of each individual harvest with stems removed and bisulfite added (and sugar, if used) were caught in a single large container and well mixed. Then, equal amounts (about 3 L) were dipped from this container in rotation into appropriate sublots. Continuous mixing while dipping ensured comparable composition of the sublots. A total of one double and three single sublots were prepared unless the amount of fruit was too small. Each single sublot was intended to yield about 22 L of wine. The double sublot was drained and lightly pressed to remove the juice from the pomace. This juice was allowed to settle at about 5 C for about 6 hours. The top relatively clear half was siphoned off as a single sublot, and the bottom turbid half was retained as an additional single sublot. The total number of treatments was thus five for each variety, grapes permitting. All the sublots were then inoculated with actively growing yeast in grape juice, approximately 10 ml/L. Fermentation was conducted in small stainless-steel fermentors (about 45 L) closed with rubber gasketing and a waterfilled fermentation bung.

During every operation, contact with air was minimized by careful, rapid transfer; flushing settling tanks, fermentors, and bottles with carbon dioxide; etc. To avoid the introduction of extraneous differences, SO_2 was not added at subsequent stages. The three sublots which contained pomace were allowed to ferment a total of 24 hours, 48 hours, or 120 hours, and then the liquid was drained and lightly pressed from the pomace. This liquid must was returned to the fermentor, and all lots were allowed to ferment to dryness at 16 C. In the second harvest of 'Steen' and the harvest of 'Green,' insufficient grapes were obtained and the sublots with 48 and 120 hours of pomace contact were eliminated.

During fermentation the progressive decrease of Brix was checked at intervals (usually daily until near the end of fermentation) with a hydrometer, and the samples containing pomace were mixed with the cap twice per day. After each sublot of a given harvest had fermented dry and most of the lees had settled, the wine was racked and filled into glass containers of about 4 L capacity, sealed, and placed in a cold room at about 5 C. All wines were in the cold room by 15 April. Their chemical and sensory analyses were begun on 28 April, at which time the

wine was decanted without disturbing the small amount of lees and sealed into standard 750-ml wine bottles. These samples were prepared and the analyses conducted in the same order that the wines were prepared, so as to give approximately the same time after fermentation. All chemical and sensory analyses were completed by 12 June 1969, and statistical analysis and summary were completed later in California.

Analysis for total phenol content was by the method of Singleton and Rossi (4), and other chemical analyses were by methods in standard use for wines.

A preliminary sensory analysis of the wines was used to see whether there were recognizable differences among the wines and to aid in the designing of score sheets and instructions. The sensory data reported are only those from ratings of each wine by a 12-individual expert taste panel, although they agreed well with preliminary ratings made by two individuals as much as one month earlier.

The wine samples were served in standard 8-oz wine glasses at ambient temperature. To retain volatile odorants, the samples, from coded capped bottles, were poured just before being presented to the tasters in glasses coded with 2-digit random numbers. The tasters received the 3 or 5 wines representing a single harvest of a single variety as a set arranged in random order, and tasted one set per day. Tastings were held in the R.I.O.V. tasting room, samples on a white-topped table with convenient spittoons, rinse water, etc.

The tasters were required to read a specific instruction sheet at the first tasting, and were handed it again at each subsequent tasting. The format was outlined and they were asked to disregard color and appearance, since all samples were in the range considered normal for young dry white table wines. Other instructions were as follows:

Desirable aroma: Give the wine with the most desirable aroma the highest rating on an arbitrary scale of 0–10. Zero should be a wine with the least desirable aroma likely to be encountered, and 10 should be a wine with as highly desirable aroma as is likely to be encountered.

Astringency: Rate the persimmon-like "puckering" mouth surface effect of tannin in the wines on a scale of 0 (absent) to 10 (as high as likely to be found in a wine). Be careful, since that effect tends to be tiring to judge (first impressions are sometimes better than retrials) and tends to carry over from one sample to the next.

Bitterness: Rate the quinine-like bitterness of the wines. Make an effort to rate bitterness separately from astringency, acidity, or other fractors on a scale from 0 (absent) to 10 (as high as likely to be encountered in wine). Bitterness is often more noticed in the back of the throat, and also tends to carry over from sample to sample.

Quality: In the over-all quality rating, 17–20 indicates outstanding wine; 13–16 indicates commercial wines without appreciable defects; 9–12 indicates wines that have defects but can be blended or readily treated to make them commercially acceptable; and 0–8 indicates wines which are seriously defective or spoiled and cannot be made salable easily if at all.

Each taster recorded his impressions on a score sheet which listed in advance the date, grape variety, his name, and the wine codes, and had blanks for his ratings of desirable aroma, astringency, bitterness, and quality, and his comments.

There were 8 tasting sessions on as many days, one for each harvest. 'Steen' (II) samples were presented first for training (these results were discarded although similar to the second tasting), and again at the end of the series. Tasters were allowed to come at their convenience, between about 10 and 12 AM with one or two exceptions. All usual precautions were enforced, such as prohibition of discussion of samples among tasters prior to or during rating. About 20 minutes was the typical time that most tasters took in carefully rating a set of 5 samples. If more wine was desired a complete new set was provided.

RESULTS AND DISCUSSION

Chemical analyses of the various wines are listed in Table 1. Analyses of variances showed very highly significant differences related to grape variety or harvest for all of the constituents measured. The differences in alcohol content related to treatments were also significant beyond the 99% level of confidence. However, the few tenths of a percent differences in alcohol content would not be expected to have direct sensory effects since about 4% difference in ethanol concentration is the minimum recognizable by tasters in wines (1).

The wines from the clear and turbid juices were essentially identical within the reproducibility of such samples in each component measured by these analyses (Table 1). The wines made with pomace contact during fermentation were significantly (95% confidence-level) lower in acidity and higher in pH than the wines made from juice, but additional pomace contact time beyond 24 hours made no significant further reduction in acidity. This is consistent with the well known fact that the juice contained in grape skins is higher in cations and lower in pH than that readily expressed as free-running juice.

Extract (Table 1) was significantly (99% confidence-level) higher with pomace contact than without. Increasing time of pomace contact generally gave further increases in extract, as expected, though the differences were not significant.

Total phenols (Table 1) calculated as gallic acid were significantly different by treatments, and ac-

Table 1. The composition of dry table wines from several white grape varieties with increasing grape solids contact during fermentation.

Analysis and treatment[b]	Variety[a]							
	C.B.	Co.	G.	R.	S(I).	S(II).	W.F.	Av.
Must Brix[c]	21.6	20.1	21.6	19.5	20.0	21.7	19.6	
Alcohol (% v/v @ 60°F)								
C	12.4	11.4	12.6	10.8	11.2	12.3	11.6	11.74
T	12.2	11.3	12.6	10.7	11.2	12.2	11.6	11.68
1D	11.8	10.8	12.3	10.6	11.2	12.2	11.2	11.44
2D	11.8	10.8	—	10.4	11.1	—	11.2	11.05
5D	11.6	10.8	—	10.4	11.0	—	11.1	10.95
Acid (g tartaric/100 ml)								
C	.52	.96	.56	.66	.44	.67	.38	.60
T	.57	.95	.55	.68	.46	.66	.35	.60
1D	.53	.87	.52	.64	.41	.38[d]	.30	.52
2D	.51	.90	—	.64	.37[d]	—	.29	.54
5D	.32[d]	.92	—	.65	.42	—	.33	.53
pH								
C	3.54	3.19	3.58	3.43	3.78	3.48	3.64	3.52
T	3.47	3.18	3.54	3.38	3.73	3.46	3.61	3.48
1D	3.62	3.32	3.64	3.50	3.94	3.80[d]	3.80	3.66
2D	3.69	3.35	—	3.52	4.04[d]	—	3.84	3.69
5D	3.91[d]	3.35	—	3.52	3.99	—	3.86	3.73
Extract (g dissolved solids/100 ml)								
C	1.88	2.17	2.08	1.86	1.99	1.99	1.52	1.93
T	2.01	2.17	2.12	1.99	1.76	1.99	1.55	1.94
1D	2.07	2.25	2.19	2.09	1.88	2.01	1.57	2.01
2D	2.09	2.32	—	2.14	2.12	—	1.60	2.05
5D	1.99	2.45	—	2.19	2.19	—	1.76	2.12
Total phenol (mg gallic acid equiv./L)								
C	220	162	182	258	151	194	279	207
T	250	164	178	242	158	184	276	207
1D	311	211	225	327	196	219	341	261
2D	360	234	—	358	238	—	370	312
5D	484	279	—	469	323	—	472	405

[a] C. B. = 'Clairette blanche'; Co. = 'Colombar' ('French Colombard'); G = 'Green' ('Sémillon'); R = 'Reisling'; S = 'Steen' ('Chenin blanc'); W.F. = 'White French' ('Palomino').

[b] C = clarified juice from the top half of a settled sample was fermented. T = the turbid half of the same settled sample. 1D = 24 hours, 2D = 48 hours, and 5D = 120 hours between inoculation with yeast of the whole crushed grape must and separation of the fluid from the pomace.

[c] Prior to addition of sugar the Brix of the must was C.B. and Co. 17.8 and W.F. 16.8.

[d] A malo-lactic fermentation is indicated in these 3 samples.

cording to the Duncan range test at the 99% confidence level they were grouped, all varieties combined, clear-turbid, 24-48 hr, and 120 hr, in which those not underlined by the same lime differ significantly. Thus, fermentation with the pomace at a low temperature for 24 hours produced a definite increase in the wine's phenol content, and by 5 days a further highly significant increase was produced.

Table 2 shows the quality ratings given by the panel to the wines. Wines made from must clarified by settling were invariably rated as highest in quality by a large margin. That is brought out further by Table 3, which shows that the clarified sample was higher in quality rating than all other

Table 2. Mean ratings for quality by a sensory panel of a series of dry white table wines made by variation in grape solids contact.

Grape variety	Treatment					
	Clear juice	Turbid juice	24-hr pomace contact	48-hr pomace contact	120-hr pomace contact	Variety means
'Clairette blanche'	15.3	11.6	13.4	12.9	10.8[a]	12.80
'Colombar'	14.9	11.1	13.5	12.8	11.7	12.80
'Green'	14.7	12.8	11.2	—	—	12.89
'Riesling'	14.5	13.0	13.5	12.2	9.9	12.62
'Steen' (I)	12.7	10.3	12.1	9.9[a]	10.9	11.18
'Steen' (II)	12.8	10.6	9.8[a]	—	—	11.08
'White French'	13.5	10.2	9.2	10.6	11.6	11.02
Treatment means	14.06	11.37	11.82	11.68	10.97	12.06

[a]A malo-lactic fermentation is indicated in these 3 samples.

Table 3. Analyses of quality ratings of wines made by different treatments by the Duncan multiple-range test. (Values not derlined by the same line differ significantly at 95% confidence.)

Grape variety	Quality rating by treatment in decreasing order, left to right				
'Clairette blanche'	Clear	24 hr	48 hr	Turbid	120 hr
'Colombar'	Clear	24 hr	48 hr	120 hr	Turbid
'Green'	Clear	Turbid	24 hr		
'Riesling'	Clear	24 hr	Turbid	48 hr	120 hr
'Steen' (I)	Clear	24 hr	120 hr	Turbid	48 hr
'Steen' (II)	Clear	Turbid	24 hr		
'White French'	Clear	120 hr	48 hr	Turbid	24 hr
All Varieties	Clear	24 hr	48 hr	Turbid	120 hr

wines in every harvest except for 'Steen' (I), where only the 24-hour pomace contact brought the quality close enough to eliminate a significant difference. That is an unusually large and consistent quality difference to be produced in acceptable wines by simple changes in winemaking practices. Van Wyk and de Waal (6) also have reported increased sensory quality and stability produced by must clarification. The nature of this effect is considered further below.

Analyses of variances showed that differences in quality with respect to treatment were significant at 99.9% confidence except for the 'Steen' (I) set, which were significant at 99% confidence. As usual there was a highly significant difference among tasters and among grape variety samplings. Treatment x tasters interaction was not significant when only the clear, turbid, and 24-hour samples were compared, and barely so (95% confidence) if all 5 treatments were considered. Thus, the tasters agreed well on the relative merit of the wines but their scaling differed sufficiently to produce the significant difference among tasters. There was a highly significant interaction (p< 0.01) of grape variety or harvest with treatments and with tasters. If scaling differences were corrected by some normalizing process and statistically corrected for the differences of taster opinion with regard to varieties, agreement by the panel on the effect of the treatments would clearly be exceptionally near unanimity.

Note (Tables 2, 3) that wines from turbid juice were rated much lower in quality than wines from clarified juice and often lower than wines with pomace contact. The turbid sample had started with *twice* the fine solids that were present in unclarified juice. It therefore seems reasonable that the samples from turbid juice were generally rated as lower in quality than some of the samples with pomace present during fermentation and thus from unclarified juice. The samples with pomace contact decreased significantly (p < 0.05) in quality with increasing time of pomace contact, with two exceptions: in 'White French' this order was reversed, and the 48-hour sample was lowest in quality in the 'Steen' (I) set.

The low quality of the 'Steen' (I) sample with 48-hr pomace contact may be due to a malo-lactic fermentation (see Table 1). All three wines which evidently had undergone acid lowering by a malo-lactic fermentation were rated at the bottom of their set. Studies in Germany (2) have indicated that malo-lactic fermentation generally lowers the quality of white table wines. Comments by a few sensory panelists indicated that these three samples had some "oxidized" or "beeswax" character, but comments were similar on a few other samples and no bacterial character was noted.

The quality ratings for these wines were generally low, reflecting in part the poor 1969 vintage. The growing season was wet, with the first widespread and serious downy mildew attack known in South Africa. Rains were frequent during the vintage, and grapes were picked underripe. It should be emphasized, however, that these wines were all in the range considered by the panel as standard commercial quality or readily correctable to that level by blending or normal cellar treatments. Furthermore, this manner of scoring was relatively new to these experienced wine judges and it is believed that they tended to rate wines somewhat lower for

Table 4. Three quality ratings by a 12-member sensory panel for dry white wines made with differing contact with grape solids.

Quality factor and grape variety	Mean ratings (0 low–10 high) in decreasing desirability order[a]				
Astringency					
'Clairette blanche'	3.2 (C)	3.6 (1D)	3.6 (2D)	4.5 (T)	4.9 (5D)
'Colombar'	3.2 (C)	3.8 (1D)	4.0 (2D)	4.3 (T)	4.5 (5D)
'Green'	3.8 (C)	4.5 (T)	4.7 (1D)		
'Riesling'	3.6 (C)	3.8 (1D)	4.2 (2D)[b]	4.3 (T)	4.9 (5D)
'Steen' (I)	3.1 (C)	3.1 (2D)	3.2 1D)	3.8 (T)	4.4 (5D)
'Steen' (II)	4.0 (1D)	4.1 (T)	4.2 (C)		
'White French'	2.2 (C)	3.7 (T)	3.9 (5D)	4.1 (2D)	4.2 (1D)
All varieties	3.4 (C)	3.8 (1D)	3.8 (2D)	4.2 (T)	4.5 (5D)
Means by variety	3.4 (SI), 3.7 (WF), 3.8 (CB), 3.8 (Co), 3.9 (R), 4.1 (SII), 4.3 (G)				
Bitterness					
'Clairette blanche'	3.2 (C)	3.4 (T)	3.5 (1D)	3.5 (5D)	3.8 (2D)
'Colombar'	2.7 (5D)	3.0 (C)	3.0 (1D)	3.0 (2D)	3.8 (T)
'Green'	4.0 (C)	4.2 (T)	5.7 (1D)		
'Riesling'	3.4 (C)	3.4 (T)	3.4 (1D)	3.7 (5D)	3.8 (2D)
'Steen' (I)	3.5 (C)	3.9 (2D)	3.9 (5D)	4.0 (T)	4.0 (1D)
'Steen' (II)	3.4 (C)	4.1 (1D)	4.6 (T)		
'White French'	3.6 (C)	3.8 (5D)	3.8 (1D)	3.9 (T)	4.2 (2D)
All varieties	3.3 (C)	3.5 (5D)	3.6 (1D)	3.7 (T)	3.7 (2D)
Means by variety	3.2 (Co), 3.4 (CB), 3.4 (R), 3.8 (WF), 3.8 (SI), 4.0 (SII), 4.6 (G)				
Desirable aroma					
'Clairette blanche'	7.1 (C)	5.8 (1D)	5.8 (2D)	4.4 (T)	3.9 (5D)[b]
'Colombar'	7.5 (C)	6.0 (1D)	5.6 (2D)	4.6 (T)	4.3 (5D)
'Green'	6.7 (C)	5.1 (T)	3.8 (1D)		
'Riesling'	6.8 (C)	5.4 (1D)	5.3 (T)	4.7 (2D)	3.0 (5D)
'Steen' (I)	5.5 (C)	4.8 (1D)	4.1 (5D)	3.2 (T)	2.9 (2D)[b]
'Steen' (II)	5.8 (C)	3.5 (T)	3.2 (1D)[b]		
'White French'	6.0 (C)	5.0 (5D)	4.2 (2D)	3.5 (T)	2.4 (1D)
All varieties	6.5 (C)	4.9 (1D)	4.6 (2D)	4.2 (T)	4.1 (5D)
Means by variety	6.0 (Co), 5.9 (R), 5.8 (CB), 5.2 (G), 4.5 (SI), 4.2 (SII), 4.0 (WF)				

[a] C = clear, T = turbid, 1D = 24 hr, 2D = 48 hr, 5D = 120 hr pomace contact. Values not underlined by the same line differ significantly at the 95% confidence level.

[b] A malo-lactic fermentation is indicated in these three samples.

quality than do panels who are more familiar with this type of scorecard.

Of considerable interest is the fact that even the samples fermented 120 hours on the pomace were generally considered within the commercial range for dry white table wines. Fermenting white grapes as if they were red (albeit at a cool temperature) does not make a wine which would be mistaken for a red wine by blindfolded expert tasters, as observed previously (Singleton, unpublished) in California at warmer fermentation temperature. This seems to be an important observation and shows that red grapes differ from white grapes in ways which affect the character of the wine more than by just the visual effect of the red color.

Table 4 shows the mean sensory ratings by the 12-member panel for astringency and the significant differences among them by analysis of variance and the Duncan range test. There were statistically significant differences in astringency with 4 of the individual variety-harvest sets. The general trends plus the combined data showed that the wines made from the clarified juice were rated lowest in astringency and those receiving 120 hours of fermentation on the pomace were rated highest. The 24-hour samples tended to be slightly lower in astringency than the 48-hour samples, but the differences were small and not significant in any instance.

The only known astringent substances in wines and most other food products are the polymeric phenols, the tannins. If one compares the sensory data of Table 4 with the total phenol analyses in Table 1, it appears that the additional phenol at 51 mg/L introduced on the average in increasing pomace contact time from 24 hours to 48 hours was not large enough to produce a recognizable difference in astringency. The average further increase in total phenol of 93 mg/L from extending the grape solids contact time from 48 hours to 120 hours was sufficient to produce a significant increase in average astringency ratings. These data are strongly reinforced if further comparisons are made within individual varietal sets. 'Clairette blanche,' 'Steen' (I), and 'Riesling' were all significantly more astringent after 120 hours of pomace contact than one or both of the samples with less pomace contact. The 120-hour samples of these sets had total phenol respectively 124, 102, and 111 mg/L higher than the highest of the samples with shorter pomace contact. On the other hand, all comparisons without significant differences in astringency had differences in total phenol of 85 mg/L or less. It thus appears that pomace contact in the must of seeded white grapes sufficient to produce an increase in gallic acid equivalent total phenol in the wine of about 100 mg/L also produced a threshold difference in astringency.

Taking all samples and comparing astringency ratings with total phenol analyses gave a rather disperse scatter diagram and a correlation coefficient of only 0.41. It was, however, significant at 95%

confidence. If the samples from turbid juice were left out, the correlation coefficient increased to 0.55, along with an increased confidence level.

Wines from clarified juice were rated as significantly less astringent than the corresponding 24-hour pomace contact sample even though phenol content differences were less than 100 mg/L, the indicated difference threshold. Conversely, the wines from turbid juice were judged nearly as astringent as those fermented 5 days on the pomace, and yet the total phenol content was essentially the same as that of the clear-juice wines judged significantly lowest in astringency. These anomalies are believed to result from additional factors which influenced the judges. The wines from clarified juice were much the best quality (Tables 2, 3) and had a much more desirable aroma. It is believed that astringency was rated lower in these wines because they were fruity and higher in quality. This suggests that astringency is considered a negative quality factor in dry white table wines but may be more acceptable if the wine is otherwise of high quality. The high astringency rating for the wines from turbid juice is believed to result from the fact that the wines were harsh or less attractive for reasons other than phenol content, notably hydrogen sulfide content.

The differences among grape varieties in average astringency rating were significant ($p < 0.05$) but not large. It seems notable that the 'Green' ('Sémillon') wines were rated most astringent even with the low pomace contact conditions and in spite of one of the lowest average phenol contents (Table 1). Again the rather herbaceous flavor of this variety is believed to have influenced the judges toward a higher rating for astringency (and also for bitterness).

The mean ratings of the wines for bitterness are shown in Table 4. It is of considerable interest that the ratings for bitterness did not parallel those for astringency, since both can be contributed by grape phenols to wine (3) and they are often confused by inexperienced tasters. The range in ratings among treatments was small and not significant for the combined data. Again there was a tendency for the turbid-juice sample to be rated more bitter than that from clarified juice, perhaps for the same reasons discussed for astringency.

The tendency for the wines with 48 hours or 24 hours of pomace contact to be rated as more bitter than those with either less or more pomace contact is believed real although not statistically significant. This is believed to be the first experimental evidence for observations described by winemakers as soft tannin versus hard tannin. Wines with a high level of astringency and not bitter are said to have soft tannin, whereas bitterness with astringency is described as hard tannin. We believe that this is because larger astringent tannin polymers are less bitter (3) and that the astringency interferes with recognition of the bitterness whereas the smaller bitter phenols become more obvious as the astringency decreases.

These data tend to confirm these hypotheses, but certainly do not prove them. It is well known that tannin molecule size is astringency-related and that larger molecules diffuse more slowly from cells.

The outstanding differences in these wines, however, were in their odors, which accounted for much of the differences in quality rating that have already been discussed. Table 4 also shows the ratings for desirable odor or aroma. In every instance the scores for desirable aroma of the wines were considerably higher for the clear-juice wines than for any others, and statistically significantly so in all but one instance. The combined ratings for desirable aroma decreased with grape solids contact in the order 24 hours, 48 hours, turbid juice, and 120 hours, with some variation in individual sets and generally significant differences among some members of the sets. The three wines which appeared to have had a malo-lactic fermentation (Table 1) were rated at the bottom of their group in Table 4.

The significant differences found among varieties in overall desirability of odors (Table 4) partly reflect varietal character, with the 'Riesling' and 'Sémillon' types high, and 'Chenin blanc' and 'Palomino' types low. The main characteristic, however, that made the odor of the wines from clarified juice better was common to all varieties. This was a distinctive delicate fruitiness described by the tasters in Afrikaans as *blommetjie* ("little blossoms") and termed fermentation bouquet. This specific and attractive odor resulted in all 7 instances of cold fermentation (16 C) of white juice clarified by settling and protected from air. This odor, somewhat labile, was largely lost in bottled samples of the wines stored a few weeks at warm temperatures. It was reduced or absent in samples prepared by contact with grape solids, thus giving a less desirable odor.

The odor of the wines prepared from the turbid half of the same juice which gave the clarified samples was not only much lowered or lacking in this attractive fermentation bouquet but also usually had more or less off-odor. This off-odor was frequently recognized as hydrogen sulfide but sometimes was too low to be recognized as a specific chemical, being called "organic," "stale," "like a stuffy room," etc. Hydrogen sulfide was not noted in any of the clear-juice wines and was noted less often in the pomace wines than in wines from the turbid juice. A "grape-skin" odor noted by some judges in some samples with pomace contact appeared to be considered undesirable.

SUMMARY AND CONCLUSIONS

Large sensory differences, but no chemical differences in the components measured, were produced in dry white wines made from juice clarified of the readily settled solids as opposed to juice with double the suspended solids of free-run juice. The wines were fermented under the same conditions at low temperature. The wine from the clarified juice was much higher in ratings for quality and desirable odor and was rated as less astringent and less bitter than the wine from turbid juice. These differences were relatively uniform among the 6 different grape varieties tested and two harvests of one of them.

The higher quality of the wines from clarified juice was evidently largely the result of the increased desirable odor. This odor was similar in all samples and is described as a fresh distinctive fruitiness termed fermentation bouquet. The wines from turbid juices were rated as much less desirable in odor not only because they appeared to lack this fruitiness but also because they tended to have additional off-odors, including hydrogen sulfide.

Wines made with increasing pomace contact, up to a total of 5 days of fermentation of the entire crushed grape mass of these seeded white grapes before fluid separation, were characteristically changed in composition. Particularly noteworthy was the increase in total phenol content. This increase, compared with ratings for astringency, indicated that contact with seeded white grape pomace sufficient to produce additional total phenol of about 100 mg or more per liter would produce a recognizable increase in astringency in white table wine whereas less would not.

Bitterness ratings did not parallel astringency ratings, increases in pomace contact, or total phenol content. The data suggest that high astringency can mask bitterness and that the more bitter samples tended to be those with intermediate times of pomace contact and total phenol content.

Pomace contact was generally antagonistic to desirable aroma and overall quality in these dry white table wines. Pomace contact reduced or eliminated the desirable fermentation bouquet odor, and the extra astringency, etc., was apparently considered less desirable in wines of this type.

LITERATURE CITED

1. Hinreiner, E., F. Filipello, H. W. Berg, and A. D. Webb. Evaluation of thresholds and minimum difference concentrations for various constituents of wines. 4. Detectable differences in wine. Food Technol. 9:489-90 (1955).

2. Radler, F. Über die Häufigkeit des bakteriellen Säureabbaus in deutschen Weissweinen. Wein-Wiss. 25:418-24 (1970).

3. Rossi, J. A., Jr., and V. L. Singleton. Flavor effects and adsorptive properties of purified fractions of grape-seed phenols. Am. J. Enol. Viticult. 17:240-6 (1966).

4. Singleton, V. L., and J. A. Rossi, Jr. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Viticult. 16:144-58 (1965).

5. Singleton, V. L., P. de Wet, and C. S. du Plessis. Characterization of populations of grapes harvested for wine and compensation for population differences. Agroplantae 5:1-12 (1973).

6. Van Wyk, C. J., and C. T. de Waal. Die invloed van sekere mos- en wynbehandelings op die stabiliteit van droe wit wyne. Die Wynboer 31 (364):17-19 (1962).

CHARACTERIZATION AND GROWTH-DEPRESSING ACTIVITY FOR CHICKENS OF SEVERAL NATURAL PHENOLIC MATERIALS[1]

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ABSTRACT   Several natural phenolic materials were analyzed for gallic acid and were further characterized for dialyzability and flavonoid content. These materials were added to a diet and fed to broiler strain chicks for 4 weeks. The depression in growth caused by these phenolic materials was compared with that of tannic acid on a gallic acid equivalency basis. The activities relative to tannic acid (≈ 100) were as follows: methyl gallate, 195; valonia, 117; wattle, 114; allepo tannic acid, 107; eucalyptus, 105; myrobalan, 98; tara crystals, 76; mangrove, 72; quebracho, 39; gallic acid, 30; and ellagic acid, 0. It was impossible to clearly relate the growth depressing activity of the materials to hydrolyzability or relative content of smaller phenols.

POULTRY SCIENCE 54: 2124–2127, 1975

CHANG and Fuller (1964) tested several varieties of grain sorghums and noted that growth retardation was directly related the level of tannin supplied by the various samples and Rostagno et al. (1973) found bird resistant sorghum to give poorer growth than varieties with lower tannin. Vohra et al. (1966) and tannic acid to be the most growth depressing of the several plant tannins which were studied. Metabolites of tannic acid, pyrogallol and pyrocatechol, are much more with depressing that the parent compound (Potter and Fuller, 1968; Rayudu et al., 1970). Kratzer and Williams (1951) reported that ground carob pods severely depressed chick growth. Nachtomi and Alumot (1963) found that the tannins in carob pods are complex and that degradation was necessary before they could be extracted by the usual tannin solvents. Joslyn et al. (1968) separated the phenolic compounds from green and ripe carob pods into five or four components respectively and characterized them chemically. Hydrolyzable tannins were much more

prevalent in green than ripe carob pods. When polyphenol fractions were fed to rats (Tamir and Alumot, 1970) growth depression resulted which was thought to be related to the binding of protein by tannins which resulted in insoluble protein in the feces. Joslyn and Glick (1969) had fed several tannin containing materials to rats and observed that condensed tannins were less growth depressing than was galloyl glucose.

In the present study a number of tannin containing materials have been characterized chemically and tested for growth depression in chicks to try to determine a chemical basis for the adverse effect of polyphenolic compounds on chickens.

EXPERIMENTAL

The tannin and phenolic materials were all obtained commercially except ellagic acid which was synthesized (Singleton et al., 1966).

The samples were dispersed and steeped in 20% aqueous ethanol at 400 mg./l. If any insoluble residue was present an effort was made to dissolve it by warming, grinding and stirring and the solution was then filtered. Portions were assayed for total phenol in gallic acid equivalents (Singleton and Rossi, 1965), and flavonoid in gallic acid equivalents by difference after precipitation with for-

TABLE 1.—Dialyzability, flavonoid content and chick growth depressing activity of various tannins

Tannin	Gallic acid equivalent mg./g.	Soluble non-dialyzable %	Dialyzable %	Insoluble non-dialyzable %	Flavonoid %	Non-flavonoid %	Chick growth depressing activity relative to tannic acid By wt.	By gallic acid content	Description
Tannic acid	870	36	29	35	0	100	100	100	Gallic, hydrolyzable
Allepo tannic acid	718	24	40	36	0	100	88	107	Gallic, hydrolyzable
Tara cystals	548	9	49	42	0	100	48	76	Gallic, hydrolyzable
Valonia	572	14	48	38	0	100	77	117	Ellagic, hydrolyzable
Myrobalan	550	24	37	39	0	100	62	98	Ellagic, gallic, hydrolyzable
Mangrove	545	58	22	20	85	15	45	72	Condensed
Quebracho	685	32	45	23	48	52	31	39	Condensed
Eucalyptus	593	40	33	27	36	64	72	105	Condensed
Wattle	623	15	47	38	45	55	82	114	Condensed
Gallic acid	1000	0	100	0	0	100	30	30	
Methyl gallate	1110	0	100	0	0	100	216	195	
Ellagic acid	529	—	—	100	0	100	0	0	

...yde by the method of Kramling and
...on (1965). Dialyzability was deter-
...by analysis of the dialysate (through
...phane membrane in 7 days at 22° C.
...4 changes of water) and the retentate
...dialysis tubing. Owing to precipitation
...e of the material during dialysis and,
...s oxidation, there was a significant
...n of the phenols not accounted for in
...fraction and is termed "% insoluble
...n-dialyzable."

...various materials were added at 1 or
...a chick starting diet (Rayudu et al.,
...vhich already contained approximately
...tannin. Day-old male Arbor Acres
...+ were divided into groups of 10 birds
...f equivalent weight and were housed
...trically heated batteries with raised
...oors. Duplicate groups of chicks were
...e experimental diets *ad libitum* for 4

RESULTS

...amounts of phenolic material and the
...al characterization of it for the various
...containing products are shown in Table
...o included is the chick growth depress-
...ivity relative to tannic acid which was
...rily given a value of 100. Each value
...tablished as an average of at least two
...ith duplicate groups of chicks in com-
...n with 0, 1 or 2% tannic acid. Since
...terials varied in gallic acid equivalence,
...owth depressing activity was also ex-
...ed in relation to the gallic acid equiva-

...po tannic acid, valonia, myrobalan,
...ptus and wattle had approximately the
...growth depressing activity as tannic
...Tara crystals and mangrove were ap-
...nately 3/4 as active as tannic acid while
...acho had only 39% as much activity

...Arbor Acres Farm, Inc., Nipoma, California

as tannic acid. Gallic acid was 30% as active
as tannic acid while methyl gallate was over
twice as active. Ellagic acid was without
growth depressing activity.

DISCUSSION

One can conclude that phenolic prepara-
tions have considerably different physiolog-
ical effects. Methyl gallate was considerably
more growth depressing than gallic acid.
Since phenols are commonly detoxified in
animals by conversion to more acidic deriva-
tives, sometimes by conversion to carboxyl
derivatives, this appears reasonable. Fur-
thermore, hydrolysis of the methyl ester in
the chick would give methanol, a further toxic
load, and probably not a methyl group con-
tributor for 4-methoxygallate conversion, a
possible detoxification product. Ellagic acid,
which can also be considered a derivative
of gallic acid, was inactive in growth inhibi-
tion presumably because it is very poorly
soluble.

Most of the tannins were about equally
growth inhibitory when dosages were
computed on an equal total phenol content
regardless of hydrolyzable or condensed tan-
nic nature and relative content of smaller
phenols (% dialyzable). Tara crystals (a gal-
loyl quinate tannin) and mangrove tannin
appeared slightly less growth inhibitory than
the others in spite of the fact that they had
a fairly high percentage of small (dialyzable)
phenols. It is interesting to note that the
tannins considered hydrolyzable in the litera-
ture (Haslam, 1966) tested 100% non-flavo-
noid in our assays but the condensed tannins
were apparently mixtures of flavonoid and
non-flavonoid phenols.

REFERENCES

Chang, S. I., and H. L. Fuller, 1964. Effect of tannin
content of grain sorghums on their feeding value
for growing chicks. Poultry Sci. 43: 30-35.
Haslam, E., 1966. Chemistry of Vegetable Tannins.
Academic Press, New York, pp. 179.
Joslyn, M. A., and Z. Glick, 1969. Comparative effects
of gallotannic acid and related phenolics on the
growth of rats. J. Nutrition 98: 119-126.
Joslyn, M. A., H. Nishira and S. Ito, 1968. Leu-
coanthocyanins and related phenolic compounds of
carob pods (*ceratonia Liliqua*). J. Sci. Fd. Agric.
19: 543-550.
Kramling, T. E., and V. L. Singleton, 1969. An
estimate of the non-flavonoid phenols in wines.
Amer. J. Enol. Viticult. 20: 86-92.
Kratzer, F. H., and D. E. Williams, 1951. The value
of ground carob in rations for chicks. Poultry Sci.
30: 148-150.
Nachtomi, E., and E. Alumot, 1963. Tannins and
polyphenols in carob pods (*ceratonia Liliqua*). J.
Sci. Food Agric. 14: 464-468.
Potter, D. K., and H. L. Fuller, 1968. Metabolic fate
of dietary tannins in chicks. J. Nutrition, 96: 187-
191.

Rayudu, G. V. N., R. Kadirvel, P. Vohra and F.
H. Kratzer, 1970. Toxicity of tannic acid and its
metabolites for chickens. Poultry Sci. 49: 957-960.
Rostagno, H. S., W. R. Featherston and J. C. Rogler,
1973. Studies on the nutritional value of sorghum
grains with varying tannin contents for chicks.
Poultry Sci. 52: 765-772.
Singleton, V. L., G. L. Marsh and M. Coven, 1966.
Identification of ellagic acid as a precipitate from
loganberry wine. J. Agr. Food Chem. 14: 5-8.
Singleton, V. L., and J. A. Rossi, Jr., 1965. Colorime-
try of total phenolics with phosphomolybdicphos-
photungstic acid reagents. Amer. J. Enol. Viticult.
16: 144-158.
Tamir, M., and E. Alumot, 1970. Carob tannins growth
depression and levels of insoluble nitrogen in the
digestive tract of rats. J. Nutrition, 100: 573-580.
Vohra, P., F. H. Kratzer and M. A. Joslyn, 1966.
The growth depressing and toxic effects of tannins
to chicks. Poultry Sci. 45: 135-142.

BROWNING OF WHITE WINES AND AN ACCELERATED TEST FOR BROWNING CAPACITY

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ABSTRACT

A standardized test for the browning capacity of white wines was developed. A portion of the wine is treated with 1 g of bentonite per 100 ml under nitrogen so that the developed brown pigment will stay in solution. Portions of the treated wine are incubated in sealed tubes with 25% or more headspace well sparged with oxygen and separately with nitrogen for 5 days at 55°C. The absorbance at 420 nm remains nearly unchanged for the N_2-sparged samples and under oxygen measures wines' relative capacity to brown oxidatively. Evidence is presented that a wine well protected from oxidation is largely prevented from browning whereas a wine treated by adding d-catechin is considerably increased in its capacity to brown by this test.

There have been a sizable number of studies upon the browning of white wines. Most have used arbitrarily chosen conditions to produce and measure the brown, without studying the variables.

Berg (1) used 200 ml of wine in a screw-capped bottle large enough to provide air equivalent to 600 ml O_2/l. Samples were held 7 days at 49°C before filtering (if necessary) and comparing absorbance at 425 nm with the original value. Wright (2) used absorbance at 470 nm after storage of vermouth samples for 4 months at 2, 20, or 30°C to estimate the relative browning of different wines. De Villiers (3) used a portion of each wine in a sealed full flask at 4°C as the control and measured browning at 425 nm at 10-day intervals after storing 150 ml of wine with 200 ml of air at 49°C. Fuller and Berg (4) heated 20 ml of wine plus 12 ml of air in closed glass tubes until increment of absorbance at 440 nm reached 0.150 in a 12-mm colorimeter tube. Caputi and Peterson (5) compared nitrogen-stripped and oxygen-saturated bottled wine after 10 days at 40°C or 100 days at room temperature. Du Plessis and Uys (7) heated samples in 30-ml screw-capped bottles, leaving 2 ml airspace and centrifuging if necessary, and determined absorbance periodically in 1-cm cells at 425 nm. Sapis and Ribéreau-Gayon (8) stored wines with excess oxygen in sealed bottles for 7 and 21 days at 40°C before measuring browning at 440 nm. Peri et al. (9) estimated (450 nm) enzymatic browning at 5°C and nonenzymic browning at 50°C in 100 ml of wine in 500-ml conical flasks for up to 15 days.

Berg and Akiyoshi (10) studied the parameters of browning of white wines in some detail. They showed that increases in oxygen up to but not beyond 60 ml/l of wine gave greater browning, as did also higher pH or temperature. They concluded that enzymic oxidation was not significant in the browning of white wine.

It appeared to us desirable to investigate further the conditions for a reproducible accelerated test to determine the capacity of a wine to brown. If such a test could quantitate differences among wines and clarify browning reaction conditions, it should be very useful. Standardization of test conditions would also be very helpful in comparing various worker's results.

MATERIALS AND METHODS

White wines with alcohol levels of table wines were prepared by standard methods either at Davis or Stellenbosch and used in these experiments. Absorbance was determined at 420 nm with either a Spectronic 20 (Davis) or a Spekker photometer (Stellenbosch) in 18-mm-ID test tubes. The samples

Am. J. Enol. Vitic., Vol. 27, No. 4, 1976

157

to be tested for browning were placed in the test tubes (7-10 ml) sparged with N_2 or O_2 gas, firmly sealed with either a rubber stopper held in place by electrician's plastic tape under tension or by rubber-lined screwcaps, and heated in racks in a regulated oven. The samples were removed periodically, cooled to room temperature, absorbance measured, and returned to the oven.

RESULTS AND DISCUSSION

Oxygen content: For oxidative browning to proceed as rapidly as possible, excess oxygen must be available throughout the reaction period. For this reason O_2 was used in the headspace rather than air. To be sure that O_2 transfer into the solution was not limiting the reaction rate, wine samples were browned with and without agitation. Agitation was in a tissue-culture rotor, a 2-foot-diameter wheel turning at 1 rpm at about 60° from vertical, versus the static samples vertical in a rack. Experiments were conducted also upon the minimum headspace of O_2 which gave maximum browning rate and produced maximum brown pigment levels after prolonged treatment. Typical data are shown in Table 1; this and other tests gave no significant differences in brown color level in relation to agitation or O_2 headspace as large as or larger than 5-ml/22-ml-capacity tube (23%). Thus, the oxygen content and transfer rate into the wine do not appear to be in any danger of limiting the amount or rate of the reaction under the conditions in use here.

Wavelength: The absorbance was measured at 420 nm. This is commonly measured in red wine, along with 520 nm absorbance, to estimate brown versus red shades of color and is thus a usual value for an enologist. Some browned wines exhibit a shoulder or broad maximum near 445 nm, though not all do so, and such higher wavelengths would be more subject to interference from possible pinking of leucoanthocyanidins. Owing to the general fact that browns are "spillover" visible color from more intense absorbance in the ultraviolet, many wavelengths in the 400-480-nm range have been used to measure browning in various products. Nevertheless, 420 nm is preferred for wine for the reasons given and to provide standardization.

Sugar, temperature, and amino acids: The effects of sugar and different temperatures upon browning of a Chardonnay wine are shown in Table 2. Nearly identical data were obtained with a Thompson Seedless wine. These data showed that at 52°C or below, glucose content was not an appreciable factor in accelerated browning, whereas at 80°C extra browning was caused by the presence of sugar. Further testing with intermediate temperatures gave oxidative browning absorbance at 14 days in

Table 2. Effect of temperature, oxygen, and glucose on browning of a Chardonnay wine.

	Absorbance (420 nm) at indicated days							
	No glucose				10% glucose			
°C	0	2	8	14	0	2	8	14
Nitrogen-sparged								
31	.180	.128	.133	.165	.180	.127	.135	.190
52	.181	.146	.160	.210	.176	.138	.154	.210
80	.183	.420	.375	.520	.181	.255	.900	1.50
Oxygen-sparged								
31	.180	.185	.212	.330	.180	.182	.205	.310
52	.178	.230	.380	.530	.171	.224	.360	.530
80	.180	.430	.690	.900	.183	.420	1.50	2.10

Table 3. Effect of amino acid addition on the ability of a white wine to brown under nitrogen at 52°C.

Amino acid added	Amount added (mg/l)	Absorbance at 420 nm[a]	
		26 days	44 days
Control	—	.278	.355
L-arginine monohydrochloride	250	.295	.360
L-glutamic acid	250	.292	.362
Glycine	250	.270	.368
L-histidine monohydrochloride monohydrate	250	.290	.390
L-proline	500	.310	.380
L-hydroxyproline	500	.278	.365
L-threonine	250	.330	.365

[a] Absorbance at 0 days for all samples was .175.

Table 1. Effect of headspace oxygen and roller tube agitation on browning of a white table wine (18 x 150-mm test tubes, 52°C).

Headspace	Agitated	Absorbance at 420 nm		
		0 days	3 days	7 days
5 ml	no	.133	.270	.460
10 ml	no	.133	.310	.450
15 ml	no	.133	.280	.460
5 ml	yes	.133	.290	.460
10 ml	yes	.133	.300	.480
15 ml	yes	.133	.300	.480

Chardonnay plus 10% glucose that at 31°C was 1.06 times the absorbance of the same wine without glucose, 1.00 times at 52°C, 1.24 times at 65°C, 1.39 times at 72°C, and 2.88 times at 80°C. It thus appears that sugar is of negligible effect at 55°C or below but is increasingly important as temperature rises, particularly at 80°C or higher. This caramelization effect is evident also under nitrogen (Table 2). The addition of amino acids under oxidative conditions or under nitrogen (Table 3) gave little or no increase in browning in a dry table wine over that in the controls.

Bentonite treatment: Proteins or polypeptides evidently interfere in browning measurements by reacting with and precipitating the brown pigment. That is prevented by adding a relatively heavy dose of bentonite. To avoid dilution problems the bentonite was added dry to the wine sample, mixed in until thoroughly wetted, and the wine held under N_2 at room temperature for about 2 hours with occasional shaking. The wine was then freed of bentonite by centrifugation and filtration through a membrane (0.45-micron) filter. Fig. 1 shows that without bentonite the maximum brown color is less and increases rapidly as the bentonite level is increased to 0.384 g/100 ml (32 lb/1000 gal) but very little beyond. Levels as high as 3.84, 7.68, and 15.36 g of bentonite/100 ml of wine gave little or no further increase in browning rate or extent in any wine tested except as the pH began to be increased slightly at these extreme levels. The ben-

tonite treatments did not affect the total phenol content appreciably, e.g., an untreated wine with 163 mg GAE/l total phenol assayed 160 mg GAE/l after 3.84 g/100 ml of bentonite and 168 mg GAE/l after 7.68 g. Further tests indicated that 3.84 g of bentonite per 100 ml may lower white wine total phenol about 5%, and that 7.68 g/100 ml usually lowered it about 10%. Considering that different lots of bentonite may differ considerably, the treatment should be as high as possible to allow for less effective bentonite. Fig. 1 shows that levels of 0.38, 0.77, and 1.54 g/100 ml give similar results, and the other tests showed that these should have little or no effect on total phenol content or pH. For routine use, 1 g of bentonite per 100 ml of wine is recommended.
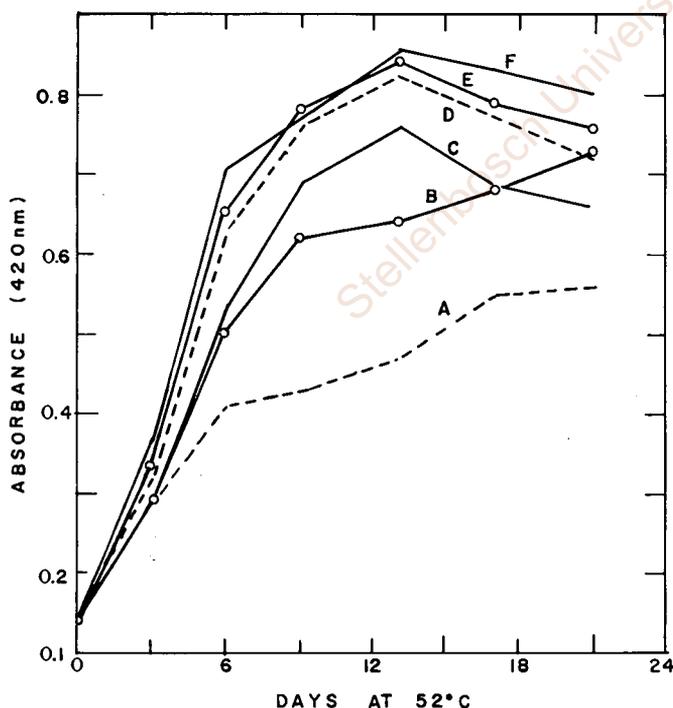
Reproducibility: If no bentonite is used, some wines produce precipitates of browned material and the development of brown in solution is decreased (Fig. 1). If heavily bentonited, these wines produce brown pigment at a uniform rapid rate (Fig. 1) but eventually reach a maximum of soluble brown pigment. In some samples the brown pigment then begins to decrease by polymerization and precipitation. The maximum brown is a reproducible value if either no precipitation occurs or the brown precipitate is dissolved by accurate dilution with 60% aqueous ethanol at pH 3.3. Table 4 shows that reproducible values were obtained in triplicate trials with two different wines.

With wines having considerably different browning rates the time to reach maximum brown in these tests can be different (Fig. 2) besides requiring two weeks or more for a test. During the first day or two of heating of a heavily bentonited white wine in the presence of oxygen the wine may even decrease in brown, may remain unchanged, or, more commonly, may brown, though at a different rate than later (Fig. 2). From about 3 to 11 days, however, the rate of brown absorbance increase is

Fig. 1. The effect on browning caused by prior treatment of a white wine with different levels of bentonite. a = 0 bentonite, b = 8 lb/1000 gal (0.1 g/100 ml), c = 16 lb/1000 gal (0.19 g/100 ml), d = 32 lb/1000 gal (0.38 g/100 ml), e = 64 lb/1000 gal (0.77 g/100 ml), f = 128 lb/1000 gal (1.54 g/100 ml).

Table 4. Reproducibility of the accelerated method used to induce browning in white wines (0.77 g bentonite/100 ml, 52°C).

Sample	Absorbance at 420 nm[a]		
	0 days	21 days	27 days
White Riesling	.167	1.30	1.32
White Riesling	.165	1.30	1.31
White Riesling	.171	1.28	1.31
Chardonnay	.240	1.19	1.18
Chardonnay	.241	1.20	1.20
Chardonnay	.228	1.21	1.20

[a] For 21 and 27 days the absorbance value is calculated from the absorbance reading of one part sample diluted with three parts of 60% aqueous ethanol adjusted to pH 3.3.
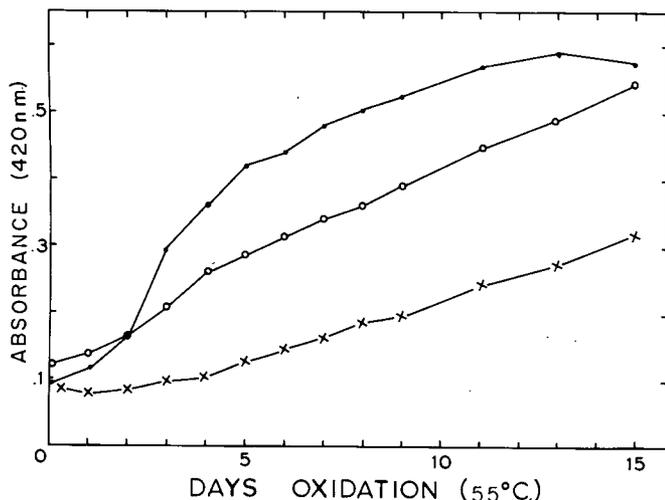
Fig. 2. Browning of three different wines with time under oxygen.

Fig. 3. Increase in browning caused by the addition of d-catechin to a white wine. a = Chardonnay wine alone, b= 200 ppm d-catechin, c = Chardonnay wine plus 200 ppm d-catchin.

essentially linear (Fig. 2) with time for each wine. Thus the browning capacity of different wines can be estimated by comparing their relative brownness at a constant time. We chose five days.

Browning test: The chosen procedure, then, for comparing browning capacity is: mix 100 ml of the wine thoroughly with 1.0 g of dry Wyoming bentonite, stopper it under nitrogen, and wait (with occasional shaking) for at least 2 hours. Settle and centrifuge, then filter through a 0.45-micron membrane filter enough to prepare four replicate test tubes. We have used 7 ml in 18 x 150-mm test tubes. Sparge two of the tubes thoroughly with N_2 free of O_2, and the other two with O_2. Seal each tube for the duration of the experiment with taped-down rubber stoppers or tight screw caps as the sparging tip is removed. Hold at 55°C for 5 days and read the brown absorbance developed in each tube at 420 nm. If desired, the sample can be held until maximum brown is reached, and that determined separately.

Minimal browning under nitrogen: The nitrogen-sparged samples serve as controls indicating any nonoxidative browning. Duplicates are recommended because samples that are completely depleted of oxygen by nitrogen generally do not brown. If more than one sample is N_2-sparged, an incorrectly high sample owing to incomplete removal of O_2 or loosening of a stopper can usually be detected. The O_2-treated samples usually duplicate each other very closely, but, again, can indicate leaks. Under the conditions indicated, the browning of dry white wines is almost completely oxidative. This is illustrated by the fact that a series of 5 different wines heated under N_2 at 60°C gave original and 8-day absorbances at 420 nm of, respectively, 0.058-0.057, 0.107-0.123, 0.086-0.097, 0.074-0.084, and 0.094-0.100. The same wines under O_2 for the same treatment gave absorbances of 0.414-0.712. Furthermore, if a sample browning under O_2 is then sparged with N_2 and reheated, the increase in
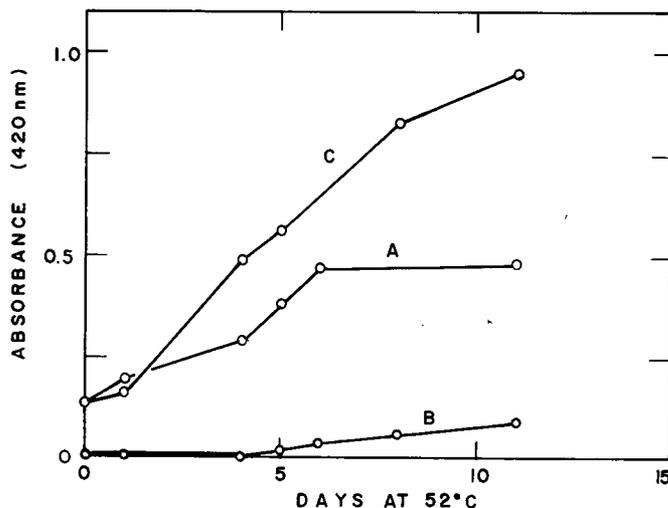
brown ceases and, in fact, the absorbance may decrease slightly.

Practical applications: Caputi and Peterson (5) did not find good correlation between their accelerated browning test and wine browning on the shelf. That may very well be true of the test procedure suggested here. Nevertheless, the test does distinguish among wines with differing capacity to brown. As an example, Fig. 3 shows the browning of a Chardonnay wine and the same wine with 200 ppm of d-catechin added, as might be the case with increased pressing for example.

LITERATURE CITED

1. Berg, H. W. Varietal susceptibility of white wines to browning. II. Accelerated storage tests. Food Research 18: 407-10 (1953).

2. Wright, D. Factors affecting the color of dry vermouth. Am. J. Enol. Vitic. 11:30-34 (1960).

3. de Villiers, J. P. The control of browning of white table wines. Am. J. Enol. Vitic. 12:25-30 (1961).

4. Fuller, W. L., and H. W. Berg. Treatment of white wine with Nylon 66. Am. J. Enol. Vitic. 16:212-18 (1965).

5. Caputi, A., Jr., and R. G. Peterson. The browning problem in wines. Am. J. Enol. Vitic. 16:9-13 (1965).

6. Peterson, R. G., and A. Caputi, Jr. The browning problem in wines. II. Ion exchange effects. Am. J. Enol. Vitic. 18:105-12 (1967).

7. du Plessis, C. S., and A. L. Uys. Browning in white wines. II. The effect of cultivar, fermentation, husk, seed, and stem contact upon browning. S. Afr. J. Agric. Sci. 11:637-48 (1968).

8. Sapis, J. C., and P. Ribéreau-Gayon. Etude de brunissement des vins blancs. I. Transformation des composés phénoliques au cours du brunissement. Connaissance Vigne Vin 2:323-48 (1968).

9. Peri, C., C. Pompei, G. Montedoro, and C. Cantarelli. Maderization of white wines. I. Influence of pressing of the grapes on the susceptibility to oxidative browning. J. Sci. Food Agric. 22:24-8 (1971).

10. Berg, H. W., and M. Akiyoshi. Some factors involved in browning of white wines. Am. J. Enol. 7:1-7 (1956).

The Phenolic Cinnamates of White Grapes and Wine

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An isolation procedure was developed for the recovery of the cinnamate derivatives from Müller–Thurgau white wine by means of adsorption on charcoal, elution with acidic methanol and repeated chromatography on Sephadex LH-20 with dilute acetic acid as the eluent. The crystalline products recovered were *cis*- and *trans-p*-coumaroyl-(+)-tartaric (coutaric) acid and *trans*-caffeoyl-(+)-tartaric (caftaric) acid, as shown by spectral data, hydrolysis, chromatography, etc. The corresponding quinates were not detected and neither were ferulic acid derivatives nor the free cinnamic acids in this young white wine. The data are briefly discussed in relation to previous reports on these compounds in grapes or wines and their significance as wine constituents.

1. Introduction

Ribereau-Gayon[1] reported that the leaves, grape skins and red wine from the *Vitis vinifera* grape var. Cabernet Sauvignon contained caffeoyl-, *p*-coumaroyl- and feruloyl-tartaric acids as the phenolic cinnamoyl derivatives. Previous workers, as well as some later ones, had identified the grape compounds as the chlorogenic analogues with quinic rather than tartaric acid as the non-phenolic moiety.[2]

It was considered desirable for several reasons to reinvestigate this group of compounds from grapes. The original identification[1] of the tartaric derivatives was solely by paper chromatography without actual isolation of significant amounts of the compounds. Only one variety was studied and the samples examined were extracts of the solid tissues (leaves, berry skin and red wine fermented on the skins). It has been shown subsequently[3] that the non-flavonoid phenolics of grapes are largely confined to the freely expressed juice. They therefore constitute a much higher proportion of the total phenols of white wines than of red and the cinnamates appear to be the major class of phenols in grape juice and white wines made without pomace extraction.[2,4] Furthermore, the concentration of potassium hydrogen tartrate and the pH are known to be much higher in the grape skin as compared to the freely expressed juice of the berry centre, and migration of acyl groups[5] might produce artefacts or normal biochemistry might allow both types (quinic and tartaric) of phenolic esters to be present.

In consequence the objectives of this research were to isolate the cinnamate derivatives from white wine or juice and determine their number and nature.

2. Experimental

2.1. Wine processing

Müller–Thurgau grapes grown in the experimental vineyard of this research station were harvested at 12.8° Brix on 8 October 1976. The juice was expressed and fermented by standard methods to give a dry white table wine. For other research, the wine was passed through a very large column of Sephadex LH-20 in a displacement development mode. The column eluate was monitored by its

ultraviolet absorbance. Eluate absorbing within the range 300–330 nm indicative of cinnamates was collected until the more strongly bound flavonoid phenolics (280 nm) began to break through. The wine collected up until this point (about 50 gallons) was fully aromatic and was essentially a normal table wine of good flavour (Riesling type) minus the flavonoids and condensed tannins which were retained on the Sephadex LH-20. It provided the starting material for isolation of the cinnamate substances. Subsequent tests indicated that fresh juice or white wine without pretreatment behaved similarly in the isolation procedure.

2.2. Isolation of cinnamates

The best results were obtained by adsorption on activated charcoal[6] (BDH decolourising powder, product 33032 lot 1825670).

After preliminary tests, batches of the wine were stirred for 30 min with charcoal plus an equal amount of Celite 545 diatomaceous earth and were filtered on a large Buchner funnel. The cake was washed three times with small amounts of water and extracted by stirring for 30 min with methanol, containing 1 % acetic acid using 20 ml g^{-1} charcoal. The extraction was repeated at least twice. Efforts were made to prevent air from drawing through the charcoal in order to minimise charcoal-catalysed oxidation.[7] The charcoal could be re-used.

The methanolic extracts were combined, concentrated *in vacuo* $< 35°C$ to a syrup, water was added and concentration was continued until the residue was free of acetic acid odour. The concentrated eluates, generally representing 10 litres of wine were added to a column of Sephadex LH-20 (6 g dry wt swelled in water, packed by gravity flow in a 13 mm diameter glass column to give a void volume of 9.7 ml) and developed with water. Fractions were collected, their u.v. absorbance spectrum determined and absorbance recorded at 310 and 275 nm. The maximum absorbance was at first near 275, then near 310 and finally again near 275 nm after about 20 V_o. The column was washed with methanol then with water, in preparation for re-use.

The fractions from different runs, eluted from the LH-20 column by water and shown to be similar by absorption maxima, retention volume and paper chromatography (2 % acetic acid on Whatman No. 1), were combined and rechromatographed on LH-20. Difficulties were encountered in peak shape, purity and reproducibility which were solved as indicated separately.[8] The best results were obtained when the samples were chromatographed in the dark to prevent *cis–trans* isomerism and eluted first with 1–2 void volumes of 0.05N HCl in 0.2 % HOAc in water to remove salt-forming bases or cations and then with HCl-free dilute acetic acid until the cinnamates had been collected. The column generally used was an LKB 4200 precision column 12×600 mm packed with 18 g of Sephadex LH-20 and a void volume of 21.5 ml. About 80 mg cinnamates, as estimated by chlorogenic acid extinction at maximum absorbance, was the most that could be chromatographed to give peaks without excessively flattened tops (overloading).

Column fractions were collected (in the dark as far as possible) in an LKB fraction collector with flow controlled by an LKB varioperpex 12 000 peristaltic pump (about 0.5 ml min^{-1}). Elution was monitored at 280 nm by an LKB Uvicord II 8300.

Because the method was being developed during this work, all fractions were chromatographed three and some five times on Sephadex LH-20 columns. Provided care is taken not to overload the columns, complete purification can be accomplished efficiently in not more than three sets of chromatographic runs. The first serves to separate the cinnamates from other substances, the second to separate and purify the cinnamates and the third separates the purified isomers following u.v. irradiation.

Spectra of the effluent fractions, 200–350 nm, were determined at appropriate dilutions in an SP800B Unicam spectrophotometer. Fractions from different runs corresponding to a peak and shown by spectrum, retention volume and paper chromatography to have the same single cinnamate were combined. Fractions containing more than one cinnamate were rechromatographed.

The purified fractions were concentrated in as little light as possible *in vacuo* at 35°C or less in a Büchi rotary evaporator to a syrup, water was added and concentration continued until free of acetic acid odour. The samples were seeded with crystals from preliminary runs and the syrup

was allowed to evaporate until the dry crystalline substance could be scraped from the flask. Some brown, contaminating material could be removed with a very small amount of cold water. Three crystalline, white or light tan products that represented single cinnamates were obtained on paper chromatography.

2.3. Hydrolysis

Complete hydrolysis was accomplished by refluxing some of each cinnamate ester in 1N-NaOH for 30 min in a stream of nitrogen. A portion of the hydrolysate was acidified strongly with HCl, extracted with ether and the extract chromatographed on Whatman No. 1 paper in toluene, acetic acid, water (4:1:5) solvent and in 2% aqueous acetic acid. R_F values were identical with caffeic and p-coumaric acids, as were fluorescence characteristics, caffeic acid (white) becoming more intense yellow in ammonia fumes and p-coumaric acid (faint) becoming dark bluish violet with ammonia. The unhydrolysed esters were similar to their respective cinnamic acids except that substance D (Figure 1) was more intensely bluish white in ammonia.
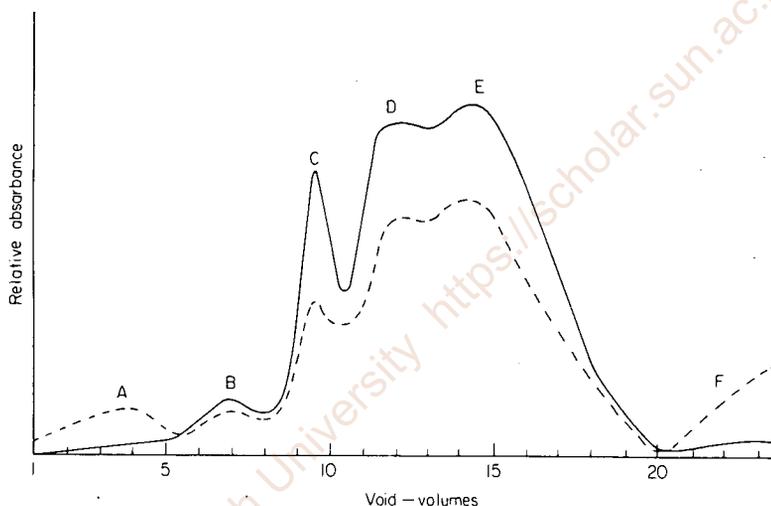
Figure 1. A typical chromatogram of grape phenolic cinnamates on Sephadex LH-20 developed with one void-volume of 0.05N-HCl and then 0.2% AcOH in water. The solid line indicates absorbance at 310 nm and the broken line absorbance at 275 nm.

A second portion of the hydrolysate was passed through a short column of Zerolit 225 SRC14 cation exchanger in the H^+ form and chromatographed on paper in n-propanol, concentrated NH_4OH (60/40%) with authentic tartaric and quinic acids.

3. Results and discussion

3.1. Charcoal adsorption and elution

The results of charcoal treatment of wine portions are given in Table 1. Adsorption of the cinnamates (absorption at 320 nm) was nearly quantitative, but not the elution. Selective rejection of cinnamates was possible, but considered unlikely in view of the similar chromatographic pattern obtained before and after charcoal treatment. The pattern was identical with that of the substances eventually isolated, viz. the *cis* and *trans* forms of p-coumaroyl- and caffeoyl-tartaric acids (for convenience in the rest of the paper these are called coutaric and caftaric acids).

Based upon the molar absorbance[9] of pure *trans*-chlorogenic acid, 19 200 litre mol^{-1} cm^{-1}, the base wine contained 102 mg of total cinnamates per litre or a total of 4.49 g. The combined eluates

Table 1. Adsorption and elution with charcoal

| Wine volume (litre) | Charcoal wt (g) | % of original wine absorbance | | | | No. of extractions |
| | | adsorbed | | eluted | | |
		280 nm	320 nm	280 nm	320 nm	
2.0	5.8	72	80	37	46	Chromatographic elution
2.0	5.8	74	80	*b*	35	4
2.0	5.8*a*	*b*	61	*b*	45	4
10.0	57	*b*	93	*b*	40	4
8.0	57*a*	*b*	88	*b*	61	4
10.0	65	*b*	95	*b*	23	3
10.0	122*a*	*b*	89	*b*	55	4

a Reused charcoal.
b Not determined.

from charcoal available for further processing contained about 1.93 g of total cinnamates on the same basis, after some losses were incurred by using samples for analysis, etc.

3.2. Chromatography on Sephadex LH-20

Variability was originally encountered in chromatography on Sephadex LH-20 of the concentrated, acetic acid-free eluates from charcoal. This is the subject of a separate communication[8] showing that continuing *trans-* to *cis*-isomerisation of the cinnamates in ambient light and on the glass column is part of the problem. The preferred procedure involved protecting the samples on and off the column from light, supplemented with deliberate u.v. isomerisation when appropriate in the final stages of purification. Initial unprotected LH-20 chromatograms indicated as many as seven partially-separated peaks which had u.v. spectra suggesting cinnamate content. However, when these peaks were individually separated and rechromatographed, multiple peaks resulted.

The peak ultimately identified as *trans*-caftaric acid was the last cinnamate eluted from LH-20 chromatograms representing fresh wine samples even when the eluent was changed to methanol or aqueous acetone and the Sephadex completely freed of adsorbed visible or u.v.-absorbing components. This was true in subsequent rechromatography unless samples had been left in aqueous solution under conditions allowing hydrolysis, in which case the cinnamic acid, e.g. caffeic, was produced and appeared as a separate peak elutable very slowly and well behind the cinnamate esters with dilute (0.2%) acetic acid, or more rapidly (but still behind the esters) with dilute methanol. Since free cinnamic acid derivatives were found after but not before hydrolysis was encouraged, it again appears that the natural condition in grapes, as expected from other plants, is the absence of the free cinnamates in favour of the acyl forms and this remains true in fresh, cool-fermented, white wines.

The preferred LH-20 chromatographic procedure has been described in section 2.2. A representative chromatogram is shown in Figure 1. All the carbon eluates were so processed or reprocessed and the fractions shown to be identical by spectra, retention volume, and paper chromatography were combined. Overlapping fractions with more than one cinnamate were rechromatographed. The relative retention volumes indicated in Figure 1 are somewhat subject to the absolute and relative amounts present. With smaller amounts the separation between Peaks C and D reached baseline and the amount generally used for best resolution was equivalent to about 4 mg of total cinnamates estimated from u.v. absorption per g of dry Sephadex LH-20 employed. Adequate separation could usually be obtained with about twice this amount.

Fractions equivalent to Peaks A and F (Figure 1) were free of shoulders or maxima in the 305–325 nm region and were therefore free of cinnamates (as also indicated by paper chromatograms, etc.). However, such fractions had some end absorbance in the 305–325 nm region and would have

contributed to the apparent cinnamate content of the wine. The spectra of fractions A suggested nucleic acid derivatives and fractions F contained gallic acid as indicated by paper chromatography, spectra and other tests. These 'contaminants' can be greatly reduced by preliminary chromatography of the crude charcoal eluates on relatively small, heavily loaded columns of Sephadex LH-20.

Combined fractions equivalent to B, C, D or E of Figure 1 were rechromatographed until separation was complete. Fractions B were present only in relatively small amounts, particularly after the use of an initial portion of dilute HCl. When rechromatographed, the B fractions diminished and yielded further portions of C, D and E plus fractions resembling nucleic acids in spectra, suggesting labile complexes or salts not completely converted to the free tartaric acid ester. Fractions C, D and E were crystallised. All three substances were very acidic without other obvious tastes in the very small amounts sampled.

3.3. Chemical composition

Partial hydrolysis indicated only one cinnamate moiety and one acid moiety in each substance (C, D, E above). After complete hydrolysis, *p*-coumaric was identified in C and D, and caffeic acid in E.

The acid moiety in all three substances was identified as tartaric acid, and quinic acid was shown to be absent by paper chromatographic comparison with the known substances in n-propanol: concentrated NH_4OH (60/40%) and by reaction with *meta*-vanadate to give an orange spot for tartaric acid; reagents specific for quinic acid gave no reaction. Interestingly, the unhydrolysed tartrate esters did not give the characteristic tartaric-*meta*-vanadate reaction. From these data it follows that C and D were *p*-coumaroyl tartaric acids and E was caffeoyl tartaric acid.

3.4. Data regarding *cis-trans* forms

Since C and D were both *p*-coumaroyl tartrates the difference between them required explanation. U.v. absorbance per unit weight was less for C than for D.[9] The molar absorbance precluded the presence of two units of *p*-coumaric acid per unit of tartaric acid in either. C was faster moving on paper in 2% acetic acid than D and, when irradiated by u.v. in solution, C or D became an equilibrium mixture of C and D capable of again being separated by chromatography in the dark on Sephadex LH-20. Thus, crystalline C was *cis*- and D was *trans*-coutaric acid.

The caftaric acid represented by Peak E of Figure 1 is the *trans*-isomer, since it moved more slowly on Whatman No. 1 paper in 2% acetic acid than the isomer produced by its irradiation. Furthermore, at equilibrium there was considerably more of the E(*trans*)-form than the *cis*-form of caftaric acid, which is consistent with other studies.[9–11] That the proportion of coutaric acid in the *cis*-form at equilibrium is much higher than of caftaric acid is consistent also with studies with other cinnamates.[10,11] At pH 3.5 *p*-coumaric acid is 62% *cis* at equilibrium but caffeic is 21% *cis*.[11]

The *cis*-caftaric acid was not evident in chromatograms such as Figure 1. It was hidden in the later portion of the much larger amount of *cis*-coutaric acid represented by Peak C. *Cis*- or *trans*-coutaric acid free of any caftaric acid or incidental contaminants, were prepared by u.v. irradiation of a solution of the one form and rechromatography in the dark on Sephadex LH-20 as before to isolate the opposite isomer in its pure form. Pure *cis*-caftaric acid was similarly produced from the pure *trans*-form, but not yet in sufficient amount for separate study.

Since the order of elution from the Sephadex was *cis*-coutaric, *cis*-caftaric, *trans*-coutaric and *trans*-caftaric, mobility on Sephadex is clearly more affected by geometry than by an additional phenolic OH, as has also been shown for cinnamates of quinic acid.[8] Feruloyl tartrates, had they been present, would have complicated the separation further, since they should display intermediate adsorption characteristics. If isomerisation is allowed to occur on the column,[8] complete purification becomes impossible by this technique. Furthermore, the protection of grape and wine samples from light is seen to be important to simplify the chromatograms by retaining the initial *trans*-forms and is essential if it is desired to assess the degree of previous exposure to light or to study the effects of processing.

We assume that the natural form of both coutaric and caftaric acids is *trans* and that the *cis*-isomer arises in wine on exposure to light. A few hours in a bare test tube on a cloudy winter day

or 30 min under an u.v. lamp cause equilibration. The evident ease with which light caused isomerism may explain difficulties not only in isolating and identifying these components, but also in obtaining exactly reproducible chromatograms of sequential samples of the same wine when 'wine-profiling' has been attempted by the method of Somers and Ziemelis.[12]

3.5. Preliminary characterisations

Since additional studies are planned on the characterisation of these compounds, only preliminary data are given here.

3.5.1. Mass spectra

In mass spectral studies (160°C probe, 1.8 e volts), *cis*- and *trans*-coutaric acids gave weak molecular ions at *m/e* 296. Caftaric acid did not give a satisfactory peak at *m/e* 312, but showed a peak corresponding to the loss of a unit of water at *m/e* 294; the coutaric acids gave one at *m/e* 278. These results were consistent with parallel studies with the corresponding quinic acid derivatives, as were the more intense peaks at *m/e* 164, 147 and 119 for the coutaric acids and 180, 163 and 135 for *trans*-caftaric acid.

3.5.2. Spectra

The absorbance spectra in water of *cis*- and *trans*-coutaric acid and their change with isomerisation are shown in Figure 2. Maxima are at 306 nm for *cis*- and 311 nm for *trans*-coutaric acid. The peaks at 209 and 225 nm are about equally intense for the *trans*-isomer, but the 225 nm absorbance is reduced to a shoulder for the *cis*-isomer. Isosbestic points occur at 220, 240 and about 265 and 350 nm.
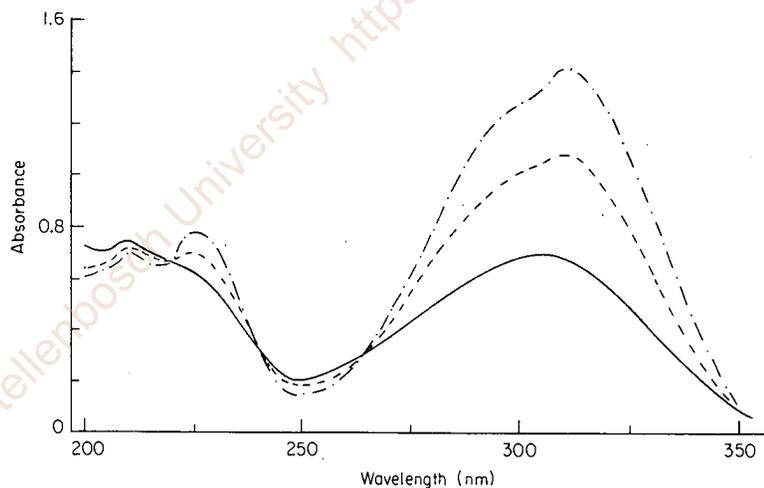
Figure 2. Absorption spectra of coutaric acid in water at about 2 mg/100 ml: ——, *cis*-; —·—, *trans*-; ——— *cis–trans* equilibrium mixture.

Table 2 summarises some of the spectral and optical rotation data obtained. The spectral data agree rather well with expectations based on chlorogenic acid and *p*-coumaroylquinate.[9–11] Kahnt[10] reports the extinctions at the longest wavelength maxima for the *cis*-isomers as 50–55% of those for the *trans*-forms; Table 2 indicates 63% for the coutaric pair. He reports $\epsilon = 18\,600$ litre mol^{-1} cm^{-1} for *trans*-chlorogenic acid in 50% ethanol; halfway between the water and ethanol values for *trans*-caftaric acid (Table 2) is $\epsilon = 18\,300$. He also reports higher molar absorptivity at the maxima for the *trans*-form of *p*-coumaric compared to caffeic acid derivatives, the ratio being about the same as found here for the coutaric and caftaric acids.

Table 2. Ultraviolet spectral features and optical rotations of coutaric and caftaric acids

	Absorbance					
	Water		Ethanol		Ethanol	
					Water	Specific rotation $[\alpha]_D^{20}$ (2, H_2O)
	λ_{max} (nm)	ϵ_{max} (litre mol^{-1} cm^{-1})	λ_{max} (nm)	ϵ_{max} (litre mol^{-1} cm^{-1})		
cis-coutaric acid	306	11 800	313	16 000	1.35	+24.4°
	209	12 600				
trans-coutaric acid	311	18 700	315	25 100	1.35	−29.7°
	225	10 800	228	14 000	1.30	
	209	10 600				
trans-caftaric acid	325	16 000	332	20 600	1.29	−30.3°
	217	13 600	220	17 600	1.30	

3.5.3. Optical rotation

The optical rotation sign difference between the *cis*- and *trans*-coutaric acids was surprising (Table 2). The optical form of the tartaric acid present in the cinnamoyltartaric acids was reported by Ribereau-Gayon[1] to be the 'natural' L-(+)-tartaric acid expected in grapes based upon precipitation of the calcium racemate when (−)-tartaric acid and calcium ions were added to the hydrolysate from the cinnamate esters. Scarpati and D'Amico[13] reported a specific rotation of +37° for, presumably, the *trans*-isomer of their caftaric acid from chicory and −12.6° for the tartaric acid portion. Scarpati and Oriente[14] had also identified the 'unnatural' (−)-tartaric in dicaffeoyltartaric acid with a specific rotation in methanol of +383.5° isolated from the same plant. They synthesised the analogous dicaffeoyl compounds from (−)-, (+)- and *meso*-tartaric acids and reported specific rotations of +384.6°, −384.2° and 0° in methanol respectively. Thus, their natural product was shown to involve the 'unnatural' D(−)-tartaric acid. The same dicaffeoyl-(−)-tartaric acid has been isolated from endive and lettuce with +374° specific rotation.[15] Since their monocaffeoyltartaric acid had a specific rotation of +37° and ours −30.3°, we conclude in agreement with Ribereau-Gayon[1] that grape coutaric and caftaric acid contain the natural grape (+)-tartaric. The only other reported isolation of cinnamoyltartrates is coutaric acid from spinach leaves.[16] It contained *meso*-tartaric acid and was found as the monoammonium salt.

3.6. Wine and grape significance

The totals isolated and recovered in crystalline form were 380 mg *cis*-coutaric, 620 mg *trans*-coutaric and 680 mg *trans*-caftaric acids. The total absorbance at 320 nm in the combined charcoal eluates and calculated as chlorogenic was 1.93 g. Recalculation as 40% *trans*-caftaric and 60% *trans*-coutaric from the molar absorptivities of Table 2 gives an original content of 1130 mg of total coutaric acids and 640 mg of total caftaric acids; values which are very close to the amounts isolated. Not only does this show that the processing and recovery were efficient, but it also indicates the absence of significant portions of other cinnamate derivatives such as the ferulates, free cinnamic acids or cinnamoylquinic acids.

The substances isolated are the major non-flavonoid phenols of grapes and wine and as such their contribution to wine flavour, oxidation, browning, etc. is potentially important.[2-4] The fact that chlorogenic acid analogues are replaced in grapes and wine by cinnamoyltartrates seems at first sight directly connected with the high tartaric acid content of grapes; nevertheless, coutaric and caftaric acids may have some special roles since they are present also in chicory, endive and spinach, plants not known for their tartaric acid content. Furthermore, the tartaric acids involved include all three stereoisomers, a specific one in each plant.

Ribéreau-Gayon[1] reported feruloyl tartaric acid in red grapes, which was not found by us in white grapes. In losing their ability to synthesise their highly methoxylated anthocyanins (wild grapes are red) white grape mutants may have also lost the ability to methylate caffeic to ferulic acid.

Acknowledgements

References

1. Ribéreau-Gayon, P. *C. r. hebd. Séance Acad. Sci., Paris* 1965, **260**, 341.
2. Singleton, V. L.; Esau, P. *Phenolic Substances in Grapes and Wine, and their Significance* 1969, Suppl. 1 Adv. Food Res. 282 pp., New York, Academic Press.
3. Kramling, T. E.; Singleton, V. L. *Am. J. Enol. Vitic.* 1969, **20**, 86.
4. Singleton, V. L.; Noble, A. C. In *Phenolic, Sulfur and Nitrogen Compounds in Food Flavors* 1976, Symp. Series No. 26, p. 47, (Charalambous, G.; Katz, I., Eds) American Chemical Society, Washington, D.C.
5. Haslam, E.; Makinson, G. K.; Naumann, M. O.; Cunningham, J. *J. Chem. Soc.* 1964, 2137.
6. Hulme, A. C. *Biochem. J.* 1953, **53**, 337.
7. Singleton, V. L.; Draper, D. E. *Am. J. Enol. Vitic.* 1962, **13**, 114.
8. Singleton, V. L.; Timberlake, C. F.; Whiting, G. C. *J. Chromat.* 1977, **140**, 120.
9. Whiting, G. C.; Coggins, R. A. *J. Sci. Fd Agric.* 1975, **26**, 1833.
10. Kahnt, G. *Biol. Zbl.* 1966, **85**, 545.
11. Kahnt, G. *Phytochemistry* 1967, 6, 755.
12. Somers, T. C.; Ziemelis, G. *J. Sci. Fd Agric.* 1972, **23**, 441.
13. Scarpati, M. L.; D'Amico, A. *Ricerca Scient.* 1960, **30**, 1746.
14. Scarpati, M. L.; Oriente, G. *Tetrahedron* 1958, **4**, 43.
15. Wöldecke, M.; Herrman, K. *Z. Naturforsch.* 1974, **29C**, 360.
16. Tadera, K.; Suzuki, Y.; Kawai, F.; Mitsuda, H. *Agric. Biol. Chem., Tokyo* 1970, **34**, 517.

OXIDATION OF WINES. I. YOUNG WHITE WINES PERIODICALLY EXPOSED TO AIR

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ABSTRACT

Chardonnay and Sauvignon blanc dry white table wines and a dry sherry from French Colombard plus Thompson Seedless were bottled in screw capped bottles holding 750 ml of wine with 25.0 ml of headspace and stored at 23.5°C (75°F). At weekly intervals the headspace air was replaced with the result that the wine was exposed to about 7 ml of oxygen per liter each week. Analyses of samples covering 10-12 weeks and oxygen exposure of 0 to 80 ml per liter were made. As oxygen exposure increased, fruitiness sensory rating and general quality decreased (for sherry it increased) and oxidation flavor rating increased. Pinking and browning spectral changes are discussed. Total phenol decreased 10-14 mg gallic acid equivalents per liter in 10 weeks exposure to oxygen and this decrease involved primarily the nonflavonoid components — not the flavonoids. Vicinal dihydroxyphenols accounted for only 27-54% of the total phenols oxidized in 10 weeks. Although decrease in total oxygen capacity as measured by uptake under alkaline conditions was only of the order of 4-6 ml over a 10-week exposure to oxygen, total exposure to 60 ml of O_2/l or so appears to be required to make the transition from table wine to sherry. Exposure to zero oxidation appears best for young white table wine quality although some indication of quality regeneration presumably with increased complexity at about 24 ml O_2/l exposure was found with the Sauvignon blanc samples.

Although more prevalent with respect to red than white table wines, there has been a belief that limited oxidation associated with aging can give improvement in wine quality. Appreciable oxidation of white table wine is, of course, a serious negative quality factor, but oxidation is an important positive factor in desirable sherry character. Work in this laboratory and elsewhere (3,6) has indicated that the primary oxidizable substrate in normal wines is the constituent phenols of the wine. Not all phenols of wine are equally autoxidizable nor do they contribute equally to browning or other quality effects. Autoxidation of the primary phenolic substrate leads to the coupled production of an active oxidant, probably hydrogen peroxide, which can then produce other oxidations (11). The experiments to be reported here were intended to help clarify the oxidation of young white wines with three major objectives: a) to analyze the wines for changes during oxidation in as many different ways as appeared likely to give useful information, b) to estimate whether or not limited oxidation gives improved wine quality, and c) to estimate the minimum oxidation to produce sherry character.

The wines studied were dry table wines from Chardonnay from a cool vineyard area, Sauvignon blanc from a warm area, and a shermat blended from French Colombard and Thompson Seedless from a warm area. Thus, it was hoped to represent the range of commercial white wine types with a minimum of samples.

MATERIALS AND METHODS

The wines were made by standard procedures in the University experimental winery with grapes from the University's experimental vineyards at Davis or at Oakville, Napa County. The must and wine analyses are indicated in Table 1. The musts for the dry white wines were clarified by settling overnight and racking prior to inoculation with Montrachet yeast. The shermat was not settled and was fermented dry and racked before fortification with high proof grape spirits.

The wines were settled, filtered, and then sterile filtered through E K (Seitz) pads directly into bottles. The bottles were a single lot of new screw capped metric bottles holding 752 ± 1 ml of wine with 25.0 ml headspace (based on actual trial with several bottles). The bottles were sterilized in a hot oven at about 80°C

49

for five hours. The caps, pump tubing, pipettes to be used were wrapped and autoclaved. The filter setup was sterilized by passing a 2000 ppm SO_2 solution through it followed by water and then the wine. The bottles were filled to the top and then exactly 25.0 ml of wine removed with a sterile pipette and discarded. The headspace for the control samples was flushed with nitrogen gas passed through a 0.45 μm pore membrane filter. The caps were applied taking care to maintain sterility. The capped bottles were dipped through about 1000 ppm SO_2 solution, allowed to dry and labeled. Enough bottles of each wine were prepared to allow four bottles to be removed each sampling period for an extended period of time.

Table 1. Analyses of experimental wines and their musts.

Grape variety	Vineyard	Must			Wine			
		°Brix	pH	Acid	Alcohol	Extract	Acid	pH
Chardonnay	Oakville	23.0	3.48	0.63	12.5	2.05	0.63	3.39
Sauvignon blanc	Davis	23.1	3.81	0.80	12.9	1.93	0.66	3.42
TS/FC Shermat	Davis	21.5	3.49	0.87	20.5	1.91	0.68	3.36

The control set (zero weeks) of each wine was removed to the cellar 13°C (55°F) and the remaining bottles were left in cases in an air conditioned room controlled at 23.5°C (75°F). The cases were tipped back and forth daily to equilibrate headspace oxygen and dissolved oxygen. At one week intervals each bottle remaining on test was opened, the headspace flushed with air passed through a sterile 0.45 μm pore membrane filter, and the cap replaced under aseptic conditions. Microbial contamination did not develop in any sample.

The headspace of 25.0 ml was calculated to contribute 6.7 ml of oxygen per liter of wine. Based on previous studies of oxygen uptake at wine pH, saturation levels of oxygen in wine, rate of consumption of oxygen, and oxidation-reduction potential lowering, it was estimated that 6-7 ml of oxygen per liter of wine (about the saturation solubility) would be consumed per week at room temperature. A set of four bottles of each wine were not aerated at each weekly sampling date and were left an additional week at room temperature with shaking to ensure complete consumption of the available oxygen. The set of four was then removed to the cellar and held until the completion of the studied period (10-12 weeks of oxygen exposure).

As soon as all the sample sets had been collected, analysis was started. The bottles were opened as a group for each wine. Two bottles were used in sensory and chemical analysis with care being taken to use freshly opened samples. When brief storage was necessary, the wine was kept covered with N_2 either in the original bottle or in capped small full sample bottles with care to avoid oxygen pickup during transfer. A third bottle of the set was used in instances requiring repeat of analytical values and the fourth bottle of each set held as a reference for later study.

Sensory analyses were conducted with a 10-member experienced wine judging panel. The wines were presented in wine glasses painted black on the outside to mask color differences. The wine types (i.e., dry white Chardonnay table wine, dry white Sauvignon blanc table wine, and dry white fortified Thompson Seedless-French Colombard blend) were identified to the panelist. All treatments (weeks of oxygen exposure) of a single wine type were presented at a sitting in random order and each set was repeated once at a subsequent sitting so that a total of 20 ratings were obtained for each wine sample. The panelists rated each sample (presented with a random two digit numerical code only) on a 1 = low to 10 = high scale for general quality, fruity aroma and oxidation flavor. Comments were also invited.

Spectral changes were monitored via standard spectrophotometry. The browning capacity was estimated by the accelerated browning test of Singleton and Kramling (7). Total phenol content was determined by Slinkard and Singleton's (8) automated version of the Singleton-Rossi (Folin-Ciocalteu) method. The flavonoid-nonflavonoid fractions were determined by a modification of the Kramling and Singleton (2) formaldehyde precipitation method. An addition was made of phloroglucinol (0.5 mg/20 ml final dilution) determined by experiment to be the minimum to yield a small amount of precipitate (removed by centrifugation and membrane filtration) even when the wine itself had too low flavonoid concentration to precipitate. The vicinal dihydroxyphenols were assayed by the phosphotungstic acid procedure of Behrman and Goswami (1) as further studied by Stern (10). This procedure determines the most readily oxidized phenols (both *ortho* and *para*-dihydric) in the presence of monophenols and *meta* diphenols. Since *p*-dihydric phenols are absent, or nearly so in wines, the values obtained are called vicinal (i.e. *ortho*-) diphenols and are calculated as gallic acid equivalents.

RESULTS AND DISCUSSION

The experiments can be criticized because the amount of oxidation observed is assumed to result from the complete consumption of the oxygen admitted to the headspace. This might not be true and no way to prove this was readily available to us. The oxidation under similar circumstances of a model solution of sulfite, for example, would have no assurance of matching a wine's reactivity. Indwelling oxygen electrodes, oxidation-reduction measurements, subsampling techniques all have serious experimental problems. Since the wine could take up *no more* oxygen than that admitted and the amount admitted should, based on previous experiments, be largely consumed in the time allotted, the values can be considered approximately correct and representing the *maximum* oxygen necessary to produce the observed changes.

The sterilization treatments and handling were successful in that the wines remained without any detectable microorganism growth throughout.

Spectral effects of oxidation: The spectra in the visible region were void of evident maxima, but the relative absorbances at 520 nm (red) and 420 nm (brown) were revealing. All wines visibly darkened as

oxidation proceeded. The Sauvignon blanc wine alone became faintly pink to the eye and this was verified by the absorbance of the undiluted wine at 520 nm (Fig. 1). This was true pinking and not just increased browning because the 420 nm absorbance was very similar for all three wines and did not increase more for the Sauvignon blanc samples.
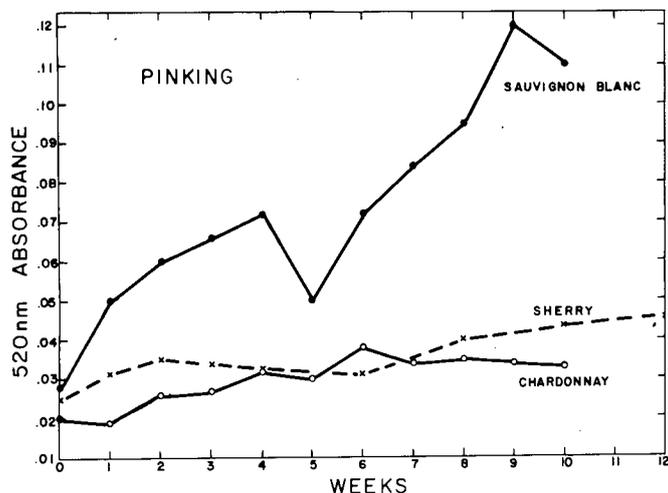

Fig. 1. Absorbance at 520 nm (pinking) of two dry table wines and a shermat upon weekly exposure to 25.0 ml of fresh headspace air per 750 ml bottle.

While pinking of white wines was not an intended part of this study, two interesting conclusions can be made from the results. Pinking of white-grape wine as previously indicated (4,5) involves oxidation of a colorless precursor to an anthocyanin-like form. However, the degree of oxidation required for complete conversion of the precursor is much more than the minimal contact previously suggested. In this case, of the order of nine weeks with a total exposure of 60 ml of oxygen per liter of wine was apparently required for complete pinking. Observations of subsequent Sauvignon blanc samples (not shown here) indicated decreasing pink and reversion to simple brown color after the tenth week. The second notable point is that the fifth week Sauvignon blanc sample which appears anomalously low in pink color was anomalously high in brown color (Fig. 2) suggesting that pinking and browning during oxidation are somewhat competitive. This has been previously suggested as the reason that pinking only became a problem as white wines were stored in stainless steel and better protected from air (5). The amount of red pigment produced was very small. Estimation from absorbance indicated less than 3 mg anthocyanin per liter, roughly equivalent to a 20 fold dilution or more of a rosé wine.

As expected, increasing oxygen exposure increased the brown color of all three wines (Fig. 2). The data were best fitted by linear regression equations. The intercepts were similar in all wines and the slopes of the sherry and Sauvignon blanc were identical. The correlation coefficients were 0.62, 0.65, 0.88 respectively for the Sauvignon blanc, Chardonnay and sherry and were significant in all cases.
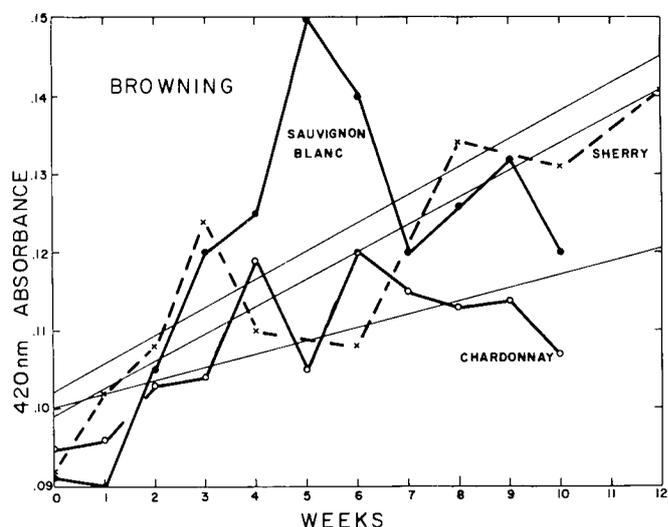
Fig. 2. Absorbance at 420 nm (browning) of two dry table wines and a shermat (with regression lines) upon weekly exposure to 25.0 ml of fresh headspace air per 750 ml bottle.

The browning capacity was similar in all three wines. However, it was not systematically affected by oxidation in these experiments as shown by variable regression and low, not significant, correlation in the different sets. The mean and standard deviation of the 420 nm absorbance produced in Singleton and Kramling's (7) test for browning capacity was 0.251 ± 0.032 for the Chardonnay, 0.264 ± 0.035 for the Sauvignon blanc and 0.278 ± 0.021 for the sherry. In each wine the initial brown was about 33% and the final about 50% of the value indicated by the browning capacity test. This shows that the brown color produced in 10-12 weeks of renewed exposure to air was not the maximum possible as also indicated by the visual appearance of the wines relative to highly oxidized wines. More importantly these data strongly suggest that the browning under the heated accelerated test and that under the prolonged room temperature exposure were similar in that they operated on the same precursors, approached similar end points, and partial browning represented consumption of a portion of the available precursors of each wine.

The ultraviolet spectra of all samples showed a maximum near 270 nm and a shoulder at 320 nm. The averages for each set of the absorbance (times dilution, 1 cm cells) at 320 nm and at the maximum with the wave length of the maximum were for the Chardonnay 4.98, 8.26, 274 nm; for the Sauvignon blanc 3.81, 7.03, 265 nm; and for the sherry 3.11, 6.81, 268 nm. The absorbance at 320 nm increased during the oxidation 3.6-5.9% and at the maximum near 270 nm 2.2-2.6%. These small apparent increases were not the result of evaporation because the loss in wine by evaporation during flushing of the headspace with fresh air once per week for 23 weeks was 0.2 ml per bottle of 750 ml of wine or 0.03%.

The upward trend of the 320 nm absorbance with oxidation exposure was significant with correlation coefficients of 0.74 and 0.68 for the Chardonnay and

sherry sets, respectively, but was not significant, 0.23 correlation coefficient, for the Sauvignon blanc. Correlation coefficients between the absorbance at the maximum near 270 nm and weeks of oxidation ranged between 0.15 and 0.52, but were not significant. The absorbance at 320 nm is due to phenols in higher proportion, whereas the 270 nm peak in white wines is one-half or more from nucleic acid derivatives (9) not known to participate in oxidation of wines. Furthermore, as phenols are oxidized to quinones their absorbance increases in the 320 nm region.

Sensory effects, other than color, of oxidation: The wines were rated in black glasses to prevent color influencing the judges. Mean scores for the panel judgments are shown in Figs. 3, 4, and 5. With oxidation, general quality decreased significantly for the Chardonnay and highly significantly for the Sauvignon blanc, but increased very highly significantly for the sherry. The sherry was low in fruitiness and did not change significantly in this rating with treatment.

However, the Sauvignon blanc and the Chardonnay did decrease highly significantly and very highly significantly, respectively, in fruitiness. The sherry and the Sauvignon blanc significantly and the Chardonnay highly significantly increased in rating for oxidation flavor as air exposure continued.

The analyses of variance also showed for every wine and every category of rating very highly significant differences among tasters indicating the usual differences in the portion of the 1-10 scales employed by individual tasters. That tasters used different ranges on different days is indicated by differences among replicate tastings which also involved two separate bottles of each treatment. These differences were significant for Chardonnay and sherry in all ratings, but in none for Sauvignon blanc. Tasters, however, were in agreement as to the nature of the changes produced by oxidation and the bottle to bottle variation was evidently low as indicated by the lack of significant interactions tasters times treatments or treatments times replicate tastings. The one exception was the fruitiness rating for sherries where the small differences among treatments allowed more disagreement among the tasters.

With the sherry (Fig. 3) and Chardonnay (Fig. 4) the linear regression lines are plotted, but with the Sauvignon blanc (Fig. 5) the regression lines are omitted to emphasize apparent differences in the ratings of each sample. Note (Fig. 4) that the panel was in agreement that high fruitiness correlated closely and positively with general quality and that oxidized flavor ratings varied oppositely to fruitiness or general quality. At the acknowledged risk of inferring too much from the limited data, it appears that the Sauvignon blanc samples (Fig. 5) varied in a complex manner. Chardonnay and sherry responded (Figs. 3,4) more simply and this more uniform response lends support to the idea that the Sauvignon blanc sample differences are meaningful. Four weeks of exposure to oxygen (24-28 ml O_2/l) appeared to give increased quality

over more or less (but not zero) oxidation. This may be an indication of increased complexity and improved quality by limited oxidation. Of course, the value of limited oxidation for more robust (skin contact) white wines intended for more aging has not been tested by these experiments.
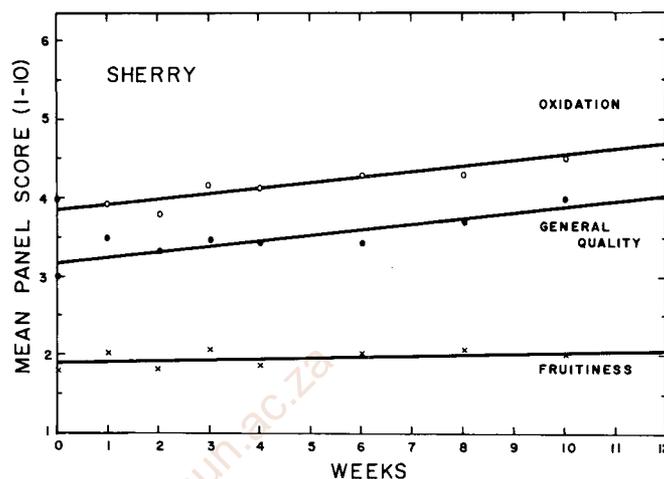
Fig. 3. Mean panel scores for general quality, fruitiness aroma, and oxidation flavor with linear regression lines for the shermat set upon weekly exposure to 25.0 ml of fresh headspace air per 750 ml bottle.
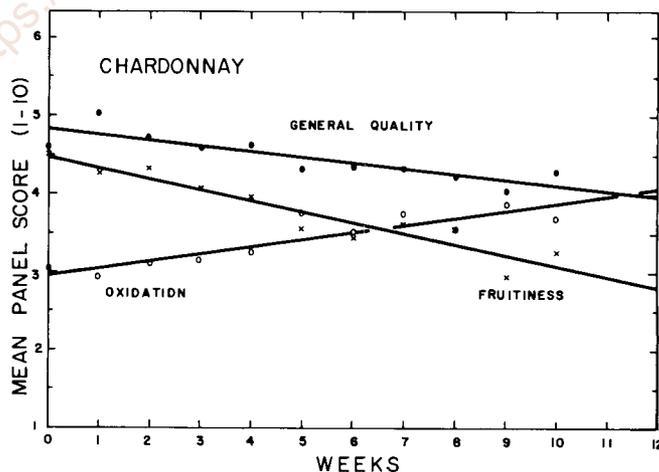
Fig. 4. Mean panel scores for general quality, fruitiness aroma, and oxidation flavor with linear regression lines for the Chardonnay set upon weekly exposure to 25.0 ml of fresh headspace air per 750 ml bottle.

Phenol composition and oxygen uptake: The data for the phenolic composition and oxygen uptake are given in Tables 2, 3, and 4 for, respectively the Chardonnay, Sauvignon blanc, and sherry sets. In each instance with increasing exposure to oxygen there is a significant decreasing trend in total phenol (respectively, r = 0.89, 0.72, 0.84), nonflavonoid (r = 0.76, 0.93, 0.73) and vicinal dihydroxy phenols (r = 0.72, 0.78, 0.87). The flavonoid content had no apparent trend for Chardonnay or sherry samples, but appeared to significantly increase in the Sauvignon blanc set as oxidation continued. This is believed to be because of

polymerization increasing the apparent flavonoid content by increasing the precipitability at the low levels in that wine. Similar effects should be operating in the other sets, but were too small to produce significant differences.
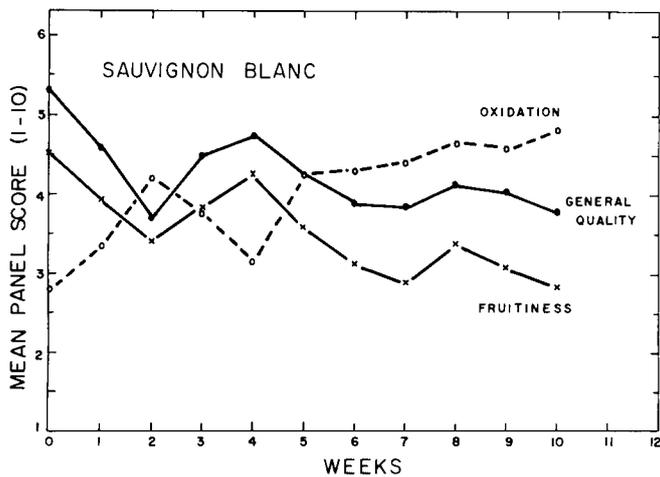
Fig. 5. Mean panel scores for general quality, fruitiness aroma, and oxidation flavor for the Sauvignon blanc set upon weekly exposure to 25.0 ml of fresh headspace air per 750 ml bottle.

Table 2. Chardonnay wine oxidation and phenol composition.

Oxygen exposure weeks	Phenol mg GAE/l				O₂ uptake (alkaline) cm³/l
	Total	Flavonoid	Nonflavonoid	Vicinal dihydroxy	
0	266	40	226	64	101
1	264	30	234	64	102
2	260	46	214	63	101
3	262	43	219	58	94
4	244	28	216	59	99
5	254	42	212	54	99
6	251	34	217	58	98
7	253	46	207	57	107
8	250	36	214	55	97
9	249	42	207	58	96
10	252	40	212	58	102

Table 3. Sauvignon blanc wine oxidation and phenol composition.

Oxygen exposure weeks	Phenol mg GAE/l				O₂ uptake (alkaline) cm³/l
	Total	Flavonoid	Nonflavonoid	Vicinal dihydroxy	
0	212	0	212	48	90
1	206	2	204	44	93
2	204	4	200	43	81
3	201	4	197	44	91
4	202	5	197	44	95
5	200	6	194	40	91
6	197	2	195	42	92
7	200	4	196	42	95
8	196	8	188	39	92
9	194	10	184	38	92
10	198	12	186	42	88

Because the flavonoid fraction was not decreased by oxidation the decrease in the total phenol content was due to the decrease in the nonflavonoid portion as

Table 4. Sherry oxidation and phenol composition.

Oxygen exposure weeks	Phenol mg GAE/l				O₂ uptake (alkaline) cm³/l
	Total	Flavonoid	Nonflavonoid	Vicinal dihydroxy	
0	288	20	268	38	106
1	279	25	254	37	113
2	278	24	254	36	111
3	277	24	253	37	109
4	277	26	251	35	110
6	276	20	256	36	110
8	272	22	250	35	111
10	274	24	250	35	107
12	271	25	246	34	112

shown in Tables 2, 3, and 4. The decrease in 10 weeks exposure to oxygen as taken from the linear regression equation was 13.9, 13.5, and 10.0 mg GAE/l total phenol and 18.3, 22.7, and 10.8 mg nonflavonoid respectively for the Chardonnay, Sauvignon blanc and sherry sets. The relatively constant oxidative consumption of 10-14 mg GAE of total phenol in these three wines during a 10-week period indicates that the conditions were comparable and the three wines behaved nearly identically. The fact that the amount of total phenol consumed by oxidation was inversely proportional to the total phenol level of the table wines suggests that with the lower total phenol the proportionate effect of regenerative polymerization becomes less and the substrate can become limiting. The apparently greater decrease in nonflavonoid content with oxidation than in the total was partly explained by the tendency of the apparent flavonoid content to increase at the expense of the nonflavonoid content, but is probably also due to the nature of the analysis. There is also greater possibility of additional oxidation and other losses occurring during the fairly prolonged precipitation and filtration steps of nonflavonoid estimation.

The vicinal dihydroxyphenol content, calculated as gallic acid, decreased in 10 weeks exposure to oxidation 7.3, 6.5, and 2.6 mg GAE/l respectively for the Chardonnay, Sauvignon blanc, and sherry sets based on the linear regression. The decrease in total phenol was 1.8, 2.1, and 3.8 mg per mg of vicinal diphenol (all as gallic acid). Thus, it appears that the total phenol decreases more than the consumption of vicinal diphenols and indicates that monophenols or nonvicinal polyphenols are being consumed during oxidation. Considering the three possibilities of accounting for high oxygen consumption, and high total phenol relative to diphenol: the polymerization of an *ortho*-quinone from a diphenol with a monophenol to regenerate the oxidizable substrate, the production of coupled oxidation (11), and hydroxyl insertion by such reactions as the Fenton reaction (6), these data seem reasonable. They also indicate the magnitude of such reactions overall. About 0.8-2.8 mg of total phenol oxidized in addition to direct oxidation of 1 mg GAE of vicinal diphenol, i.e., up to nearly three times as much total disappears as does identifiable vicinal diphenol.

Oxygen uptake capacity under alkaline conditions did not appear to decrease (Tables 2-4) as oxygen expo-

sure continued. Previous studies in this laboratory (10) have shown that this value does decrease in more severely oxidized wines. The total oxygen uptake under alkaline conditions divided by the total phenol content indicates in all cases about 0.4 cm^3 of O_2 is consumed per mg GAE total phenol. Thus, the typical consumption for a decrease of 10 mg GAE of total phenol in a liter of wine would be 4 ml of oxygen at standard temperature and pressure or too little to be proven reliably in these tests considering the variability in O_2-uptake determinations (Tables 2-4).

There is an apparent discrepancy between the indications that the wine has been exposed to and presumably consumed about 60 ml of O_2 (ambient conditions) per liter, and yet the indicated decrease in total phenol is only equivalent to about one-tenth of that amount of oxygen under alkaline uptake conditions. We believe that this apparent discrepancy is explained by the fact that when a phenol is oxidized to a quinone the quinone can dimerize with another phenol regenerating both total phenol and the oxidizable substrate. This process can continue as long as there are unsubstituted positions available for polymerization. Under alkaline conditions the oxidation reaction is rapidly taken to completion without time for much regenerative polymerization. Thus, total oxygen consumption over 10 weeks in pH 3.4 wine can be and evidently is much greater than in an alkaline wine oxidized rapidly to completion. Furthermore, the coupled oxidation of ethanol and other substrates (11) by the coproduced potent oxidant would be a considerable contributor to oxygen disappearance in the slow acidic wine oxidation, but probably a lesser contributor under rapid alkaline uptake. Therefore, a higher proportion of the total oxygen consumed would go directly to phenol oxidation in the alkaline assay.

Chemical versus sensory effects: The sensory estimations in some instances showed differences not clearly brought out by chemical analyses. As has often been noted before, the sensory panel was able to find consistent differences which, although in agreement with the chemical data, could not easily be replaced by the chemical data. However, several conclusions not evident from the sensory data are made clear by the chemical data. Further information on the nature of the pinking, browning, and phenolic substance participation in oxidation have been shown. One of the objectives of this work was to estimate at what point the transition from table wine flavor to sherry flavor occurred. This cannot be determined precisely. However, tentative conclusions seem possible. The quality of the sherry increased regularly with oxygen exposure (Fig. 3) approaching a rating of 4 out of 10 by 12 weeks. From these data and panelist's comments it is clear that by this time the wine was clearly sherry-like, but still was improving. The table wines started at standard or better quality and decreased to about a rating of 4 for general quality by 10 weeks of oxygen exposures (Figs. 4,5). By this time they were somewhat sherry-like in flavor and certainly defective in terms of brown color and oxidation flavor from a table wine viewpoint. As a rough estimate, then, it appears that exposure to 60-70 ml of oxygen per liter was required to produce the transition from a table wine with maximum "acceptable" oxidation to a sherry with minimum acceptable oxidation.

LITERATURE CITED

1. Behrman, E. J., and M. N. D. Goswami. Quantitative determination of o- and p-dihydric phenols in presence of monohydric phenols by use of phosphotungstic acid reagent. Anal. Chem. 36(11):2189-91 (1964).

2. Kramling, T. E., and V. L. Singleton. An estimate of the nonflavonoid phenols in wines. Am. J. Enol. Vitic. 20:86-92 (1969).

3. Rossi, J. A., Jr., and V. L. Singleton. Contributions of grape phenols to oxygen absorption and browning of wines. Am. J. Enol. Vitic. 17:231-9 (1966).

4. Simpson, R. F. Oxidative pinking in white wines. Vitis 16:286-94 (1977).

5. Singleton, V. L. Common plant phenols other than anthocyanins, contributions to coloration and discoloration. In: Chemistry of Plant Pigments, C. O. Chichester, ed., Adv. Food Res. Suppl. 3:143-91 (1972), Acad. Press, New York.

6. Singleton, V. L., and P. Esau. Phenolic substances in grapes and wine, and their significance. Adv. Food Res. Suppl. 1:282 p. (1969), Acad. Press, New York.

7. Singleton, V. L., and T. E. Kramling. Browning of white wines and an accelerated test for browning capacity. Am. J. Enol. Vitic. 27:157-60 (1976).

8. Slinkard, K., and V. L. Singleton. Total phenol analysis: automation and comparison with manual methods. Am. J. Enol. Vitic. 28:49-55 (1977).

9. Somers, T. C., and G. Ziemelis. Interpretations of ultraviolet absorption in white wines. J. Sci. Food Agric. 23:441-53 (1972).

10. Stern, P. M. Comparison of methods for determination of oxidizable substrates in wine, particularly phenols. M.S. thesis, Univ. Calif., Davis. 98 p. (1968).

11. Wildenradt, H. L., and V. L. Singleton. The production of aldehydes as a result of oxidation of polyphenolic compounds and its relation to wine aging. Am. J. Enol. Vitic. 25:119-26 (1974).

THE NONFLAVONOID PHENOLIC FRACTION OF WINE AND ITS ANALYSIS

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THE NONFLAVONOID PHENOLIC FRACTION OF WINE AND ITS ANALYSIS

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ABSTRACT

Chromatography of typical California white table wine on Sephadex G-25 indicated about 6% of the total phenol assayed by colorimetry with the Folin-Ciocalteu reagent was large protein-tannin complexes excluded from the gel and about 20% was flavonoid rather strongly absorbed and not eluted with 3% acetic acid. About 20% was caftaric (caffeoyl tartaric) acid and related hydroxycinnamates and other known wine phenols. Evidence is presented that about another 32% is tyrosol plus complexes involving hydroxycinnamates (320 nm absorbance) possibly with nucleic derivatives (260 nm absorbance). The remainder, about 20%, appears to include reaction products between oxidized phenols and sulfhydryl compounds. These substances retain fluorescence and other properties suggesting derivation from caftaric acid and related compounds via the action of polyphenol oxidase. The reaction of nucleic acid derivatives with the Folin-Ciocalteu reagent was investigated. Most of these compounds do not react appreciably, although purines with *meta* disubstitution of hydroxyl or amine groups did react equivalent to a monophenol (½ gallic acid's reactivity). The total nucleic acid content from previous analyses or by spectral calculation on wines tested here would account for only 10% or less of the apparent total phenol content. The validity of analyses for nonflavonoid phenols is considered strengthened.

The importance in grapes and wines of their phenolic components both as constituents and as reactants is well documented (5). It is also well known that some phenols have more color, more astringency, more bitterness, more browning capacity, or more oxidizability than others (5,6). In our laboratory as well as in several others methods are being sought and applied to selectively analyze for important classes of phenols (4). Kramling and Singleton (2) developed a method for selectively precipitating the flavonoid components and determining the phenol content before and after this treatment as total phenol, nonflavonoid and, by difference, flavonoid content. The method depends upon formaldehyde reaction limited in strongly acid solution to *meta*-polyhydroxyphenols such as the phloroglucinol A-rings of flavonoids without coprecipitation of mono or vicinal dihydroxyphenols (2,4). It has been used to show that the nonflavonoids are essentially confined to the juice and similar in red or white wine (2), that wood extraction during aging increases the nonflavonoid content of wine (8) and that browning capacity of white wines is greater when flavonoid content is higher (3,4).

The nonflavonoid fraction consists of the smaller phenols of grapes and wine notably the hydroxybenzoic and hydroxycinnamic acid derivatives. It also could include analytically, but can be corrected for by separate analysis, potentially interfering substances such as free sulfur dioxide, ascorbic acid, or ferrous ions. These interferences have been separately considered and in the usual grape must or wine situation do not interfere significantly or can be separately determined and subtracted. Somers (11), in the development of a gel-chromatographic method of characterizing wines, reported that a large portion of the total apparent phenol content of white wine by colorimetric assay was in fact not phenolic although this conclusion was partially based on the assumption that no phenols moving faster than vanillin on his columns were present in wine. The majority of the ultraviolet absorbance preceding vanillin off the column was from nucleic acid derivatives (11). It is recognized that flavonoids are held back strongly on such columns and small or very polar nonflavonoid phenols would be required to precede vanillin off the column unless they were so large as to be effectively excluded from the gel (10,11).

This consideration cast considerable doubt on the use of the nonflavonoid assay as a strictly phenol measurement. This research was initiated in an effort to clarify this situation.

MATERIALS AND METHODS

Nucleic acid interference in the Singleton-Rossi (7) (Folin-Ciocalteu) assay for total phenol: Various nucleic acid derivatives representing all natural structural variations and most of the specific substances reported in wines were obtained from commercial sources. Each was made to a solution of known concentration.

Aliquots representing a range of concentrations were reacted with the Folin-Ciocalteu reagent according to the method of Singleton-Rossi (7) in 100 ml total reaction volumes. After full color development at room temperature each was read in a Bausch and Lomb Spectronic 20 colorimeter at 765 nm.

The absorbance values were plotted against concentration for each nucleic acid derivative. From such plots the molar absorptivity of the color generated was calculated (4).

Gel column chromatography: As specified by Somers (11), Sephadex G-25, fine, was used and eluted with 3% aqueous acetic acid. The elution was continuously monitored and recorded at 280 nm with an ISCO UV analyzer. The column was 1.75 cm in diameter and the bed consisted of 7 g dry gel to give a void volume of 13.5 ml. Elution was by gravity and controlled with a Mariotte flask to give flow rates between 30-70 ml/hr. Sample sizes were ≤ 2 ml.

Authentic standards developed on the column were from commercial sources with the exception of tyrosol which was biosynthesized via yeast fermentation with tyrosine as the only nitrogen source. Its identity was confirmed by melting point, color tests, ultraviolet and infrared spectra.

Wines were prepared by standard University of California, Davis, experimental winemaking practice using University vineyard grapes. Most of the work was done with three different wines chosen to represent as wide a range as possible: Thompson Seedless bottle aged since 1965, 1977 dry Muscat of Alexandria, and 1977 dry white wine from Pinot noir. There were some differences in the relative amounts of the different individual peaks among wines, but the pattern and the contribution to the total from the different gross fractions was quite similar.

Wine samples were concentrated, under vacuum in a Büchi rotoevaporator at < 36°C, by a factor of 2.5 with 2 ml of concentrate being applied to the gel column. The elution profile was monitored and small volume fractions collected. Absorption spectra of the fractions were studied with a Bausch and Lomb 505 spectrophotometer. Where needed, two-dimensional paper chromatography of isolated peaks were carried out on Whatman #1, 10" x 10" precut paper. One dimension was developed ascendingly by BAW (n-BuOH:HOAc: H_2O, 4:1:5) and the other by 6% aqueous acetic acid. Spots were located either under UV (366 nm) light, with and without NH_3 fumes, or in some cases with ferric chloride-potassium ferrocyanide spray.

RESULTS AND DISCUSSION

Nucleic acid derivative interference in the total phenol assay: Table 1 is a listing of nucleic acid materials tested and their molar absorptivity produced with the Folin-Ciocalteu reagent. It contains representatives of all basic structural variations and most of the derivatives reported in wines.

It can be seen that a purine structure with a *meta*-di substitution of hydroxyls or amine groups (guanine, xanthine) appears essential to obtain a significant reaction. Such compounds react equivalent to a monophenol, which gives a molar absorptivity of 11,000-14,000. All other structures, pyrimidines and monosubstituted purines, give a negligible molar absorptivity. The table shows that the sugar moiety of a nucleoside or nucleotide decreases the reactivity considerably.

Tercelj (12) has reported the amounts of nucleic acid derivatives in wine. Based upon these reported concentrations and the molar absorptivities determined here, nucleic acid materials would contribute less than 5 mg of gallic acid equivalent per liter of any wine. This is within experimental error in white wines (200-250 GAE/l) and certainly negligible in a red wine (1,000 and up GAE/l). Thus according to the data available, nucleic acid materials would not significantly interfere with the Folin-Ciocalteu analysis of wines.

Gel column chromatography: Table 2 contains a list of standard phenolic constituents that were eluted on Sephadex G-25 and their elution behavior in terms of void volumes. Some of these compounds were not examined by Somers. He reports that no phenolic compound he tried eluted before 3.4 void volumes except tyrosine and vanillin. As can be seen tyrosol elutes much earlier than 3.4 void volumes and well into the area he considered nonphenolic. Vanillic and gallic acid elute after 3.4 void volumes, but their esters elute earlier than the free acid form and in the case of ethyl vanillate elution is well before 3.4 void volumes. Esters of other benzoic acids have been reported in wine and since the mechanism of adsorption is hydrogen bonding (9) the mobility of p-hydroxybenzoate and perhaps syringate esters should be faster than vanillate esters.

When wine samples are eluted from Sephadex G-25 and monitored at 280 nm, a wine profile is obtained. Fig. 1 shows typical wine profiles obtained by Somers and here. He reported peak 1 to be a complex of protein and tannin, thereby partially refuting his own argument that all materials eluting before 3.4 void volumes are nonphenolic. He reported Peak 5 contained derivatives of caffeic acid. As was noted in Table 2 authentic cinnamoyl tartrate esters isolated from grapes were found to elute in this region. The spectrum of caftaric (caffeoyl tartaric) acid matches that of fractions taken from this peak (Fig. 2) and the major spot obtained from two-dimensional paper chromatography of the same fractions supports the conclusion that peak 5 contains cinnamoyl tartrate esters.

Peaks 2, 3, and 4 are of major interest to this study since they constitute a large portion of the total gallic

Table 1. Nucleic acid derivatives tested and their molar absorptivity in "total phenol" colorimetry as used for wine.

PURINE BASES

DERIVATIVE	STRUCTURE OF BASIC MOIETY	MOLAR ABSORPTIVITY
ADENINE		207
ADENOSINE-DI-(P)	"	48
GUANINE		14160
GUANOSINE	"	300
GUANOSINE-TRI-(P)	"	351
HYPOXANTHINE		120
INOSINE-TRI-(P)	"	44
XANTHINE		10337
URIC ACID		8854
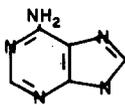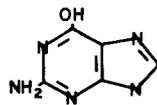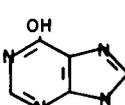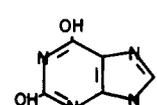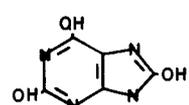
PYRIMIDINE BASES
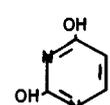
URACIL		17
URIDINE	"	9
URIDINE-TRI-(P)	"	55
THYMINE		12
CYTIDINE-TRI-(P)		10
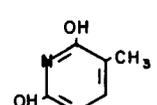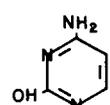
Table 2. Relative elution rate (in void volumes) of known phenols on Sephadex G-25 columns developed with dilute acetic acid.

Compound	Elution volume on this laboratory's G-25 column[a] (void vols)	Elution volume on Somers' G-25 column[b] (void vols)
L-Tyrosine	2.6	2.1
Tyrosol	2.7	
Ethyl vanillate	3.1	
Tryptophan	3.4	3.1
Vanillin	3.4	3.2
p-Coutaric acid - *cis*	3.6	
p-Coutaric acid - *trans*	3.7	
Vanillic acid	3.9	3.8
Caftaric acid - *trans*	4.2	
Methyl gallate	4.3	
p-Coumaric acid - *trans*	4.8	4.3
Gallic acid	5.5	
Potassium salt of coutaric acid - (H_2O eluted)	2.7	

[a]Eluant 3% HOAc, flow rate 15-40 ml/hr.
[b]Eluant 3% HOAc, flow rate 150 ml/hr.
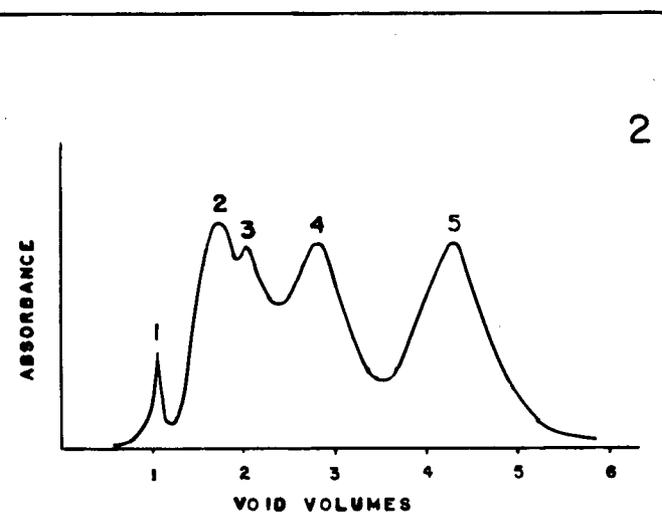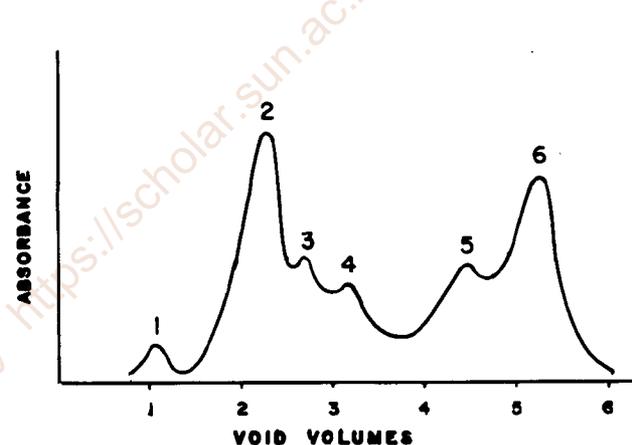
Fig. 1.Chromatographic elution profiles of representative wines from Sephadex G-25 as obtained by 1: Somers and Ziemelis (11) and 2: in this research.
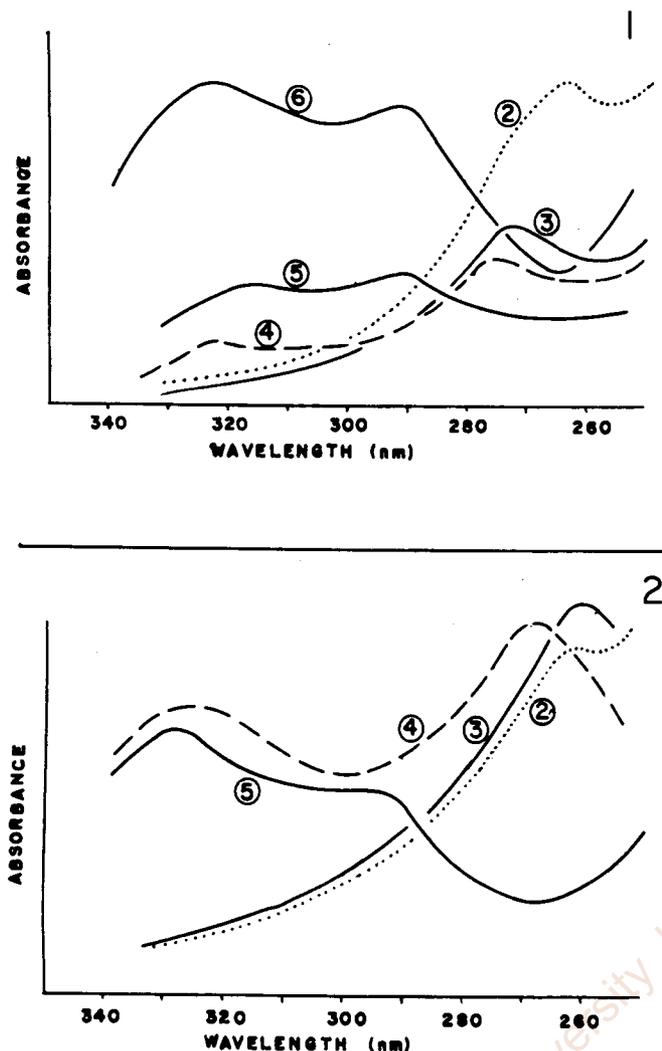
Fig. 2. Ultraviolet absorption spectra of the column fractions from Fig. 1 as obtained by 1: Somers and Ziemelis (11) and 2: in this research.

acid equivalent (GAE), yet were regarded as nonphenolic by Somers. All three peaks show a maximum absorbance between 260-270 nm. However, peak 4 has a significant secondary maximum at 320-325 nm which is indicative of cinnamate content. When fractions from this peak were chromatographed two dimensionally, a spot fluorescing yellow under 366 nm light was observed. This spot moved rapidly in 6% acetic acid (R$_f$ .86), but very little in BAW (R$_f$ .08) for which reason it was termed 'polar X'. The spot was reactive to ferric ferrocyanide spray. Eluted from the chromatogram it retained the spectrum of both 260 nm and 320 nm absorbance though the 320/260 ratio was diminished. Polar X fractions were highly reactive with the Folin-Ciocalteu reagent.

From this evidence it seems probable that polar X could be a complex with a cinnamate portion and a nucleic acid portion. A similar complex for similar reasons was postulated by Somers (11).

Peaks 2 and 3 are similar to each other in absorption spectra (Fig. 2). Because of the similarity between these two peaks and the incomplete separation they were combined for further investigation being referred

to as polar Y. Fractions from the polar Y region were positive to ferric chloride-ferrocyanide spray and highly reactive with Folin-Ciocalteu reagent. When polar Y was chromatographed two-dimensionally on paper, two fluorescent spots were resolved. One was similar to polar X, moving fast in 6% acetic acid and slow in BAW (R$_f$'s 0.95, 0.10). It was yellow fluorescing under 366 nm light, but when eluted from the chromatogram displayed only single peak absorption at 270 nm. The second polar Y spot was less polar (R$_f$'s BAW-0.47, 6% HOAc-0.78) and blue fluorescing with NH$_3$ fumes, but otherwise was colorless under 366 nm light.

The absorption spectra of the polar Y peaks initially seemed to rule out the possibility of cinnamate content, although benzoic acid derivatives could absorb in the area observed. However, Henze (1) in studying inhibition of enzymatic browning of chlorogenic acid solutions with cysteine and glutathione obtained sulfhydryl addition products with oxidized chlorogenic acid which had only 270-275 nm absorbance and not 320 nm. Henze's description of the products in terms of chromatographic behavior, spectra, and fluorescence correlate well with the fractions of polar Y. Thus the absence of 320 nm absorbance in the polar Y peaks does not preclude the presence of cinnamate derivatives.

This idea was pursued by determining if caftaric acid would form a similar addition product. A mixture of caftaric acid, cysteine and tyrosinase was incubated for four hours at pH 5. When the mixture was chromatographed a caftaric spot plus an additional fluorescent spot were resolved compared to the caftaric spot alone for the control consisting of the mixture with no enzyme. The new product was yellow fluorescing and slow moving in BAW (R$_f$ 0.15) which is as Henze described his. When the spot was eluted from the paper it showed absorbance at 252 nm and lower and at 320 nm which is not like Henze's. The product when eluted on a column of G-25 eluted at about 3 void volumes. Thus, while this specific product is not a prime candidate to account for the phenolic character of the polar Y fractions, there are many other sulfhydryl-cinnamate combinations possible. Both Henze's and this work suggest that related types of compounds may be present in the early eluting fractions, having arisen from the oxidative action of polyphenoloxidase during grape processing and winemaking or even from nonenzymic production of the quinone followed by reaction with the sulfhydryls.

Finally, the wine profile was analyzed for total phenols fraction by fraction so as to ascertain how the total GAE for the wine was distributed among the peaks. Table 3 is a balance sheet resulting from the analysis. Tyrosol and polar X are treated as a unit as tyrosol elutes on the forward edge of the polar X peak.

The balance sheet shows that, as Somers reported (8), about 60% of the total GAE of the wine elutes before the 3.4 void volume demarcation point. This would mean that only 40% of the GAE of this typical white wine would be phenolic if his allegation were correct that nothing ahead of 3.4 void volumes was phenolic. However, an additional 32% is polar X plus tyrosol and

6% is in the protein-tannin peak. All this is proven to be phenolic or highly believed to be phenolic based on the properties reported. The remaining 22% of the total GAE is contained in the polar Y peaks. This remainder can also possibly be accounted for in terms of phenolics in the form of either benzoic acid derivatives or, more likely, sulfhydryl addition products of oxidized cinnamates.

If, hypothetically, all the GAE of polar Y were due to nucleic acid materials, then for the two wines used here with an average of 190 mg GAE/l the polar Y peaks would contribute (190 x 22%) = 42 mg GAE/l. This value must be compared to the 5 mg GAE/l which nucleic acid derivatives were previously estimated to contribute.

Table 3. Summary of analyses of specific wine profile as percent of total gallic acid equivalent (GAE).

33%	Tyrosol plus "Polar X"
7%	Protein-tannin complex
23%	"Polar Y"
63%	Elutes from Sephadex G-25 before 3.4 void volumes
20%	Cinnamoyl tartrate esters (coutaric, caftaric acids)
83%	Recovered from Sephadex G-25 columns
17%	Flavonoid materials adsorbed and not recovered
100%	

Perhaps, there is eight times more nucleic material in the polar Y peaks than Tercelj's data would allow. To check this possibility an estimation of the maximum nucleic acid material present in the polar Y peaks was made based upon 260 nm absorbance. This was done through the use of an average (ϵ_m 10,000), an average MW (250), and Beers Law. By this method the polar Y peaks of a Muscat wine were estimated to contain 97 mg/l nucleics and a Ruby Cabernet 91 mg nucleics/l. Tercelj reported 119 mg nucleic/l for an intact Cabernet Sauvignon wine. Thus this estimation from the spectra supports Tercelj's reported value and eight times that value could not be present in the polar Y peaks. Based on this it was concluded that the amount of nucleic acid material that could potentially be present in the polar Y peaks could not account for the GAE apparent phenol contained therein.

Also of interest is the gallic acid equivalent distribution after the 3.4 void volume demarcation. As can be seen from the table approximately 20% of the wine's GAE is contained in the area of peak 5. This peak contains the cinnamoyl tartrate esters as mentioned before.

The remaining 20% of the GAE total for the wine could not be recovered from the Sephadex G-25 column. It has been reported that catechins and other flavonoids are irreversibly bound to Sephadex G-25 gels and not eluted by solvents tolerated by the adsorbent. Thus the difference between the total GAE for the wine and the GAE which was recovered from the column was attributed to flavonoids, mainly monomeric, but including any dimeric or larger condensed tannins present.

The data above concerning the GAE distribution in an average white wine of approximately 200 mg/l GAE are summarized in Table 4.

Table 4. Generalized balance sheet of the distribution of the gallic acid equivalent (GAE) in a total phenol analysis of a typical white wine with about 200 mg/l GAE.

Compound(s)	% of total GAE
Identified cinnamates (cinnamoyl tartrates; coutaric, caftaric acids)	20
Other cinnamates (complexes, "Polar X", see text)	20
Tyrosol	10
Tannin-protein complexes	5
Flavonoids (monomeric and larger)	20
"Polar Y" fraction (small phenols, cinnamate derivatives, see text)	20
Nucleic acid derivative interference	2.5
Other interference (free SO$_2$, ascorbic acid, etc.)	2.5
	100%

LITERATURE CITED

1. Henze, R. E. Inhibition of enzymatic browning of chlorogenic acid solutions with cysteine and glutathione. Science 123:1174-5 (1956).

2. Kramling, T. E., and V. L. Singleton. An estimate of the nonflavonoid phenols in wines. Am. J. Enol. Vitic. 20:86-92 (1969).

3. Singleton, V. L. Browning of wines. Die Wynboer (No. 445):13-14 (1969).

4. Singleton, V. L. Analytical fractionation of the phenolic substances of grapes and wine and some practical uses of such analyses. In: Chemistry of Winemaking, A. D. Webb, ed. Adv. Chem. 137:184-211 Am. Chem. Soc., Washington, D.C. (1974).

5. Singleton, V. L., and P. Esau. Phenolic substances in grapes and wine, and their significance. Adv. Food Research, Suppl. 1:282 p. Acad. Press, New York. (1969).

6. Singleton, V. L., and A. C. Noble. Wine flavor and phenolic substances. In: Phenolic, sulfur, and nitrogen compounds in food flavors, G. Charalambous and I. Katz, eds. Am. Chem. Soc. Symposium Series 26:47-70 (1976).

7. Singleton, V. L., and J. A. Rossi, Jr. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic. 16:144-58 (1965).

8. Singleton, V. L., A. Randopoulo Sullivan, and C. Kramer. An analysis of wine to indicate aging in wood or treatment with wood chips or tannic acid. Am. J. Enol. Vitic. 22:161-6 (1971).

9. Singleton, V. L., C. F. Timberlake, and G. C. Whiting. Chromatography of natural phenolic cinnamate derivatives on Sephadex LH-20 and G-25. J. Chromatogr. 140:120-4 (1977).

10. Singleton, V. L., C. F. Timberlake, and A. G. H. Lea. The phenolic cinnamates of white grapes and wine. J. Sci. Food Agric., 29:403-10 (1978).

11. Somers, T. C., and G. Ziemelis. Interpretations of ultraviolet absorption in white wines. J. Sci. Food Agric. 23:441-53 (1972).

12. Tercelj, D. Étude des composés azotés du vin. Ann. Technol. Agric. 14:307-19 (1965).

WHITE TABLE WINE QUALITY AND POLYPHENOL COMPOSITION AS AFFECTED BY MUST SO$_2$ CONTENT AND POMACE CONTACT TIME

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ABSTRACT

Dry white table wines were made from Chardonnay, Chenin blanc, French Colombard, and Semillon grapes following 0, 3, 6 and 12 hours holding of the destemmed, crushed grapes before juice separation and with additions at crushing of zero, low (50 mg/L), intermediate (100 mg/L) and high (200 mg/L) levels of SO$_2$. A fifth series, making 20 wines per variety, had zero added SO$_2$ and was bubbled with oxygen gas throughout holding.

These oxygenated musts browned severely, but when finished the wines tended to be slightly less brown, more resistant to further browning, less fruity, and lower in general quality than the comparable (zero SO$_2$) nonoxygenated wines. Deliberate oxidation of musts is concluded to be undesirable, even though it was possible to decrease the phenolic browning substrate in the wine by this procedure.

Added SO$_2$ was bound during fermentation, of course, and the free SO$_2$ in the wine was nearly the same whether SO$_2$ had been added or not. Furthermore, during pomace contact the total SO$_2$ was rapidly lowered. Increased pomace contact and increased SO$_2$ increased extraction of total phenol, specifically the flavonoid fraction, from the pomace into the wine and increased absorbance at 280 and 320 nm by the wines. Absorbance at 420 nm and the capacity to brown further were directly related to pomace contact and inversely to added SO$_2$. Increased pomace contact increased the wines' pinking upon exposure to oxygen and low levels of added SO$_2$ encouraged, while high levels discouraged, such pinking. Astringency or bitterness differences were not large or consistent enough to be meaningful except for increased bitterness at the highest level of pomace contact. Fruitiness and general quality were affected similarly, being improved by low to intermediate levels of SO$_2$ addition to the must and generally harmed by appreciable pomace contact. However, the optimum pomace contact appeared to be zero only for Chenin blanc and increased for the others in the order French Colombard, Chardonnay, and Semillon.

It is an ancient tenet of good winemaking that no more sulfur dioxide should be added than necessary. It is not practical to eliminate it entirely from wine because yeasts commonly manufacture some bisulfite in the course of reducing sulfate to produce essential sulfur-bearing cellular constituents such as cysteine and methionine (2,4). The desirability of minimizing the addition of SO$_2$ is periodically reemphasized in the technical literature and both the permitted and recommended levels have been lowered over the years (6,7) without universal agreement upon the optimum level under all circumstances. Suggestions for complete elimination of SO$_2$ additions have generally emanated from areas with a cool climate producing high acid, low pH, and slow ripening grapes from early varieties. Such elimination would appear much more risky for high final wine quality in warmer areas producing fully ripe grapes with higher pH, less acid,

greater tendency to brown, etc.

There has been also some disagreement upon the relative desirability of skin contact after crushing for white table wines. Some winemakers, at least for some wines, prefer immediate separation of the juice and its clarification so as to emphasize light, elegant, clean, fruity characteristics in the wine (9,12). Others favor some holding time before pressing to improve juice yield and to emphasize increased full, robust, complex, or varietal character in the wine (3,8).

Skin contact and SO$_2$ content interact (7) because, among other reasons, phenol extraction from the skin requires first the death of the cells and SO$_2$ can kill the cells as well as inhibit phenol oxidases, etc. Skin contact and/or SO$_2$ addition to white table wine musts have been studied by several groups before (3,8,9,12) with various objectives. For example, Ough (8) com-

14

pared wines from free-run juice and after skin contact up to 48 hours for many components and sensory quality in relation to concerns about mechanical harvesting. In the present study further answers were sought to the questions: 1) Is the phenol content of wine appreciably affected by SO_2 content during skin contact? 2) Can good wine be made from warm-region grapes without SO_2 additions? and 3) How does deliberate oxidation prior to fermentation affect white wine? Deliberate oxidation of the must has been claimed by a few winemakers to result in a white table wine that is resistant to browning and yet is of good quality.

MATERIALS AND METHODS

The grapes used were Semillon (U.C. vineyard, Davis, harvested August 30, 1978, at 19.5°Brix, 0.81% total acid as tartaric, pH 3.70), Chardonnay (U.C. vineyard, Oakville, September 5, 1978, 21.1°Brix, 0.78% total acid as tartaric, pH 3.45), Chenin blanc (U.C. vineyard, Davis, September 12, 1978, 21.0°Brix, 0.85% total acid as tartaric, pH 3.40, and French Colombard (U.C. vineyard, Davis, October 5, 1978, 21.4° Brix, 1.06% total acid as tartaric, pH 3.65). The fruit of each variety was destemmed and crushed into a single large container, mixed, and dipped in 4-L portions alternately into five sublots. Each sublot was treated individually with the appropriate level of SO_2 (zero, low, intermediate, and high — 0, 50, 100, and 200 mg/L) and mixed. The SO_2 additions were made as uniformly as possible using a standard aqueous solution of SO_2 dispensed with a 50-mL graduated cylinder. The fifth sublot (with zero added SO_2) was fitted with a glass tube to the bottom of the polyethylene holding tank and bubbled slowly, but continuously, with oxygen gas during the entire pomace contact period.

The grapes were tempered overnight or processed early in the morning in a room air conditioned at 24°C (75°F). Must temperatures were not actually controlled during the contact periods, but remained within a few degrees of this temperature. At 0, 3, 6, and 12 hours of pomace contact, each sublot was mixed and about 16 L removed. This portion was drained and lightly pressed (basket press) to yield about 8 L of juice. Each juice was settled overnight in a closed container in a 0°C (32°F) room. The relatively clear supernatant was then siphoned from the sediment and about 4 L was placed

in an 8-L bottle, inoculated with Montrachet yeast culture in sterile juice (20 mL/L), the headspace sparged with nitrogen, and a fermentation bung attached. The fermentation was conducted to dryness in a room maintained at about 16°C (60°F). The wine was then racked from the yeast lees and stored at 10°C (50°F) in full, stoppered bottles until tasted and analyzed beginning at least three months after the wines were fermented. The last analysis was that for total SO_2 and it was completed on April 4, 1979.

The subsamples for tasting and analysis were decanted clear from any sediment. The sensory panel consisted of 10 experienced judges. The wines were served one at a time in coded black glasses to prevent any visual differences influencing the judges. Panelists were told the variety of grape and asked to judge the wines as dry white table wines. Each wine was judged twice for a total of twenty ratings per wine for fruity aroma, astringency, and bitterness, each on a 1 (low) to 10 (high) arbitrary scale and general quality on a 1 (low) to 20 (high) U.C. Davis scorecard scale.

The absorbance at 280, 320, and 420 nm was determined and the wines were analyzed for total phenol, flavonoid, and free and total (Ripper) SO_2 by standard methods (1,10,14). Pinking was estimated visually on an arbitrary scale (where 2 = very faint and 10 = approximately rosé intensity) after the samples had been exposed in nearly empty bottles for three to five days. Browning capacity was estimated by accelerated browning under controlled conditions (11).

RESULTS AND DISCUSSION

Several different methods of presenting the data were examined. The trends were generally very similar with all four varieties. Normalizing the data by such mathematical treatments as converting all values within a varietal set to a percentage of one treatment's (usually 50 mg/L SO_2, zero pomace contact) value gave no useful improvement over simply averaging the data for all varieties for each treatment so that the latter approach has been used. Furthermore, presentation of the data for SO_2 level and pomace contact time together minimizes the number of figures required. Table 1 gives the values determined for the sample with 50 mg/L SO_2 and zero pomace contact time for

Table 1. Analytical characteristics of the samples receiving 50 mg/L SO$_2$ and zero pomace contact.				
Characteristic	Semillon	Chardonnay	Chenin blanc	French Colombard
Free SO$_2$, mg/L	16	9	5	11
Total SO$_2$, mg/L	43	46	33	40
Total phenol, mg GAE/L	240	241	224	328
Flavonoid, mg GAE/L	128	175	146	222
Nonflavonoid, mg GAE/L	112	66	78	106
280 nm, absorbance x diln.	6.9	6.3	5.8	10.5
320 nm, absorbance x diln.	3.5	4.0	4.0	4.7
420 nm, absorbance	.17	.14	.11	.19
Browning capacity	.62	.32	.30	.75
Fruitiness, mean rating (1-10)	5.6	3.4	4.0	4.0
Bitterness, mean rating (1-10)	3.3	3.5	2.6	2.6
Astringency, mean rating (1-10)	3.2	3.4	3.0	2.8
Gen. quality, mean rating (1-20)	12.6	11.6	12.4	11.6

each variety to indicate the degree of varietal similarity or difference and additional comments on individual varieties will be made as warranted. Standard statistical methods were applied to the sensory data.

The free sulfur dioxide found in the 32 wines which had received no addition of SO_2 ranged between 3.3 and 11.0 mg/L with an average of 7.2 mg/L. There was no consistent difference in these values whether or not the must was deliberately oxygenated. The Chenin blanc wines consistently had slightly less and the Semillon slightly more free SO_2 in these series than the other two varieties. The total SO_2 was slightly higher in 14 of the 16 instances in the oxygenated samples than the others receiving no SO_2 addition, averaging 15.8 mg/L for the oxygenated and 14.5 mg/L for the nonoxygenated. The production of SO_2-binding aldehyde during oxidation is predicted (15). The "varietal" differences were small in total SO_2 with a range among all values for wines without SO_2 added of 9.4-22.8 mg/L, but Semillon and French Colombard samples were slightly higher on the average than the other two varieties.

As expected, the free SO_2 for all 48 wines which had had SO_2 added was only slightly higher after fermentation than in those which had had none added, ranging from 3.7 to 20.7 mg/L. The average content of free SO_2 for all the samples receiving the low, intermediate and high dosage was 9.2, 11.3, and 12.3 mg/L, respectively. Similarly there was a slight decrease in free SO_2 with prolonged pomace contact averaging for 0, 3, 6, and 12 hours 12.8, 10.7, 10.2, and 9.5 mg/L, respectively.

The total SO_2 (Fig. 1) remained low and constant for the samples receiving none by addition. While those with added SO_2 remained higher in proportion to greater initial additions throughout the holding periods tested, there was a large decrease of total SO_2 with increasing holding time on the pomace. Evidently a considerable portion of the added SO_2 was lost, probably by oxidation to sulfate in the course of the prolonged pomace contact. The exposure to oxidation was undoubtedly more severe than would be the case commercially because of the more frequent dipping and mixing of the must. Based on the total SO_2 of the zero-time samples, the putative 50, 100, 200 mg/L SO_2 actually averaged 40, 140, and 240 mg/L.

The total phenol content is summarized in Fig. 2. Note the very regular increments with regard to both SO_2 and pomace contact at 3 to 12 hours. This increment in total phenol was 2.7 to 3.6 mg GAE (gallic acid equivalent) per liter per hour pomace contact, averaging 3.1 mg/L. The different varieties showed very similar analyses except the French Colombard samples were higher in total phenol than the other varieties. The increment in total phenol caused by raising the added SO_2 from zero to the low level averaged 6.7 mg GAE/L, to the intermediate level averaged 17.7 mg GAE/L, and to the high level averaged 34.0 mg GAE/L. Oxygenation decreased the total phenol 17 to 33 mg GAE/L, increased pomace contact increasing the loss.

The nonflavonoid fraction was slightly different by
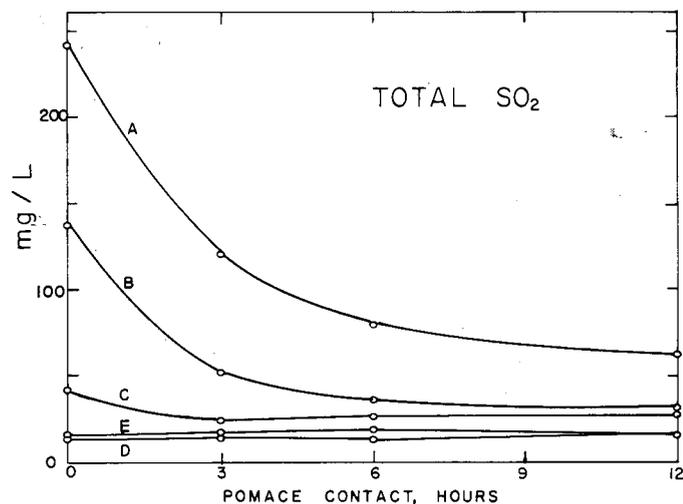
Fig. 1. Total SO_2 content (means for all four grape varieties) of wines prepared from musts with high (A), intermediate (B), low (C), zero (D,E) additions of SO_2 and oxygenated (E) after 0, 3, 6, or 12 hours of pomace contact.
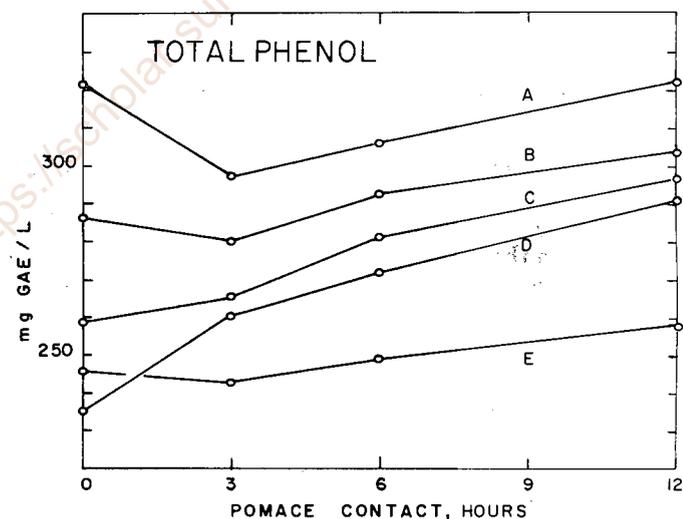

Fig. 2. Total phenol content (means for all four grape varieties) of wines prepared from musts with high (A), intermediate (B), low (C), zero (D,E) additions of SO_2 and oxygenated (E) after 0, 3, 6, or 12 hours of pomace contact.

variety; French Colombard > Semillon > Chenin blanc > Chardonnay. It was, however, nearly constant regardless of treatment as predicted (5) and therefore the variation in total phenol is explained by the variation in flavonoid extraction (Fig. 3). The fact that the zero time samples were systematically distorted in apparent phenol content for increasing total SO_2 is attributed to interference of high SO_2 levels in the phenol assay. While this could be corrected for, it was not done here to show the actual values obtained. The oxygenation decreased the total and flavonoid phenols considerably.

The absorbance of the appropriately diluted wines at 280 nm is another estimate of total phenols since many phenols and relatively few other components absorb maximally at that wavelength. As shown in **Fig.**
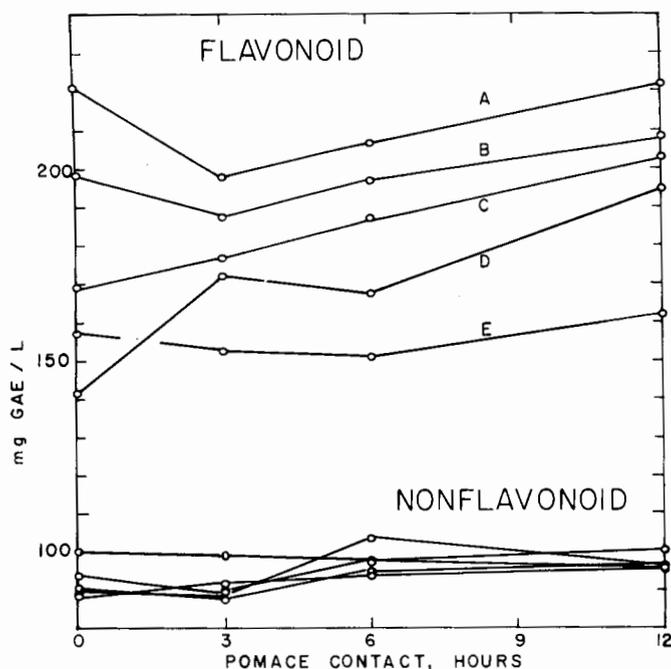
Fig. 3. Flavonoid and nonflavonoid content (means for all four grape varieties) of wines prepared from musts with high (A), intermediate (B), low (C), zero (D,E) additions of SO_2 and oxygenated (E) after 0, 3, 6, or 12 hours of pomace contact.


Fig. 4. Absorbance at 280 nm (times dilution) (means for all four grape varieties) of wines prepared from musts with high (A), intermediate (B), low (C), zero (D,E) additions of SO_2 and oxygenated (E) after 0, 3, 6, or 12 hours of pomace contact.


Fig. 5. Absorbance at 320 nm (times dilution) (means for all four grape varieties) of wines prepared from musts with high (A), intermediate (B), low (C), zero (D,E) additions of SO_2 and oxygenated (E) after 0, 3, 6, or 12 hours of pomace contact.

4, these data also indicate progressive increase with both increasing SO_2 and increasing pomace contact time. Note that the zero-time samples were consistent with the rest of the trends, thereby confirming that the colorimetric total phenol values for these samples were distorted by interference presumably by high SO_2 content. Note also that oxygenation greatly reduced the 280 nm absorbance (Fig. 4) as it did the total or flavonoid phenols (Figs. 2,3).

Absorbance at 320 nm is a potential measure of caffeoyl tartrate (caftaric acid) (13) and related compounds in wine. Fig. 5 shows progressive increase in 320 nm absorbance with increase in added SO_2 or increased pomace contact time unless the system is oxygenated. This is probably reflecting overlap from the greater absorbance at 280 nm rather than actual caftaric acid differences as shown by the failure of the nonflavonoid content (Fig. 3) to be similarly affected.

Actual brown as shown by 420 nm absorbance (Fig. 6) was quite different. As might be expected it increased regularly with increased pomace contact, but decreased with increasing initial SO_2 content. Although the oxygenated samples were considerably browner prior to fermentation than the comparable samples not oxygenated, the wines were slightly less brown for the two highest pomace contact times. This difference in reduced wine brownness following deliberate must oxidation has been previously noted by winemakers and was consistent with all four grape varieties except for the 12-hour Semillon samples. The French Colombard samples as a set were considerably browner than the other sets and the Chenin blanc slightly the least brown.

The capacity of the wines to brown additionally upon further exposure to oxygen (Fig. 7) increased greatly with longer pomace contact and was suppressed by increased SO_2 additions. Oxygenation of the must decreased the capacity of the wine to brown compared to similar samples (no SO_2 added) without oxygenation of the must, especially at the longest pomace contact times. The actual maximum brown intensity reached by accelerated browning was French Colombard > Semillon > Chardonnay > Chenin blanc and the Chardonnay samples were somewhat more suppressed in relative browning by high SO_2 than were the others.
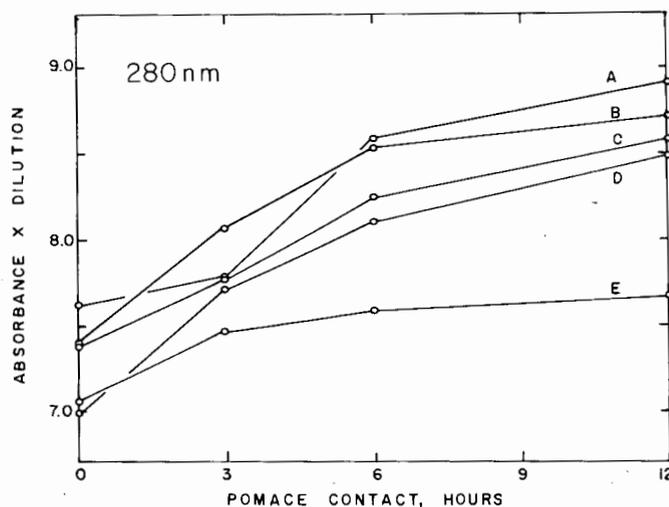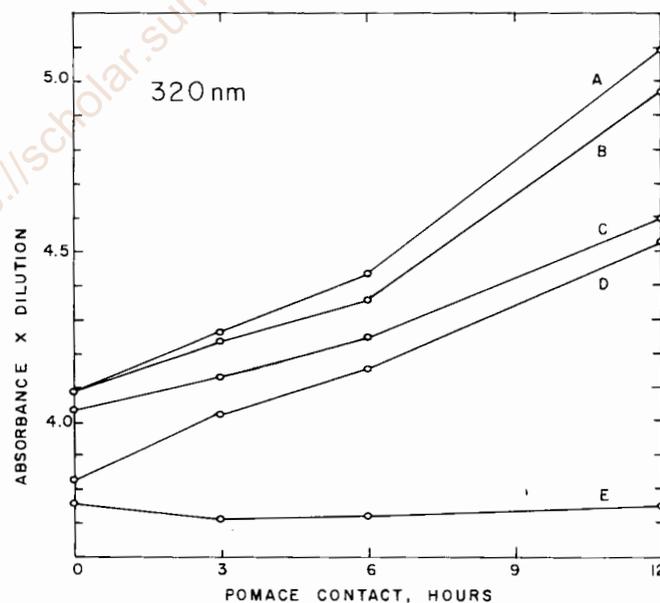
Pinking was not originally a part of the experiment, but some of the residual samples from the tast-
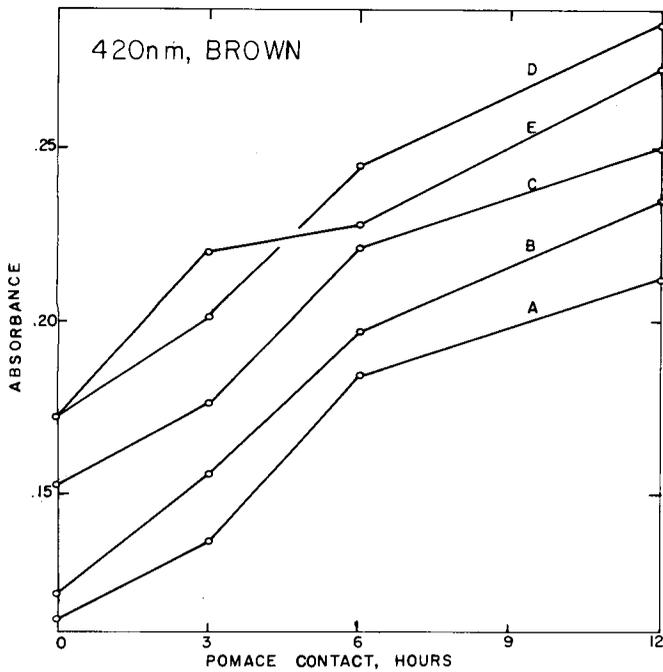
Fig. 6. Absorbance at 420 nm (brown) (means for all four grape varieties) of wines prepared from musts with high (A), intermediate (B), low (C), zero (D,E) additions of SO$_2$ and oxygenated (E) after 0, 3, 6, or 12 hours of pomace contact.
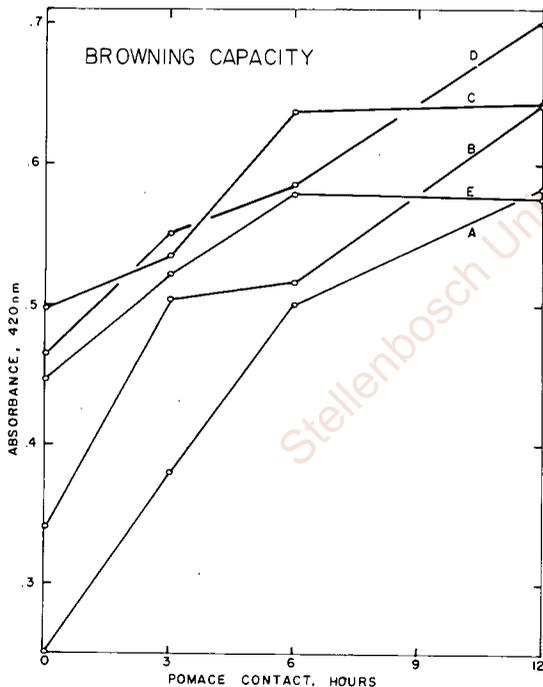

Fig. 7. The browning capacity after accelerated browning (means for all four grape varieties) of wines prepared from musts with high (A), intermediate (B), low (C), zero (D, E) additions of SO$_2$ and oxygenated (E) after 0, 3, 6, or 12 hours of pomace contact.

ing pinked intensely. Some of the Semillon samples became as pink as commercial rosés. The samples were coded, randomized, and judged visually on a 2 = very faint pink, 10 = rosé relative score basis. All sets produced some pink samples, especially in the long pomace contact samples. The mean combined ratings

for all samples are shown in Fig. 8. An attempt was made a few months later to produce pinking in fresh subsamples and evaluate it more objectively by spectrophotometry, but the samples browned rather than pinked so the relatively crude sensory data are shown. The Chardonnay samples pinked least and more erratically, the Semillon most strongly (with little brown), and the French Colombard strongly, but with more orange hue owing to browning, especially in the high pomace contact, low SO$_2$ samples.
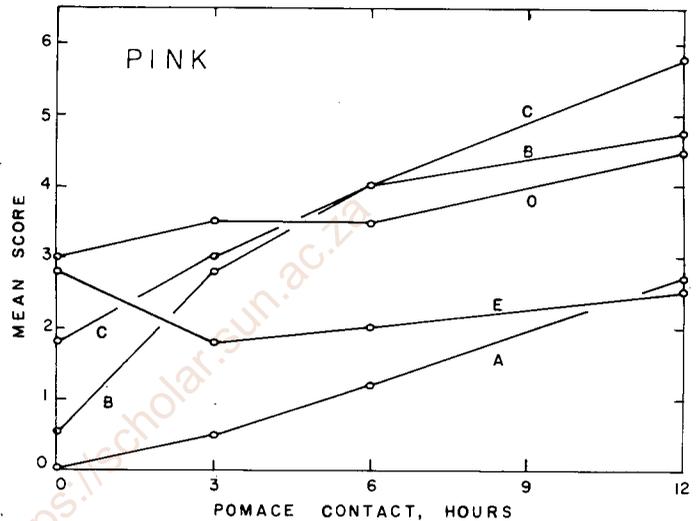

Fig. 8. Pinking (mean visual rating for all four grape varieties) after limited oxygen exposure of wines prepared from musts with high (A), intermediate (B), low (C), zero (D,E) additions of SO$_2$ and oxygenated (E) after 0, 3, 6, or 12 hours of pomace contact.

The mean sensory panel ratings for bitterness were significantly different related to pomace contact in the Chardonnay, to SO$_2$ content in the Chenin blanc and to interaction between the two treatments in the Chardonnay and French Colombard sets. Combining the data for all varieties gave mean bitterness ratings for 0, 3, 6, 12 hours of pomace contact of 3.01, 3.05, 3.07, and 3.22, respectively, a meaningful increase only for the highest level. Ratings for astringency were only significantly different for SO$_2$ levels with Chardonnay and pomace contact time and its interaction with SO$_2$ levels for French Colombard. The combined data for astringency showed no clear trends. Of course, as in all white table wines, astringency and bitterness were low in these wines. Previous studies had indicated that about 100 mg GAE/L difference in total phenol is a threshold difference amount in astringency or bitterness (12). These samples showed differences of this magnitude only in the extremes of the Chardonnay and French Colombard sets. The lack of notable differences for astringency attributed to either SO$_2$ or pomace contact and for bitterness only at the extreme of pomace contact seems, therefore, reasonable.

Fruitiness varied significantly with respect to time on the skins for all the varietal sets but Chardonnay and with respect to SO$_2$ level in all but Chenin blanc. There was a significant interaction between the two treatments, contact time versus SO$_2$ additions, in all

four sets. The combined data for all four varieties are shown in Fig. 9. A complex relationship is indicated. Mean fruitiness was increased by low additions of SO_2 and more so by intermediate, but decreased by high SO_2 addition to the must. Increased pomace contact decreased the fruitiness for wines receiving SO_2 additions. The samples receiving no added SO_2 were low in fruitiness, the oxygenated samples lowest of all, but increased slightly with pomace contact.
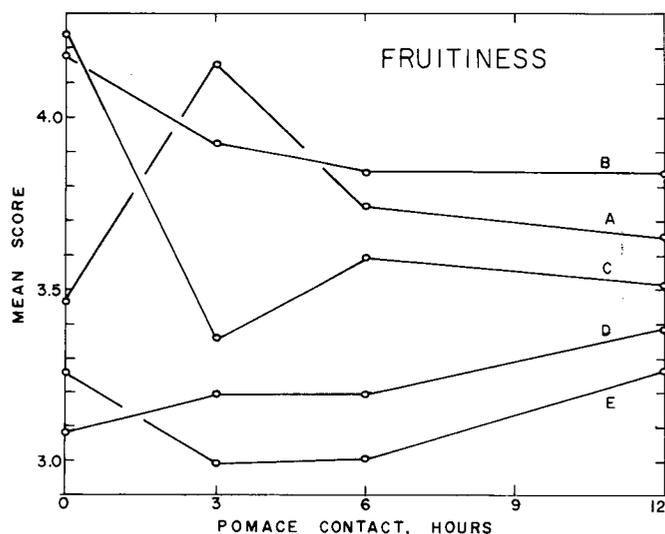

Fig. 9. Fruitiness ratings by sensory panel (means for all four grape varieties) of wines prepared from musts with high (A), intermediate (B), low (C), zero (D,E) additions of SO_2 and oxygenated (E) after 0, 3, 6, or 12 hours of pomace contact.

The combined data for general quality ratings are shown in Fig. 10. It appears that fruitiness difference (Fig. 9) was a major factor in general quality rating (Fig. 10). The differences in general quality with respect to pomace contact were significant with all four varieties. With respect to SO_2 treatment, the differences in general quality were significant except for the Chardonnay, and the interactions of both treatments and general quality were significant except in the Chenin blanc set. The quality of these wines was not very high even though the noticeable color differences were masked by presentation in black glasses. The samples were all rated slightly below 13, the minimum for commercial wines without defects on the U.C. Davis scorecard. The Semillon and Chardonnay were rated as slightly better on the average than the Chenin blanc and the French Colombard was rated lowest in general quality.

The effect of pomace contact on general quality appeared variable by variety; best mean quality was at 12 hours for Semillon, six hours for Chardonnay, three hours for French Colombard and zero hours for Chenin blanc. In his wider time range of comparisons, Ough (8) found 12 hours generally best. Owing to differences among varieties and the generally ordinary to low quality in these wines, extrapolation of these results to winery practice generally should be somewhat cautious. It is, however, evident from Figs. 9 and 10 that the wines without added SO_2 in the must were less
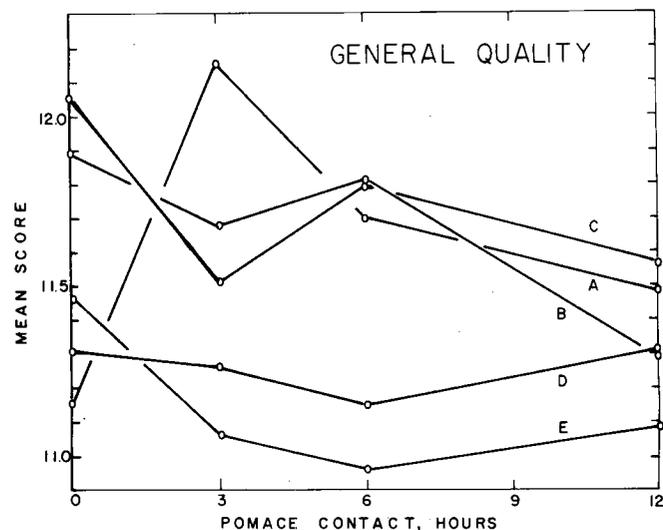

Fig. 10. General quality ratings (not involving appearance) by sensory panel (means for all four grape varieties) of wines prepared from musts with high (A), intermediate (B), low (C), zero (D,E) additions of SO_2 and oxygenated (E) after 0, 3, 6, or 12 hours of pomace contact.

good and when the musts were oxygenated became poorer. Wines with added SO_2 in the must were universally better than those without added SO_2. Collectively a low (50 mg/L) addition of SO_2 to the must was usually better than more, although this was not invariably the case. A high level of initial addition of SO_2 to the must (200 mg/L) was generally undesirable, but in the French Colombard set uniformly and some of the others sporadically this highest level was better than lower levels, particularly at the longer pomace contact times.

CONCLUSIONS

Deliberate oxygenation of musts does not necessarily produce finished wines which are browner and they may be more resistant to further browning, but no advantage is seen because the wines here were less fruity and lower in quality.

Pomace contact between crushing and juice separation may have advantage depending on the variety and the style of wine desired by the winemaker. Total phenol content increased about 3 mg GAE/L/hour of pomace contact at 24°C (75°F). It also increased in samples with three hours or more of pomace contact as SO_2 addition at the crusher was increased from zero to approximately 50, 100, or 200 mg/L by about 7, 18, or 34 mg GAE/L, respectively. Data are given relating these increases to increased pinking and browning when the wine is exposed to limited or intensive oxidation.

In general it appears from these data that better wines are made with modest amounts of initial SO_2 rather than either zero or high additions. Only a low level, perhaps 50 mg/L, of SO_2 need be added at crushing if grapes are in good condition and oxidation and microbial contamination are limited. Either more care or more SO_2 is likely to give better wine if appreciable pomace contact is allowed before juice separation. This is, of course, what enologists have recommended for

many years, but additional data were deemed desirable and informative.

LITERATURE CITED

1. Amerine, M. A., and C. S. Ough. Wine and Must Analyses. John Wiley & Sons, New York. 121 p. (1974).

2. Dott, W., M. Heinzel, and H. G. Trüper. Sulfite formation by wine yeasts. I. Relationships between growth, fermentation and sulfite formation. Arch. Microbiol. 107:289-92 (1976).

3. du Plessis, C. S. A new approach to wine quality investigation with special regard to bouquet. Proc. 3rd Int. Oenol. Symp. Paper 5:8 p. (1972).

4. Eschenbruch, R. Sulfite and sulfide formation during winemaking — a review. Am. J. Enol. Vitic. 25:157-61 (1974).

5. Kramling, T. E., and V. L. Singleton. An estimate of the nonflavonoid phenols in wines. Am. J. Enol. Vitic. 20:86-92 (1969).

6. Lüthi, H. R. To what extent can SO_2 be replaced in wine treatment? Proc. 3rd Int. Oenol. Symp. Paper 16:18 p. (1972).

7. Müller-Späth, H. Less sulfur dioxide in wine. Weinwirtschaft (Mainz) 111(1-2):27-8, 30, 32, 34 (1975).

8. Ough, C. S. Substances extracted during skin contact with white musts. I. General wine composition and quality changes with contact time. Am. J. Enol. Vitic. 20:93-100 (1969).

9. Ough, C. S., and H. W. Berg. Simulated mechanical harvest and gondola transport. II. Effect of temperature, atmosphere, and skin contact on chemical and sensory qualities of white wines. Am. J. Enol. Vitic. 22:194-8 (1971).

10. Singleton, V. L. Analytical fractionation of the phenolic substances of grapes and wine and some practical uses of such analyses. In: Chemistry of Winemaking. A. D. Webb, ed. Am. Chem. Soc., Washington, D.C. Adv. Chem. 137:184-211 (1974).

11. Singleton, V. L., and T. E. Kramling. Browning of white wines and an accelerated test for browning capacity. Am. J. Enol. Vitic. 27:157-60 (1976).

12. Singleton, V. L., H. A. Sieberhagen, P. deWet, and C. J. van Wyk. Composition and sensory qualities of wines prepared from white grapes by fermentation with and without grape solids. Am. J. Enol. Vitic. 26:62-9 (1975).

13. Singleton, V. L., C. F. Timberlake, and A. G. H. Lea. The phenolic cinnamates of white grapes and wine. J. Sci. Food Agric. 29:403-10 (1978).

14. Slinkard, K., and V. L. Singleton. Total phenol analysis: Automation and comparison with manual methods. Am. J. Enol. Vitic. 28:49-55 (1977).

15. Wildenradt, H. L., and V. L. Singleton. The production of aldehydes as a result of oxidation of phenolic compounds and its relation to wine aging. Am. J. Enol. Vitic. 25:119-26 (1974).

GRAPE AND WINE PHENOLICS; BACKGROUND AND PROSPECTS

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ABSTRACT

The phenols from the grapes (cinnamates, anthocyanins, tannins, etc.) and their derivatives are the most important group of compounds distinguishing sound wines of different types and qualities and their reactions during storage. To justify this statement and set the stage for the five following papers, some salient research findings, especially those since 1969 and by the symposium participants, are reviewed (145 refs.) and their practical meaning interpreted. Some previously unpublished results from our laboratory also are outlined, particularly on pigment-tannin relationships in red wine and oxidation-browning reactions in wine. Three aspects found in the studies on wine (autocatalysis, several atoms of oxygen consumed per catechol equivalent oxidized, and variable amounts of oxygen consumed depending on reaction conditions) can be explained by regenerative polymerization of the phenolic oxidation products.

This paper has three major objectives: 1) to develop a necessary minimum background on the importance and status of phenolic substances in grapes and wine (so that the other participants can focus on a current aspect of their research), 2) to outline some of the major research findings in this field (especially by symposium participants), and 3) to describe some of the recent or current research carried on at Davis in this field.

It is possible for the content of all phenols of wines to range from near zero to several grams per liter and grapes contain several grams per kilogram of fresh weight. Phenols constitute probably the third most abundant constituents in these products (following the carbohydrates or the alcohol derived from them and the fruit acids). They are considered the most significant source of differences in flavors as well as color among white, pink, and red wines and between light and robust styles of most specific types of wine. Finally, they include the natural ingredients of wine which react with oxygen and are crucial to its preservation, maturation and aging whether the intent is to minimize or encourage change. At least temporarily let us consider them the center of the enological universe.

The most exhaustive review of grape and wine phenols and their functions was published in 1969 (105) with the literature through 1967 covered as completely as possible. About 1,225 references were cited and nearly three times that number initially considered. In preparing for this paper, an update of that review with the publications appearing from 1968 to date was considered. A bibliography approaching completeness containing about 1,600 titles was collected by supplementing our own continuing files with a computerized search of Chemical Abstracts and additional references gleaned from major reviews appearing in the interim (e.g., 2,11,16,18,34,75,79,84,88). Incidentally, about 80% of the references were recovered from Chemical Abstracts or regular perusal of major journals in the field, but the additional 20% required checking the reviews. This 20% was about half theses, symposia, and applied technical

publications. The other half was either in journals missed by Chemical Abstracts or missed in searching. It is obviously not possible to review all this literature in detail here but some interesting facts become apparent from considering these listings.

That almost as many citable papers have appeared since 1967 as had appeared in total prior to that time shows that interest has burgeoned in this field. The publications were in English 25% (including some translations and probably better coverage), in Russian 24%, in French 17%, in Italian 10%, in German 9%, in Japanese 4%, in Spanish 2%, and 9% in all others, notably Bulgarian and other Eastern European languages. These comparisons indicate not only a widespread concern with these compounds and recognition of their importance, but also show growing contribution particularly from Italian scholars. Notably decreased in most areas was the periodic introductory paper with little new information and notably increased was the general level of sophistication in the research reported. Further improvement is expected with probably fewer but deeper papers becoming the norm.

It is slowly being accepted that the phenolic substances are the most important secondary components of plants in general. Work with grapes and wine, where their importance has long been recognized, has helped spread knowledge of the roles of phenols in plants and foods from plants. However, much good research has also developed from organic chemists, biochemists, plant physiologists, plant pathologists, food scientists, and other specialists becoming interested in these compounds and studying them in other products as well as grapes and wine. In some areas, biosynthesis for example, the grapevine is not as tractable a subject as a less complex plant, but the results from simpler systems elucidate the situation in the grape.

Even specialists may need reminding of the many ways phenols may be involved with grapes and wines. The 1968-1980 citations retrieved were 4% general references covering several topics, 5% were on by-

products, notably grape anthocyanins as safe substitutes for banned synthetic food dyes, and 5% were on nutritional and quality relationships including tristimulus color evaluation, flavor effects, and bioflavonoid effects. Viticulture and grape biochemistry were the main concern of 16% of the references and subtopics included polyphenol oxidase and related enzymes of grapes, phenol development during ripening, and the interrelationships among grape phenols and cultural practices, plant hormones, weather, and pathology.

Conversion of grapes to new wine was the main topic of 19% of the papers and subtopics ranged over the phenolic components of various grape fractions and the effects of mechanical harvesting, the use of damaged grapes, pectinase treatment, carbonic maceration, must heating, fermentor design, vinification conditions, and pressing. White, pink, and red vinifications, of course, give major differences centered on the phenolic components including but not limited to the anthocyanins.

Processing and storage reactions related to phenolics of wine or grape products were the primary subject of 20% of the reports and subtopics included browning, fining and stabilization, polymerization, and aging changes. Oak phenols and extraction from cooperage into beverages are additional subjects mostly not included in the bibliography under consideration.

Relationships with microbes totaled 3% of the references and included such diverse topics as inhibition of yeasts and bacteria by either grape phenols or added phenols in wine, microbial modification or production of phenols in wine, and removal of phenols by adsorption on yeast or bacterial cells.

Analysis totaled 15% of the citations and included, beside techniques for general or specific analysis for phenols, studies on typical phenolic composition of various wines or grapes, detection of addition of phenolic dyes or adulterants to wine, or detection of other types of blending operations, especially illicit ones.

Identification and characterization of phenolic substances in grapes or wine were the subject of the remaining 13% of the papers under consideration and included studies on volatile phenols, phenolic acids and other nonflavonoids, anthocyanins, catechins, flavonols, condensed tannins and other flavonoids and specific characterization of their properties.

Total phenols and a "phenol balance": In 1969, it was concluded (105) that comparing the qualitative and quantitative composition of grapes and wine indicated no major portion of the phenols remained unidentified. This still appears essentially correct and most of the newer studies extend or verify previous findings without discovering new kinds of phenols. Interesting exceptions include a series of stilbene derivatives appearing as phytoalexins in grape leaves in response to *Botrytis cinerea* infection or ultraviolet light damage (55,56). Jurd and Somers (54) identified xanthylium salts in the yellow degradation products from grape anthocyanogens and they were found in stored grape juice by Hrazdina and Borzell (47) and in wine by Michaud et al. (67). Based on spectral and chromatographic data pelargoni-

din glycosides (27) and pelargonidin-yielding anthocyanogens (83,125) have been reported in addition to the cyanidin, peonidin, petunidin, delphinidin and malvidin glycosides, procyanidins, and prodelphinidins considered normal for grapes.

A great need seen in 1969 was the ability to follow each phenol quantitatively in a "phenol balance" mode (original amount, final amount, other products) so that complex changes in grapes or wine could be monitored (105). That 28% of the papers published since then focused on analysis or identification of phenolics suggests agreement with this objective, but that it has not been attained. Nevertheless, considerable progress in this direction has been made. One way to determine the amount and changes in individual phenols in grapes or wine and therefore their roles is to analyze for each one separately. This necessitates precise separation procedures, usually chromatographic. High performance liquid chromatography (HPLC) has begun to justify the high expectations held for it, notably, for grapes and wines, in the hands of Nagel and co-workers (69,70,73,74,142,143). Another approach is to relate analyses for classes of phenols back to an analysis for total phenols. Until or unless HPLC can be developed to give a complete separation and quantitation of all important phenols in one chromatogram, the latter method or some combination with it seems the most practical solution.

Without going into various other methods for total phenol content in plant extracts, it seems necessary here to mention two, absorption spectra and colorimetry with molybdotungstophosphate, the Folin-Ciocalteu and related reagents. The ultraviolet and visible region absorbance can be, of course, extremely useful, but the molar extinction of different substances can vary by a considerable multiple (*cis*-coutaric acid has about half the molar absorbance of the *trans,* for example, and at equilibrium represents about 40% of the total coutaric acid) (111). The absorbance maxima also differ considerably in wavelength for different phenols. Furthermore, as first shown by Somers and Ziemelis (124), white wines have about half or more of their UV absorbance in the phenol range contributed by other substances, especially nucleic acid fragments.

The improved colorimetric method (108) has been found in several studies to be the method of choice for grape extracts, wines, and probably for general use (e.g.,24,28,39,40,92). Generally, and I think preferably, the results are expressed as mg of gallic acid equivalent (GAE) phenol per liter of wine or per kg of fresh fruit. Comparisons can then be made with any separately determined phenolic component based on the respective molar color yields predicted (or better, co-determined) in the assay (103). This Folin-Ciocalteu reagent assay does have shortcomings. It depends on the phenolic substances being relatively easily oxidized (although monohydroxyl phenols are determined) and other readily oxidized substances, notably ascorbic acid, aromatic amines, and sulfur dioxide, can interfere. However, these substances are ordinarily low or are easily determined by separate analyses and their effect can be subtracted

from the total in grape extracts or wines. A more severe problem only with musts or sweet wines, especially if color development in the assay is hastened by heating, is interference by sugar. This is apparently caused by the production of reductones behaving like ascorbic acid by the action of the alkaline final assay solution on fructose and to a lesser extent glucose. It can be allowed for by suitable sugar levels in the reference standard samples (29,103,114).

Phenols of grapes compared to wines of different types: The total phenol of grape berries is very variety dependent and appreciably influenced by the weather. It averages for seeded *Vitis vinifera* varieties in California about 4,000 for white and 5,500 mg GAE/kg for red varieties (105,107). About 200 mg/kg of this or about 5% is in the juice, about 1% is in the pulp, and the remainder is about ⅓ in the skins and ⅔ in the seeds in red grapes (105). In white grapes the mutation evidently has usually caused the loss of the ability to make the anthocyanins without significant change in the remainder of the skin phenols (105,113). Therefore, the total extractable phenols of white grape skins, particularly on a mg GAE/cm² of skin basis, is considerably less than that of red grapes (113).

The phenols of grape juice (other than tienturiers) obviously would be common to all wines. It has been shown by Kramling and Singleton using an assay they developed (62) that the juice phenols are almost entirely not flavonoids and account for most of the apparent nonflavonoid content of all wines at about 100 to 300 mg GAE/L. Montedoro and Fantozzi (68) have extended this assay to separately determining the tannins after precipitation with methyl cellulose. Properly applied these assays enable separate estimation of hydrolyzable tannins, condensed tannins, monomeric flavonoids, and nonflavonoid monomeric phenols. Two other similar methods using other precipitants gave comparable results for tannin content (23).

A very large part of the juice phenols consists of tartaric acid acylated with caffeic, p-coumaric, or ferulic acids (caftaric, coutaric, and fertaric acids) first reported in grapes by Ribéreau-Gayon (87) and recently studied further by Castino (87); Singleton, Timberlake, and Lea (110); and Nagel's group (70,73,74). In spite of many reports to the contrary, chlorogenic acids, caffeoyl quinates, appear to be absent.

The phenol content of wine rises, and the increase is essentially entirely in the form of flavonoid substances, as there is increasing contact, maceration, breakdown and extraction from the more solid grape tissues, the skins, seeds, and possibly stems. The total content of phenols in wine is always less than that possible from the grape material because usual processing does not accomplish complete extraction and such factors as coprecipitation of phenols, particularly tannins, with grape proteins, yeast cells, and fining agents removes some. The total apparent phenol content of the lightest white wines is about the same as that of clear juice, although the qualitative makeup may be considerably altered. Commercially produced red wines are seldom over 2,500 mg/

L even for rough young wines intended for aging. About 1,100 to 1,800 mg GAE/L is usual for finished red table wines on the market. Wines fermented from white grapes by red wine vinification methods generally have total phenols well below that of red wines similarly prepared, about 400-800 mg GAE/L (e.g., 26,109). Rose wines generally also analyze in the 500 mg GAE/L range, but can be quite variable.

It is obviously possible to affect the phenolic content and composition greatly by varying winemaking practices. Even considerable selectivity such as varying the flavonoid-nonflavonoid or anthocyanin-tannin ratios is quite feasible, but beyond the scope of this review. In red vinification usually about 25% to 50% of the phenols of pomace appear in the wine (e.g., 6,7,77,91,95,104). Considerable further development in this area seems possible as we learn more details. Much rather empirical application has already been made toward optimizing conditions for maximum red color without excessive tannin or aging requirement, for example. Light and popular red wines not suitable for long aging are now major products in several countries.

Phenol content of wines by classes: Some further discussion of the qualitative and quantitative phenol composition of wines does seem appropriate. An effort to estimate the typical content of typical wines by classes of phenol is given in Table 1. Every number in such a table can be questioned and exceptions described. Many of the values are compromises and some are conjectures, but it is hoped that this table conveys a useful synthesis. An effort was made to indicate not only typical amounts, but also directions of change and shifts in relative amounts, some known and some postulated.

Table 1. Gross phenol composition estimated in mg GAE/L for typical table wines from *Vitis vinifera* grapes.

Phenol class	Source[a]	White wine Young	White wine Aged	Red wine Young	Red wine Aged
Nonflavonoids, total		175	160-260	235	240-500
Cinnamates, derivatives	G, D	154	130	165	150
Low volatility benzene deriv.	D, M, G, E	10	15	50	60
Tyrosol	M	10	10	15	15
Volatile phenols	M, D, E	1	5	5	15
Hydrolyzable tannins, etc.	E	0	0-100	0	0-260
Macromolecular complexes					
"Protein"-"tannin"	G, D, E	10	5	5	10
Flavonoids total		30	25	1060	705
Catechins	G	25	15	200	150
Flavanols	G, D	tr	tr	50	10
Anthocyanins	G	0	0	200	20
Soluble tannins, derivatives	G, D	5	10	550	450
Other Flavonoids, derivatives	G, D, E, M	?	?	60?	75?
Total phenols		215	190-290	1300	955-1215

[a] D = Degradation Product; E = Environment, Cooperage; G = Grapes; M = Microbes, Yeast.

Although the nonflavonoid fraction of wines not exposed to oak cooperage totals about the same as that of the juice from which it was made, there is considerable qualitative modification and some may be gained as well as lost. Except for the hydrolyzable tannins which may be contributed by wooden cooperage, these nonflavonoids are mostly small molecules. Caftaric and related acyl phenols are partially hydrolyzed to the free cinna-

mic acids. These then can be converted in part to decarboxylated derivatives such as 4-vinylcatechol and these in turn reduced to 4-ethylcatechol and analogs by the action of yeasts or malolactic fermenters. Degradation of flavonoids is also indicated by production of more volatile phenols as red wines age (107). Although the total of volatile phenols is small, many substances are involved (30,31,136). Vanillin or vanillic acid and related lignin fragments can also be contributed by cooperage wood. Other benzene derivatives, especially gallic acid, are present in small amounts in wine that can be augmented by hydrolysis of epicatechin gallate and perhaps by other degradations as wines are processed or aged. Microbial decarboxylation of benzoic acids and the microbial conversion of quinic or shikimic acid to protocatechuic acid and catechol can occur (140). Phloroglucinol and its carboxy and methyl derivatives are reported in wine as results of flavonoid degradation by rotting of the grapes or thermal effects (14). Tyrosol is produced in wine by yeast from either sugar or tyrosine and doesn't change significantly with aging (98).

The hydroxycinnamates, caftaric acid and relatives, are the major nonflavonoids of grape juice. In white wine about ⅓ appears to be unchanged, ⅓ is evidently oxidized and linked with sulfhydryl substances, and the remaining ⅓ seems to be in some complex with nucleic acid fragments (69). With longer age, particularly with oxygen exposure, further loss and hydrolysis is expected. Red wines are similar as far as we know except that additional hydroxycinnamates would arise from hydrolysis of acylated anthocyanins.

Protein-tannin macromolecular complexes are significant in white wines and can be measured as shown by Somers after their exclusion from appropriate microporous gels (124). In these large complexes carbohydrate polymers appear to be also involved (41,134). Owing to precipitation, this fraction would be expected to decrease with stabilization and aging. However, it has been reported to increase (134). Polymerization can lead to reaction products that behave similarly but probably have a higher phenol to nitrogen ratio (phlobaphene-polypeptide) and smaller size. Such products are expected to be particularly significant in older red wines, but may grade into and be difficult to distinguish from the smaller (578 to 4000 MW) and relatively soluble tannic dimers and intermediate oligomers from grapes or in young wines.

These soluble condensed tannin oligomers are built-up of units of epicatechin or catechin for those generating cyanidin and of gallocatechins for those behaving as delphinidinogens. The identified dimers are the B_1-B_4 cyanidinogens representing the four possible combinations of epicatechin and catechin pairing through carbon 4 of one unit to carbon 8 of the other (60,137,139). The remaining natural oligomers appear to be a mixture of less than all possible combinations in further polymers of the same sort up to perhaps 14 units but apparently reaching only about 3,500 to 4,000 molecular weight (9,89,93). As red wines age this group of tannin polymers incorporates the red pigments.

The flavonoids are small in amount but important in white wine. The wine considered in Table 1 as typical represents one made from clarified juice with minimal grape solids extraction. A small amount of anthocyanogenic dimer or slightly larger condensed tannin is usually present with a slightly larger amount of catechins and only traces of other flavonoids. With increasing grape pomace contact especially during fermentation, the content of these flavonoids rises with a gradual reversal in the relative proportion of anthocyanogenic condensed tannins (e.g., 109). Freshly made red wines have several times as much catechins and much more of the tannin oligomers than white wines. The average molecular weight of these compounds increases with age. Polymerization, precipitation, and probably depolymerization are important causes of qualitative and quantitative change in these components during storage and aging. Anthocyanins are highly involved in these polymerization reactions, the catechins and flavonols apparently not or less so unless acetaldehyde crosslinking is also occurring.

The full complement of flavan-3-ols, catechins, for grapes and wines appears to be (+)-catechin, (−)-epicatechin, (−)-epicatechin gallate, and the three analogous gallocatechin derivatives (15,81,90,105,126). The gallate esters are low and decrease as the fruit ripens. The gallocatechins have been in considerably lower amount than the catechins in the ripe grapes and wines we have studied in California compared to roughly equal proportions reported from cooler countries (e.g., 15). This does not appear to be an artifact and there are varietal differences. The gallates and gallocatechins disappear from wine with time, but catechin seems relatively stable in red wine (71,145).

The flavonols of grapes have been somewhat less studied. Kaempferol, quercetin, and (in lesser amount and perhaps confined to red grapes) myricetin are present in grape skins as glucosides and sometimes also as glucuronosides (8,35,36,86,105). Isorhamnetin-3-glucoside, the flavonol analog of peonidin-3-glucoside, has recently been reported in red wine (145). The flavonol aglycones appear more readily released by hydrolysis than the anthocyanidins and the aglycones, being poorly soluble, generally decrease or disappear in red wines with time.

The "other" flavonoid derivatives indicated in wines in Table 1 would include the xanthylium salts already mentioned and the flavenes recently reported by Wulf and Nagel (145).

The anthocyanins, of course, are the red and purple pigments of grapes and young red wines and have been the most studied in wines and elsewhere because of their vivid color. The pigment in the grape skin appears to be complexed and copigmented with other flavonoids in ways just beginning to be understood (100,123,143). Self-association of the pigments, association with other phenols, effects of complexing metals, and cross-linking reactions with acetaldehyde can give considerable enhancement of the color and change in both the wavelength and intensity of maximum absorbance

(100,129,130). The *Vitis vinifera* pigments are the 3-glucosides whereas most other grape species have also the 3,5-diglucosides (105). This has been developed, following the work of Ribéreau-Gayon (85,105) into a useful means of detecting blending of more prestigious vinifera wines with that from other grapes species or crosses prohibited for viticultural or nomenclatural reasons. The grape skin pigments in all major varieties except Pinot noir and close relatives are partially acylated with acetic acid, caffeic acid, or *p*-coumaric acid on carbon-6 of the 3-glucose as shown by Webb, Kepner and their associates (3,4,37,105). Hrazdina and co-workers (50,142) showed that the acyl groups were still on the 3-glucose in 3,5-diglucosides of grape anthocyanins. Hrazdina also has taken the lead in isolation of individual pigments from grapes in gram quantities and using them for studies of analysis, stability, and other effects under wine-like conditions (45,48,49,72).

Individual grape varieties commonly have very different chromatographic patterns of grape skin anthocyanins. Such chromatograms together with spectra, color tests, degradation, etc. have been applied since early work by the Kepner-Webb group and by Ribéreau-Gayon (105) to characterizing the pigments of many varieties (e.g., 1,46,61,97). Such studies are complicated by oxidation reactions (49), the production of artifacts in chromatographic solvents (132), hydrolysis of acyl groups [especially the acetates (3)], glycoside hydrolysis followed by degradation, polymerization, and other problems. The order of decreasing stability in solution is 3,5-diglucosides, 3-glucosides, acylated-3,5-diglucosides, and acylated 3-glucosides (46). Of course, decreasing stability to oxidation is related to increasing free hydroxyl groups in the B-ring and would be expected to be in the order peonidin, malvidin, cyanidin, petunidin, and delphinidin derivatives (46,48).

The five aglycones just named (Fig. 1) are the ones almost universally found in red grapes and malvidin is the major one in dark red vinifera fruit with higher proportions of cyanidin in red fruit and delphinidin in the blue *Vitis labrusca* varieties such as Concord. In view of the variation among varieties, the characterization of a typical mixture of grape anthocyanins is difficult. Early studies were hampered by incomplete separation of the group of acylated pigments, but indicated (105) in a large number of vinifera grapes about 3% delphinidin-3-glucoside, 5% petunidin-3-glucoside, 41% malvidin-3-glucoside plus cyanidin-3-glucoside, 10% peonidin-3-glucoside, 15% mixed caffeoyl-anthocyanins, and 26% mixed *p*-coumaroyl-anthocyanins. HPLC is improving this situation (142,144) with nearly complete separation of known and some indications of small amounts of unknown components. Large differences among varieties are again emphasized (95). Wulf and Nagel (144) reported the pigments of Cabernet Sauvignon skins by HPLC (% of peak area) were 42.6% malvidin-3-glucoside plus 20.5% of its acetate, 6.4% of its *p*-coumarate, and 0.1% of its caffeate. No other pigment showed significant caffeoyl derivatives and the respective percentage of the 3-glucoside, acetate, and *p*-coumarate derivatives in the total pigment were: cyanidin 1.3, 0.1, 0.1; peonidin 5.3, 0.9, 0.6; delphinidin 10.0, 2.5, 0.5, and petunidin 6.1, 2.2, 0.4%.
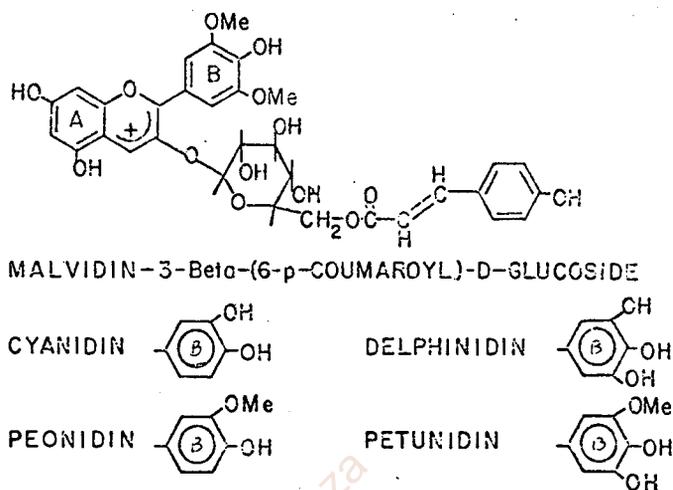

Fig. 1. Examples of typical anthocyanins from *Vitis vinifera* grapes.

It has already been mentioned that grape skin anthocyanins probably exist in the intact grape as a complex or "polymer". As grapes are extracted or made into wine, the pigments become identifiable as the individual anthocyanins just mentioned. These free anthocyanins in mildly acidic aqueous solutions like wines are in fairly complex equilibria (Fig. 2) which strongly affect the visible color and apparent anthocyanin content. In very strongly acidic media, pH 1 or less, they are in the flavylium ion form protonated to give the red cation. These benzopyrylium or flavylium carbocations are stabilized by the positive charge being delocalized over the conjugated system with carbons 2 and 4 being relatively highly positive and especially subject to nucleophilic attack (10,53,66). Brouillard and coworkers (19-22) have, with malvidin-3-glucoside from grapes, clarified the mechanisms and rates of these interactions. Briefly, the flavylium ion is in equilibrium with the quinoidal or anhydrobase (fast, pK 4.25) and, separately, with the carbinol hydrated pseudobase (slow, pK 2.60) neither of which products are charged. The carbinol base is in even slower equilibrium with an open ring *cis*-chalcone form which in the presence of light isomerizes to the *trans* form that further slows (or, without continued light, prevents) return to the pseudobase form. The equilibrium proportions are much different for the 3,5-diglucoside of other grape species but for malvidin-3-glucoside alone, the main pigment of vinifera, the main forms at pH less than 4 are the flavylium carbocation and the carbinol pseudobase, with equal proportions at pH 2.6. This would mean more than half of a young wine's anthocyanin would be in the colorless carbinol form. The quinoidal or anhydrobase form is very small in model solution, but apparently is stabilized and the proportion increased by copigmentation (100,129,130,143).

Pigment and tannin, polymerization in red wines during aging: It has been known for a long time that the pigment of red wines soon does not behave like the anthocyanins extracted from grapes (105). The solution
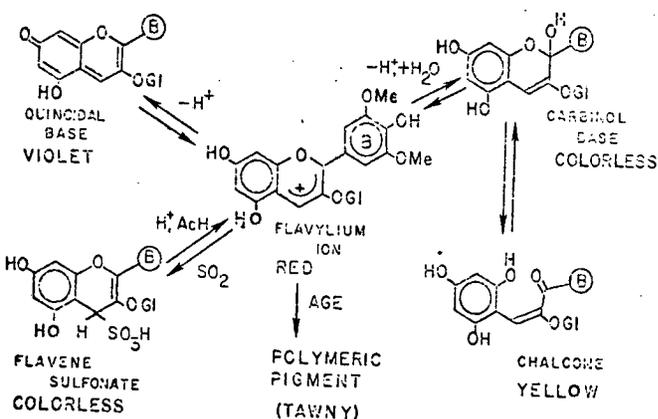
Fig. 2. Equilibria affected by pH and polymerization of the anthocyanin malvidin-3-glucoside that influence red wine color.

becomes more tawny colored, the absorbance maximum near 520 nm decreases, and the original minimum near 420 nm rises. Furthermore, the pigments become non-dialyzable and stay at the origin of normal paper chromatograms. Berg (12) noted that the pigment of red wines was of two types, pH responsive (anthocyanins) and relatively unresponsive (complexed), and followed changes increasing the latter during storage (13). Ribéreau-Gayon and Stonestreet (93) and Somers (115-117,119) investigated these red tannin polymers early and showed that they formed rapidly (a large fraction of a red wine's color was incorporated by one year) and that the polymer contained glucose that could only be removed by hydrolysis. It appeared that the anthocyanin was being incorporated into the oligomeric tannins without losing its red color entirely or its glucoside moiety.

The polymeric red tannin is also resistant to decolorization by sulfur dioxide whereas the free anthocyanins are not (105). This suggests polymerization between the

relatively electrophilic (positive) 4-position of the anthocyanin and a nucleophilic center such as positions 6 or 8 on the condensed tannin. Timberlake and Bridle (128) showed that synthetic flavylium salts with the 4-position substituted by a methyl or phenyl group were very resistant to decolorization by sulfur dioxide and sunlight. The individual free anthocyanins all decrease as the polymeric fraction increases although the rate appears slowest for malvidin-3-glucoside, perhaps partly from replenishment from hydrolysis of its acylated forms (71).

The phenolic polymers in aged red wine are undoubtedly quite heterogeneous (52,88) and the tannins can produce a red-brown color without the initial presence of anthocyanins under some circumstances. Special analyses for the polymeric pigments or fractions of the phenolic polymers of red wine have been developed and applied, with their co-workers, by Somers (118), Glories (41-43), Margheri (63-65), Bourziex (17) and others. For example, Glories (41), based on precipitations with ethanol and methanol-chloroform then selective elution from poly(vinylpyrrolidinone), reported the data in Table 2 for Bordeaux red wines of various ages.

Since this subject will be covered further by others in the symposium, I will leave it except to mention some related research, not yet published, in our laboratory by Barney T. Watson, Jr. A wine from Alicante Bouschet (high color, moderate tannin) was blended with one from Calzin (high tannin, moderate color) or with phenol fractions from grape seeds. Three sets of wines were examined for total aging times up to 26 months. Selected data from one set are shown in Table 3.

Total anthocyanin was determined by calculation from absorbance at low pH and by both the pH-shift and SO_2-bleach methods of Ribéreau-Gayon and Stonestreet (94) with excellent agreement among all three methods (e.g., when seven different grape extract preparations were compared, the set with maximum variation was

Table 2. Bordeaux red wines, phenolic polymer composition versus age. Adapted from Glories (41) in mg/L and % of total.

Vintage	Total Phenol (mg/L)	Total Anthocyanins (mg/L)	Tannin Salt and Carbohydrate Complexes (%)	Tannins			Other Flavonoids (%)	Anthocyanins	
				4000 MW (%)	2500 MW (%)	1500 MW (%)		Free (%)	Polymer (%)
1975	2760	500	20	18	10	40	12	52	48
1972	2600	110	17	25	23	23	12	30	70
1962	3850	45	24	35	22	14	5	8	92
1951	3100	40	35	20	35	10	0	0	100
1921	4250	18	41	7	40	12	0	0	100

Table 3. Phenol composition of wines after aging 4 months in bulk plus 12 months in bottle.

Sample	Phenol				Anthocyanin Pigment						
	Total (mg GAE/L)		% polymeric		Total (mg/L)		% lost	% polymeric		Oxidized after 16 mo	
	4 mo	16 mo	4 mo	16 mo	4 mo	16 mo	12 mo	4 mo	16 mo	% lost	% polymeric
										1 mo	1 mo
cante wine	1595	1556	22	28	572	219	62	16	33	17	37
cante + seed tannin	4250	3706	49	49	444	151	66	31	49	12	57
cante + Calzin	1775	1744	44	40	448	174	61	22	39	2	45
lzin wine	2025	2006	60	57	306	149	51	31	44	+5	59

respectively 203, 207, and 218 mg/L). Free anthocyanins were determined in the wines from the absorbance at low pH of the red fraction readily eluted from a polyamide column under standardized conditions. Polymeric anthocyanins and total polymeric phenols were determined, respectively, by absorbance in acid and colorimetry (108,114) on the substances retained in Spectrapor-3 dialysis tubing (nominal retention limit 3500 MW) for 72 hours against three changes of fresh model-wine solution lacking anthocyanins. We were gratified to find that the sum of the free anthocyanins and the polymeric anthocyanins by these methods almost exactly equaled (102 ± 3%) the total anthocyanin separately determined on a series of wine samples covering a wide range of residual pigment content and added tannins.

Blending with a high tannin wine or adding grape seed tannin induced polymeric pigment formation during bottle aging above that found in the control and higher tannin additions gave more red polymer. The dimer and oligomer fractions were more active than the largest phlobaphene-polymer fraction of seed tannins and the catechin fraction had little activity. About half of the pigment was polymeric by one year. The increased polymeric pigment produced much greater survival of color when the wines were then exposed to harsh conditions of heat or oxidation. The polymerization initially appeared to increase the solubility of the tannin (because of the positive charge and glucose content presumably) while stabilizing the anthocyanin against pH shift or sulfur dioxide effects on color.

The polymeric pigment and tannin increased in size with time and appeared to reach a size or configuration limiting the solubility followed by a slow loss in polymeric color due to precipitation. Total phenol, taking all the data together, decreased about 1% per month during the tests (up to 26 months). The apparent nonflavonoid content decreased considerably in the first four months and then rose slightly during further bottle age, presumably due to exhaustion of oxidation effects on the one hand and degradation of flavonoids to nonflavonoids on the other. There was no indication that the nonflavonoid phenols were involved in the anthocyanin-tannin polymerization.

Sensory effects of phenols in wines: The sensory contributions of phenols are nearly controlling for color in nearly all wines and are large for taste for red wines. Effects on clarity may be significant, and body, aroma, and off flavors may involve phenols. The flavor effects (odor and taste) of phenolic substances in wines were recently reviewed and interpreted (107). Large polyphenols account for the only known source of astringency in such food products as wine. Many phenols of wine, especially smaller ones, are bitter and the smallest include substances notable for odors. Volatile phenols are very low in young wines and are individually near or below their estimated threshold level in aged wines. They are possibly additive to contribute to sensory effects such as smokiness, vanillin-like character, pungency (the penetrating bite exemplified by capsicum, ginger or cloves), and bitterness. Other wine phenols

that are not volatile but are small appear to add appreciably to bitterness and perhaps other wine flavors. Tyrosol, hydroxybenzoates, and hydroxycinnamates are individually near or below estimated flavor thresholds, but are reported to have similar bitter or harsh effects and are probably additively important to wine flavor.

The tannins of typical young red wines are present as a group at 5 to 10 times their flavor threshold level and clearly are important to wine quality. The other flavonoids appear to be close to their flavor threshold concentrations and together certainly make direct and indirect contributions to wine quality. Pomace contact time sufficient to give a total phenol content (flavonoid) increase of 100 mg GAE/L in white table wines (107,109) and 250 mg GAE/L in red wines (102,107) produces about the minimum recognizable difference in astringency or bitterness. These values compare rather well with sensory threshold values for addition of purified whole grape seed "tannin" to water of 25 mg GAE/L or to white table wine of 80 mg GAE/L (105,107). In their excellent work on English cider, Lea and co-workers (58,59) showed oxidation reduced tannin astringency and that the most astringent component was the flavonoid tetramer. Arnold and Noble (5) have shown that increasing grape seed tannin concentration is rated by judges as increasing in astringency, but at high levels interferes and prevents increase in apparent bitterness. The same effect is evident in wine (109) and, since the larger tannin molecules are more astringent and less soluble while small phenolic molecules are bitter, shows how aging and loss of tannin could "uncover" bitterness in certain wines.

The color intensity and hue of pink and red wine are important to a wine's quality, both directly and as indicators of other effects. Purplish tinges are suggestive of such conditions as hybrid grapes, high pH musts, or anaerobic pomace heating to liberate color. Brick or tile shades suggest aging, oxidation, or related conditions and develop earlier in low phenol wines. The comparison of red wines by their relative absorbance at 420 and 520 nm as developed by Sudraud (105,127) is widely used. Ough and Amerine (78) related visual color to visual preferences and Van Buren et al. (135) also studied tristimulus color evaluation of grape anthocyanin solutions and wines.

Tromp and Van Wyk (133) concluded that color was the most important quality assessment factor among wines without abnormalities. Their panelists were well able to guess the overall quality of a red wine from its color alone, but could not guess the color of the wine from its flavor with the same ease. Generally, one with darker red color is judged as the better wine and expected to be richer, more tannic, etc. as well. However, as long as an acceptable level of red color is present, darker red may not be considered desirable in our experience (e.g., 102) and very dark red wines may be assessed by bias as younger or more tannic whether they are or not.

Somers and Evans (121) reported close positive cor-

relation between panel-assessed quality ratings and the spectral color intensity and degree of anthocyanin ionization in a set of wines from one district and one vintage. Later work (120,122) developed their methods and indicated they are generally applicable. Calculations from a set of six spectrophotometric observations indicate total anthocyanin content, total phenol content, the effect of pH and the free SO_2 present, the proportion of anthocyanin that is ionized, and the "chemical age" of the wine. Sudraud's methods (127) estimated the color intensity (sum of 420 and 520 nm absorbance) and hue (ratio of 420 and 520 nm absorbance). Total phenol came from 280 nm absorbance and the anthocyanin and other values from determining the absorbance at 520 nm in strong acid, after bleaching with SO_2, and after restoring the color with excess acetaldehyde.

Timberlake and co-workers (51,131) applied the same and similar spectrophotometric techniques with more detailed sensory studies to Beaujolais wines of two vintages. They found significant positive correlations between overall quality and wine flavor on the one hand and the content of total pigment and total anthocyanins on the other. Total pigment was that indicated by absorbance at 520 nm in dilute highly acid solution and total anthocyanin was obtained by subtracting the polymeric pigment color as indicated by residual 520 nm absorbance after addition of 0.3% sodium metabisulfite to the wine. They found that for Beaujolais, famed as young wines, increasing "chemical age" and polymeric pigment correlated negatively with quality. They discussed the approximations involved and concluded that the independent measurement of monomeric anthocyanins and polymeric pigment had not been properly solved (Watson's techniques mentioned earlier seem to be such a solution). From their own and previous data they concluded that, for the present, the method most consistent, simplest, and most free of assumptions for estimation of red wine quality by color alone is measurement of total pigment at 520 nm in the strongly acidified diluted wine. Polymeric color correlated with quality in the Australian wines but not in the Beaujolais and the most constantly high (0.55 to 0.88) correlations in the four sets of wines (and uniformly significant) were between total anthocyanin (total pigment minus polymeric pigment) and overall quality. They also point out that a correlation of r = 0.7 means only 49% ($r^2 \times 100$) of the overall quality score has been predicted by the pigment measurement.

Oxidation and browning: Oxygen is required for the browning produced in bentonited white wines below about 50°C in our accelerated browning tests (106). The capacity to brown with exposure to oxygen is increased with increasing phenolic content in the wine (105,106). The major reservoir of autoxidizable substrate and the primary source of the brown pigments of most types of wines is generally conceded to be the phenols, but different phenols have much different effects in these reactions (105). The phenols are depleted during oxidation of wine and yet enable the wine to resist excessive changes.

Oxygen uptake by wine is too slow and too prolonged for the total oxidation capacity of a wine to be determined by available techniques such as Warburg respirometry (96,105). If a sample of wine is made alkaline, the phenolate ions are much more readily oxidized and the capacity of the wine to consume oxygen in a short time averaged 0.18 cm^3 of O_2 per mg GAE/L in red wines and 0.30 cc per mg GAE/L in white wines under standardized conditions (96). In further work not yet published, Peter M. Stern, in our laboratory, compared several other methods of estimating oxidation capacity of wine and applied them to studies of various wines and model substances. He was able to develop an equation that for a series of fortified dry wines (made from Ruby Cabernet with 0 to 16 days on the pomace) predicted their oxygen uptake under alkaline conditions from their content of total phenols and vicinal dihydroxyphenols.

Fortification to 21% ethanol was used to prevent microbial growth as the same wines were treated with increasing oxygen gas headspace volumes of 0, 25, 50, and 100 mL per 400 mL bottle. Selected data are shown in Table 4. Note that exposure of the wine to oxygen decreased the phenols and the remaining oxidation capacity as would be predicted. The decrease was least in the O_2 capacity and most in the vicinal diphenols. These data suggest that the vicinal diphenols are the most readily oxidized, as expected, but also that the other phenols participate and that the exposure of acidic wine to oxygen does not decrease the remaining capacity to react with oxygen under alkaline conditions as much as might be expected. When known substances were treated the same way, the alkaline oxygen capacity was 1.08-2.18 moles of O_2 per mole of phenol for a series of vicinal diphenols including catechin and gallic acid. Uptake was zero for phloroglucinol, vanillic acid and syringic acid under the same conditions. If the reaction was only to convert the vicinal diphenol to the *ortho*-quinone, 0.5 mole of O_2/mole of phenol should be required. In further studies with exposure of bottled white wine to oxygen, Singleton et al. (112) found similar results. Only 27 to 54% of the vicinal diphenols were oxidized in 10 weeks with a cumulative oxygen exposure of about 70 cm^3/L of wine and the alkaline uptake capacity only decreased 4-6 cm^3/L. Exposure to about 60 cm^3 of O_2/L was required to make the transition from white table wine to sherry.

Wildenradt and Singleton (141) showed that autoxidation of vicinal diphenols in wine-like model solutions produced a coupled oxidation of ethanol to acetaldehyde. The apparent maximum production of aldehyde was 1 mole per mole of phenol oxidized, but was generally less as competing reactions intervened. The postulated reactions are reaction of the vicinal diphenol with one atom from O_2 to produce the *ortho*-quinone and water. The remaining activated oxygen atom combines with water to form hydrogen peroxide. This strong oxidant then attacks the next oxidizable molecule encountered, most often ethanol in wine. This sequence would explain doubling the oxygen consumption over that expected from quinone production alone, but still could not explain the four or more atoms of oxygen consumed per mole of phenol oxidized in alkaline solution in our other

Table 4. Phenol content and O_2 capacity (alkaline) in wine exposed to O_2 (acidic) in storage.

Wine	O_2 Headspace (cm³/L)	Ortho-diphenols		Other phenols		O_2 Capacity		Calc
		mg GAE/L	% loss	mg GAE/L	% loss	Exptl		
						cm³/L	% loss	cm³/L
Juice	0	57	0	208	0	60	0	59
	333	29	49	166	20	39	35	47
8 days on skins	0	182	0	1093	0	199	0	222
	67	165	9	917	16	190	5	191
	143	89	57	593	46	139	30	126
	333	68	62	495	55	103	48	107
16 days on skins	0	278	0	1134	0	255	0	244
	333	78	71	385	66	119	53	90

observations. A further explanation for this will be offered shortly.

This explanation of the source of the aldehydes long known to be produced in aging wines and spirits exposed to air is considered important. Of course, aldehyde production by aerobic film yeast growth is another source. Acetaldehyde has further importance in its ability to react with phenols much like formaldehyde and form cross linkages of one phenol to another. In acidic solution, as for the flavonoid-nonflavonoid assay (62), the reaction would be confined to the phloroglucinol type compounds particularly the A-ring of the flavonoids. This also shows a source and mechanism for the aldehyde which reacts with the anthocyanins producing more color, more stable color, and polymeric products (105,129,130).

There are too many important studies on browning reactions of wine to review in any detail (e.g., 32,33,76,80,82,99,105,138). While the fact that total phenol was only a rough measure of a white wine's relative tendency to brown has been known since early work by Berg and others (105), only fairly recently has it become clear that increased flavonoid content correlated well with increased capacity to brown if the wine is exposed to air (80,96,101).

In accelerated browning tests there is often evidence of an autocatalytic effect (106). At first, brown pigment is slow to develop or even decreases. The reaction then speeds up to a fairly rapid and constant rate for a given wine for some time. It eventually levels off as substrate is depleted or precipitation begins. Laszlo et al. (57) have shown that the rate of disappearance of oxygen increases in sequential saturations of wines. The oxygen from the first saturation of white wine disappeared in over a week, the second in 3 to 5 days, and the third in 1 to 3 days. This autocatalytic effect and the additional consumption of oxygen possible in wine are believed to be explained by the polymerization reactions of the phenols during browning.

The pertinent reactions are outlined in Fig. 3 for simple reactants. The polymerization reaction is easier to visualize as the formation of two semiquinone free radicals by two separate one-electron oxidations and then the new covalent bond forming by the pairing of the unpaired electrons of the two radicals. This is not necessary, however, as a quinone (a highly active elec-

tron acceptor) can interact similarly with a phenol (an active electron contributor). The initial dimer could be peroxide (O-O), ether (C-O), or carbon to carbon bonded and the different products should be in proportion to the relative frequency of the different resonant forms of the intermediates.
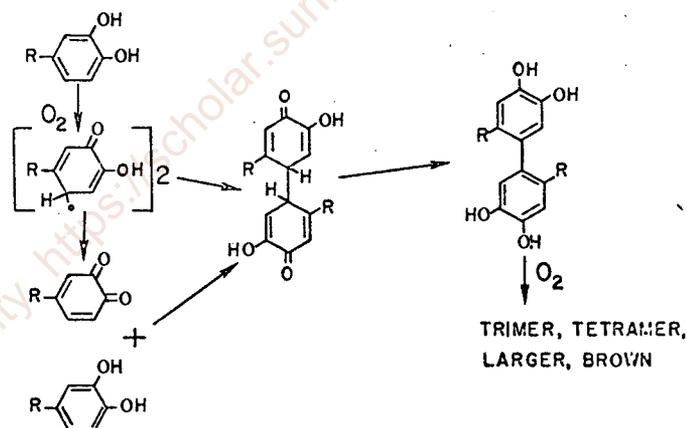

Fig. 3. Regenerative polymerization capable of forming substrates for further oxidation and eventually visibly brown products.

The result of the indicated dimerization is first a diene-one which rapidly tautomerizes (unless prevented in the particular product) to regenerate a phenolic dimer. Oxidation has occurred in that two hydrogens have been removed in converting the monomeric phenols to the dimer. The product, however, is regenerated in as autoxidizable form as the original vicinal diphenol. In fact, from theory and from examples, the new dimer would be more readily oxidized than the original autoxidizable monomer. This is because a phenyl substituent as an electron supplying group would lower the oxidation-reduction potential of the phenol to which it was attached relative to the same phenol with hydrogen in the same position. Furthermore, the extra ring would further delocalize unpaired electrons and thus stabilize intermediate free radicals. Similar reactions can occur between quinones and amines, activated methylene, or sulfur compounds to produce reoxidizable addition products.

The extra possibilities of extended conjugation through the dimer, trimer, etc. would shift the absorbance toward the visible region and lead to an heteroge-

neous family of oxidation products — the brown pigments. As the polymer complexity increases with further oxidation, quinones, quinone methides, other substitution products, and free radicals can be stabilized by stereochemical shielding and contribute to darkening color as in melanins. Electron paramagnetic resonance spectroscopy has shown unpaired electrons present in such brown pigments.

Thus, not only the autocatalytic nature of browning and oxygen uptake, but also the phenols as the source of regenerating oxidizable substrates are explained. Regenerative polymerization can account for the three puzzling observations: 1) a partially oxidized wine oxidizes faster than an unoxidized one, 2) the amount of oxygen a wine can consume is much larger than simple summing of the substrates would appear to explain, and 3) exposure of wine to air oxidation lowers the remaining oxygen capacity less than expected and variably depending on conditions affecting rate (fastest yields least capacity). Its phenols "buffer" the wine against oxidative spoilage by consuming the oxygen, keeping the rest-potential low, and reacting with the co-produced acetaldehyde. The final limit to oxygen uptake in wine would be by the exhaustion of accessible polymerization sites and by precipitation of the oxidation products. That fast alkaline oxidation consumes less O_2 than slower acidic oxidation can is attributable to rapid conversion of all potentially oxidizable phenols to quinones with less opportunity for phenol-quinone polymerization to regenerate oxidizable capacity. Some such reactions occur nevertheless as indicated by the involvement of phenols other than vicinal diphenols (Table 4) in the oxidation.

Prospects: It is with a feeling of excitement that we face the immediate future of the rest of this symposium and the future beyond. The dream of following each important phenol quantitatively during winemaking and aging is coming closer to practicality. We are becoming more comfortable in our understanding of the qualitative composition of different grapes and wine and which substances can be considered as groups in assessing quality relationships, etc. We have some understanding of polymerizations of browning and of polymeric anthocyanins. We need more, and particularly on how the two polymeric conceptions interrelate. The prospects for better wines through further improvements in both avoidance and optimization of aging changes appear bright.

Expansion of interest in viticultural aspects of phenols appears overdue, but in progress. Why are white grapes earlier ripening, generally, than reds? Is it something more fundamental than mere diversion of sugar to anthocyanins? Is it significant that the earliest important red variety is the one without acylated pigments? What are the interrelationships in pest and disease resistance with the phenols? Are they only part of the general wound-healing reactions of plants which strongly involve phenols or are there more specific effects?

Many questions have been answered at least in part about phenols in grapes and wine, many more remain to be answered satisfactorily, and still more probably remain to be posed. The future appears challenging but conquerable.

Note added in proof: The value indicated in Table 1 for the hydroxycinnamates is probably too high. It was arrived at partly by difference after subtracting all other known contributors to the nonflavonoid component. Somers and Ziemelis [J. Sci. Food Agric. 31:600-10 (1980)] have subsequently shown that sulfur dioxide does not just interfere with the Folin-Ciocalteu determination but can give considerable extra augmentation depending upon the relative amount of phenol and sulfur dioxide. This apparently would reduce the value to about 30 mg GAE/L; a value also more in line with HPLC data by Nagel's group and by Hrazdina in this volume.

LITERATURE CITED

1. Akuta, S., H. Ohta, Y. Osajima, N. Matsudomi, and K. Kobayashi. Studies on anthocyanin pigments in fruits harvested in Japan. 6. Anthocyanin pigments in *Vitis vinifera* varieties, Koshu and Gros Colman grapes. Nippon Shokuhin Kogyo Gakkaishi 24(10):521-3 (1977).

2. Amerine, M. A., and C. S. Ough. Recent advances in enology. Crit. Rev. Food Technol. 2:407-515 (1972).

3. Anderson, D. W., D. E. Gueffroy, A. D. Webb, and R. E. Kepner. Identification of acetic acid as an acylating agent of anthocyanin pigments in grapes. Phytochemistry 9:1579-83 (1970).

4. Anderson, D. W., E. A. Julian, R. E. Kepner, and A. D. Webb. Chromatographic investigation of anthocyanin pigments in *Vitis cinerea*. Phytochemistry 9:1569-78 (1970).

5. Arnold, R. A., and A. C. Noble. Bitterness and astringency of grape seed phenolics in model wine solution. Am. J. Enol. Vitic. 29:150-2 (1978).

6. Aubert, S., and C. Poux. Extraction des composés phénoliques du raisin. I. Technique d'estimation d'une vendage. Ann. Technol. Agric. 18:93-108 (1969).

7. Aubert, S., and C. Poux. Extraction des composés phénoliques du raisin. II. Taux de passage dans les vins. Ann. Technol. Agric. 18:111-27 (1969).

8. Bachmann, O. Verbreitung von Phenolcarbonsäuren und Flavonoiden bei Vitaceen. Vitis 17:234-57 (1978).

9. Bachmann, O., and R. Blaich. Vorkommen und Eigenschaften kondensiertes Tannine in Vitaceen. Vitis 18:106-16 (1979).

10. Balaban, A. T., W. Schroth, and G. Fischer. Pyrylium salts. Adv. Heterocyclic Chem. 10:241-326 (1969).

11. Beech, F. W., and C. F. Timberlake. Production and quality evaluation of table wines. J. Inst. Brew. 81:451-65 (1975).

12. Berg, H. W. Stabilisation des anthocyannes. Comportement de la couleur dans les vins rouges. Ann. Technol. Agric. Numero Hors Ser. I. 12(1):247-59 (1963).

13. Berg, H. W., and M. A. Akiyoshi. On the nature of reactions responsible for color behavior in red wine, a hypothesis. Am. J. Enol. Vitic. 26:134-43 (1975).

14. Berger, W. G., and K. Herrmann. Catechine und deren Abbauprodukte in Weintrauben, Trestern, Essigweinen und daraus hergestellten Essigen. III. Z. Lebensm.-Unters.-Forsch. 147:1-8 (1971).

15. Bokuchava, M. A., G. G. Valuiko, and A. M. Filippov. Catechols of grapes and red table wine (trans.). Prikl. Biokhim. Mikrobiol. 6(3):333-7 (1970).

16. Bourzeix, M. Les composés phénoliques du raisin et du vin. Leur transformations au cours de l'elaboration des vins et leurs

effets sur la qualité (vins rouges). Bull. OIV 49:986-1004 (1976).

17. Bourzeix, M., and N. Heredia. Estimation qualitative de la matière colorante des moûts, des moûts concentrés et des vins. Mesure de son taux de polymeres. Vignevini 6(1):19-26 (1979).

18. Bourzeix, M., and H. Saquet, eds. Les anthocyannes du raisin et du vin. Vignes et Vins Spec. No., (May):1-86 (1975).

19. Brouillard, R., and J. E. Dubois. Mechanism of the structural transformations of anthocyanins in acidic media. J. Am. Chem. Soc. 99:1359-64 (1977).

20. Brouillard, R., and B. Delaporte. Chemistry of anthocyanin pigments. 2. Kinetic and thermodynamic study of proton transfer, hydration, and tautomeric reactions of malvidin-3-glucoside. J. Am. Chem. Soc. 99:8461-8 (1977).

21. Brouillard, R., and B. Delaporte. Etude par relaxation chimique des equilibres de transfert de proton, d'hydration et de tautomerie prototropique de l'anthocyane majoritaire de Vitis vinifera, la glucoside-3-malvidine. Protons Ions Involved Fast Dyn. Phenom., Proc. Int. Meet. Soc. Chim. Phys., 30th:403-12 (1978).

22. Brouillard, R., B. Delaporte, and J. E. Dubois. Chemistry of anthocyanin pigments. 3. Relaxation amplitudes in pH-jump experiments. J. Am. Chem. Soc. 100:6202-5 (1978).

23. Burkhardt, R. Analytische Bestimmung phenolischer Inhaltsstoffe von Mosten und Weinen. II. Trennung in niedermolekulare, nicht gerbende phenolische Bestandteile und gerbende oligomere Derivate. Mitteilungsbl. GDCH-Fachgruppe Lebensmittelchem. Gerichtl. Chem. 30:206-13 (1976).

24. Castino, M. Sulla valutazione dei polifenoli totali nei vini rossi. Rev. Vitic. Enol. 32:404-13 (1979).

25. Castino, M., and R. Di Stefano. Frazionamento degli acidi fenolici dei vini bianchi per gel filtrazione. Riv. Vitic. Enol. 29:290-305 (1976).

26. Castino, M., and M. Ubigli. Preparazione di vini bianchi con macerazione di un' aliquota di parti solide. Vignevini 5(3):45-50 (1978).

27. Di Stefano, R. Identificazione di derivati della pelargonidina in un precipitavo proveniente da un mosto in fermentazione. Riv. Vitic. Enol. 32:18-21 (1979).

28. Donko, E. A borok osszesfenol-meghatarozasi modszereinek osszehasonlitasa. Szolesz. Boraszat 1:343-55 (1975).

29. Donko, E., and E. Philiotis. A cukrok zavaro hatasa az osszesfenol-meghatarozasban Folin-Ciocalteu reagens hasznalata eseten. Szolesz. Boraszat 1:357-66 (1975).

30. Drawert, F., G. Leupold, and V. Lessing. Gaschromatographische Bestimmung der Inhaltsstoffe von Garungsgetränken. XII. Wein-Wiss. 33:54-70 (1979).

31. Dubois, P., G. Boulé, M. Ilic, and J. Dekimpe. Étude des phénols volatils de deux vins rouges. Ann. Technol. Agric. 20:131-9 (1971).

32. du Plessis, C. S., Browning of white wines. Die Wynboer (499):11-13 (1973).

33. du Plessis, C. S., and A. L. Uys. Browning in white wines. II. The effect of cultivar, fermentation, husk, seed and stem contact upon browning. S. Afr. J. Agric. Sci. 11:637-48 (1968).

34. Durmishidze, S. V. Les polyphénols du raisin et du vin. Bull. OIV 44 (480):132-5 (1971).

35. Egger, K., J. Reichling, and R. Ammann-Schweizer. Flavonol-Derivate in Fromen der Gattung Vitis. Vitis 15:24-8 (1976).

36. Flanzy, M., M. Bourzeix, and N. Heredia. L'Utilisation de la chromatographie sur couches minces pour separer quantitativement les flavonols des anthocyanes des extraits et jus de raisin et des vins en vue du dosage de ces colorantes jaunes. Qual. Plant. Mater. Veg. 16:297-302 (1968).

37. Fong, R. A., R. E. Kepner, and A. D. Webb. Acetic-acid-acylated anthocyanin pigments in the grape skins of a number of varieties of Vitis vinifera. Am. J. Enol. Vitic. 22:150-5 (1971).

38. Fong, R. A., A. D. Webb, and R. E. Kepner. Acylated anthocyanins in a hybrid Vitis variety. Phytochemistry 13:1001-4 (1974).

39. Gigliotti, A. La determinazione dei composti polifenolici nei vini rossi. Riv. Vitic. Enol. 26:183-93 (1973).

40. Gigliotti, A. La determinazione dei composti polifenolici nei vini bianchi. Riv. Vitic. Enol. 27:206-14 (1974).

41. Glories, Y. Évolution des composés phénoliques au cours du vieillissement du vin. Ann. Nutr. Alim. 32:1163-9 (1978).

42. Glories, Y. Le froid et la matière colorantes des vins rouges. Rev. Fr. Oenol. 19(73):37-40 (1979).

43. Glories, Y., and M. Augustin. Recherches sur la structure et les proprietés des composes phénoliques polymerises des vins rouges. III. Fractionnement des composés phénoliques avec la polyvinylpyrrolidone (P.V.P.). Connaiss. Vigne Vin 10:51-71 (1976).

44. Gueffroy, D. E., R. E. Kepner, and A. D. Webb. Acylated anthocyanin pigments in Vitis vinifera grapes: identification of malvidin-3-(6-p-coumaroyl)-glucoside. Phytochemistry 10:813-19 (1971).

45. Hrazdina, G. Column chromatographic isolation of anthocyanidin-3,5-diglucosides from grapes. J. Agric. Food Chem. 18:243-5 (1970).

46. Hrazdina, G. Anthocyanin composition of Concord grapes. Lebensm. Wiss. Technol. 8:111-13 (1975).

47. Hrazdina, G., and A. J. Borzell. Xanthylium derivatives in grape extracts. Phytochemistry 10:2211-13 (1971).

48. Hrazdina, G., A. J. Borzell, and W. B. Robinson. Studies on the stability of the anthocyanidin 3,5-diglucosides. Am. J. Enol. Vitic. 21:201-4 (1970).

49. Hrazdina, G., and A. J. Franzese. Oxidation products of acylated anthocyanins under acidic and neutral conditions. Phytochemistry 13:231-4 (1974).

50. Hrazdina, G., and A. J. Franzese. Structure and properties of the acylated anthocyanins from Vitis species. Phytochemistry 13:225-9 (1974).

51. Jackson, M. G., C. F. Timberlake, P. Bridle, and L. Vallis. Red wine quality: correlations between colour, aroma, and flavour and pigment and other parameters of young Beaujolais. J. Sci. Food Agric. 29:715-27 (1978).

52. Jurd, L. Review of polyphenol condensation reactions and their possible occurrence in the aging of wines. Am. J. Enol. Vitic. 20:191-5 (1969).

53. Jurd, L. Some advances in the chemistry of anthocyanin-type plant pigments. In: The Chemistry of Plant Pigments. C. O. Chichester, ed. Academic Press, N.Y., Adv. Food Res. Suppl. 3:123-39 (1972).

54. Jurd, L., and T. C. Somers. The formation of xanthylium salts from proanthocyanidins. Phytochemistry 9:419-27 (1970).

55. Langcake, P., and R. J. Pryce. A new class of phytoalexins from grapevines. Experientia 33:151-2 (1977).

56. Langcake, P., C. A. Cornford, and R. J. Pryce. Identification of pterostilbene as a phytoalexin from Vitis vinifera leaves. Phytochemistry 18:1025-7 (1979).

57. Laszlo, J., T. J. von Rooyen, and A. F. Kirschbaum. The combination of molecular oxygen in grape must and young wine and its consequences. S. Afr. J. Sci. 74:95-8 (1978).

58. Lea, A. G. H., and G. M. Arnold. The phenolics of ciders: bitterness and astringency. J. Sci. Food Agric. 29:478-83 (1978).

59. Lea, A. G. H., and C. F. Timberlake. The phenolics of ciders. I. Procyanidins. J. Sci. Food Agric. 25:1537-45 (1974).

60. Lea, A. G. H., P. Bridle, C. F. Timberlake, and V. L. Singleton. The procyanidins of white grapes and wines. Am. J. Enol. Vitic. 30:289-300 (1979).

61. Liao, F. W. H., and B. S. Luh. Anthocyanin pigments in Tinto Cão grapes. J. Food Sci. 35:41-6 (1970).

62. Kramling, T. E., and V. L. Singleton. An estimate of the nonflavonoid phenols in wines. Am. J. Enol. Vitic. 20:86-92 (1969).

63. Margheri, G. Évolution de la couleur des vins au cours de leur conservation et de leur vieillissement. Ann. Technol. Agric. 27:239-52 (1978).

64. Margheri, G., and E. Falcieri. Importanza dell'evoluzione delle sostanze polifenoliche, nei vini rossi di qualità durante l'invecchiamento. Vini Ital. 14:501, 503, 505-11 (1972).

65. Margheri, G., and D. Tonon. Evoluzione del colore dei vini rossi nel corso della loro conservazione e del loro invecchiamento. Vini Ital. 19:264-70 (1977).

66. Martensson, O., and C. H. Warren. Studies of flavylium compounds. 5. On the charge distribution in pyrylium compounds. Acta Chem. Scand. 24:2745-50 (1970).

67. Michaud, J., J. Masquelier, and G. Cipolli. Présence de dérivés du xanthylium dans les vins. Rev. Fr. Oenol. 15(58):40-3 (1975).

68. Montedoro, G., and P. Fantozzi. Dosage des tanins dans les moûts et les vins à l'aide de la methylcellulose et evaluation d'autres fractions phénoliques. Lebensm.-Wiss. Technol. 7:155-61 (1974).

69. Myers, T. E., and V. L. Singleton. The nonflavonoid phenolic fraction of wine and its analysis. Am. J. Enol. Vitic. 30:98-102 (1979).

70. Nagel, C. W., J. D. Baranowski, L. W. Wulf, and J. R. Powers. The hydroxycinnamic acid tartaric acid ester content of musts and grape varieties grown in the Pacific Northwest. Am. J. Enol. Vitic. 30:198-201 (1979).

71. Nagel, C. W., and L. W. Wulf. Changes in the anthocyanins, flavonoids and hydroxycinnamic acid esters during fermentation and aging of Merlot and Cabernet Sauvignon. Am. J. Enol. Vitic. 30:111-16 (1979).

72. Niketić-Aleksić, G. K., and G. Hrazdina. Quantitative analysis of the anthocyanin content in grape juices and wines. Lebensm.-Wiss. Technol. 5:163-5 (1972).

73. Ong, B. Y., and C. W. Nagel. High-pressure liquid chromatographic analysis of hydroxycinnamic acid-tartaric acid esters and their glucose esters in Vitis vinifera. J. Chromatogr. 157:345-59 (1978).

74. Ong, B. Y., and C. W. Nagel. Hydroxycinnamic acid-tartaric acid ester content in mature grapes and during the maturation of White Riesling grapes. Am. J. Enol. Vitic. 29:277-81 (1978).

75. Otsuka, K. Phenolic compounds in wine. Nippon Jozo Kyokai Zasshi 70:533-9 (1975).

76. Ough, C. S. Substances extracted during skin contact with white musts. I. General wine composition and quality changes with contact time. Am. J. Enol. Vitic. 20:93-100 (1969).

77. Ough, C. S., and M. A. Amerine. Studies on controlled fermentation. VII. Effect of antefermentation blending of red must and white juice on color, tannins, and quality of Cabernet Sauvignon wine. Am. J. Enol. Vitic. 13:181-8 (1962).

78. Ough, C. S., and M. A. Amerine. Effect of subjects' sex, experience, and training on their red wine color-preference patterns. Perceptual Motor Skills 30:395-8 (1970).

79. Pallotta, W., and C. Cantarelli. Le catechine: loro importanza sulla qualità dei vini bianchi. Vignevini 6:19-46 (1979).

80. Peri, C., C. Pompei, G. Montedoro, and C. Cantarelli. Maderisation of white wines. I. Influence of pressing on the susceptibility of the grapes to oxidative browning. J. Sci. Food Agric. 22:24-8 (1971).

81. Piretti, M. V., M. Ghedini, and G. Serrazanetti. Isolation and identification of the polyphenolic and terpenoid constituents of Vitis vinifera. V. Trebbiano variety. Ann. Chim. (Rome) 66:429-37 (1976).

82. Pompei, C., and C. Peri. Influenza delle parti solide del grappolo sulla concentrazione in polifenoli dei vini e sulla suscettibilità alla maderizzazione. Riv. Vitic. Enol. 25:367-78 (1972).

83. Ramiah, N., N. B. R. Prasad, and P. Saradamma. Chemical components of Vitis glauca, Hook; (Syn. Cissus glauca Roxb.). J. Indian Chem. Soc. 53:1158 (1976).

84. Rapp, A., O. Bachmann, and H. Steffan. Les composés phénoliques du raisin et du vin. Leurs transformations au cours de l'elaboration des vins et leurs effets sur la qualité (vins blancs). Bull. OIV 50(533):167-96 (1977).

85. Ribéreau-Gayon, P. Différenciation des matières colorantes des raisins et des vins des cépages francais et hybrides. C. R. Acad. Agr. France 39:800-7 (1953).

86. Ribéreau-Gayon, P. Les flavonosides de la baie dans le genre Vitis. C. R. Acad. Sci., Paris 258:1335-7 (1964).

87. Ribéreau-Gayon, P. Identification d'esters des acides cinnamiques et l'acide tartarique dans les limbes et les baies de V. vinifera. C. R. Acad. Sci., Paris 260:341-3 (1965).

88. Ribéreau-Gayon, P. The chemistry of red wine color. In: Chemistry of Winemaking, A. D. Webb, ed. Adv. Chem. Ser. 137:50-87 (1974).

89. Ribéreau-Gayon, P., and Y. Glories. Determination de l'état de condensation des tanins du vin rouge. C.R. Acad. Sci., Paris, D, 273:2369-71 (1971).

90. Ribéreau-Gayon, P., and J. C. Milhe. Le flavannes du raisin. C.R. Acad. Sci. Paris, D. 268:1813-15 (1969).

91. Ribéreau-Gayon, P., and J. C. Milhe. Recherches technologiques sur les composés phénoliques des vins rouges. I. Influence des différentes parties de la grappe. Connaiss. Vigne Vin 4:63-74 (1970).

92. Ribéreau-Gayon, P., and F. Sartore. Les dosage des composés phénoliques totaux dans les vins rouges. Chim. Anal. (Paris) 52:627-31 (1970).

93. Ribéreau-Gayon, P., and E. Stonestreet. La constitution des tanins du raisin et du vin. C. R. Acad. Agr. France 50:662-71 (1964).

94. Ribéreau-Gayon, P., and E. Stonestreet. Les dosage des anthocyanes dans la vin rouge. Bull. Soc. Chim. France 2649-52 (1965).

95. Ribéreau-Gayon, P., P. Sudraud, J. C. Milhe, and A. Canbas. Recherches technologiques sur les composés phénoliques des vins rouges. II. Les facteurs de dissolution des composés phénoliques. Connaiss. Vigne Vin 4:133-44 (1970).

96. Rossi, J. A., Jr., and V. L. Singleton. Contributions of grape phenols to oxygen absorption and browning of wines. Am. J. Enol. Vitic. 17:231-9 (1966).

97. Sakellariades, H. C., and B. S. Luh. Anthocyanins in Barbera grapes. J. Food Sci. 39:329-33 (1974).

98. Sapis, J. C., and P. Ribéreau-Gayon. Étude dans les vins du tyrosol, du tryptophol, de l'alcool phényléthylique et de la γ-butyrolactone, produits secondaires de la fermentation alcoolique. II. Presence et signification. Ann. Technol. Agric. 18:207-19 (1969).

99. Sapis, J. C., and P. Ribéreau-Gayon. Étude du brunissement des vins blancs. II. Influence de certains traitments sur la teneur en composés phénoliques et sur le brunissement. Connaiss. Vigne Vin 3:215-25 (1969).

100. Scheffeldt, P., and G. Hrazdina. Co-pigmentation of anthocyanins under physiological conditions. J. Food Sci. 43:517-20 (1978).

101. Singleton, V. L. Browning of wines. Die Wynboer (455):13-14 (1969).

102. Singleton, V. L. Effects on red wine quality of removing juice before fermentation to simulate variation in berry size. Am. J. Enol. Vitic. 23:106-13 (1972).

103. Singleton, V. L. Analytical fractionation of the phenolic substances of wine and some practical uses of such analyses. In: Chemistry of Winemaking. A. D. Webb, ed. Adv. Chem. Ser. 137:184-211 (1974).

104. Singleton, V. L., and D. E. Draper. The transfer of polyphenolic compounds from grape seeds into wines. Am. J. Enol. Vitic.

15:34-40 (1964).

105. Singleton, V. L., and P. Esau. Phenolic substances in grapes and wine, and their significance. Adv. Food Res. Suppl. 1:1-282 (1969).

106. Singleton, V. L., and T. E. Kramling. Browning of white wines and an accelerated test for browning capacity. Am. J. Enol. Vitic. 27:157-60 (1976).

107. Singleton, V. L., and A. C. Noble. Wine flavor and phenolic substances. ACS Symp. Ser. 26:47-70 (1976).

108. Singleton, V. L., and J. A. Rossi, Jr. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic. 16:144-58 (1965).

109. Singleton, V. L., H. A. Sieberhagen, P. de Wet, and C. J. van Wyk. Composition and sensory qualities of wines prepared from white grapes with and without grape solids. Am. J. Enol. Vitic. 26:62-9 (1975).

110. Singleton, V. L., C. F. Timberlake, and A. G. H. Lea. The phenolic cinnamates of white grapes and wine. J. Sci. Food Agric. 29:403-10 (1978).

111. Singleton, V. L., C. F. Timberlake, and G. C. Whiting. Chromatography of natural phenolic cinnamate derivatives on Sephadex LH-20 and G-25. J. Chromatogr. 140:120-4 (1977).

112. Singleton, V. L., E. Trousdale, and J. Zaya. Oxidation of wines. I. Young white wines periodically exposed to air. Am. J. Enol. Vitic. 30:49-54 (1979).

113. Slinkard, K. Phenolic compounds in the skins of white grapes. Ph.D. thesis, Univ. Calif., Davis (1980).

114. Slinkard, K., and V. L. Singleton. Total phenol analysis: automation and comparison with manual methods. Am. J. Enol. Vitic. 28:49-55 (1977).

115. Somers, T. C. Wine tannins. Isolation of condensed flavonoid pigments by gel filtration. Nature 209:368-70 (1966).

116. Somers, T. C. Grape phenolics, the anthocyanins of *Vitis vinifera* variety Shiraz. J. Sci. Food Agric. 17:215-19 (1966).

117. Somers, T. C. Resolution and analysis of total phenolic constituents of grape pigments. J. Sci. Food Agric. 18:193-6 (1967).

118. Somers, T. C., Pigment profiles of grapes and of wines. Vitis 7:303-20 (1968).

119. Somers, T. C. The polymeric nature of wine pigments. Phytochemistry 10:2175-86 (1971).

120. Somers, T. C. Interpretations of colour composition in young red wines. Vitis 17:161-7 (1978).

121. Somers, T. C., and M. E. Evans. Wine quality: correlations with colour density and anthocyanin equilibria in a group of young red wines. J. Sci. Food. Agric. 25:1369-79 (1974).

122. Somers, T. C., and M. E. Evans. Spectral evaluation of young wines: anthocyanin equilibria, total phenolics, free and molecular sulfur dioxide, "chemical age." J. Sci. Food Agric. 28:279-87 (1977).

123. Somers, T. C., and M. E. Evans. Grape pigment phenomena: interpretation of major colour losses during vinification. J. Sci. Food Agric. 30:623-33 (1979).

124. Somers, T. C., and G. Ziemelis. Interpretations of ultraviolet absorption in white wines. J. Sci. Food Agric. 23:441-53 (1972).

125. Sturua, Z., M. A. Bokuchava, G. G. Valuiko, A. N. Sopromadze, and A. I. Siashvili. Leucoanthocyanins of grapes and wine (trans.). Prikl. Biokhim. Mikrobiol. 9(1):94-8 (1973).

126. Su, C. T., and V. L. Singleton. Identification of three fla-

van-3-ols from grapes. Phytochemistry 8:1553-8 (1969).

127. Sudraud, P. Interprétation des courbes d'absorption des vins rouges. Ann. Technol. Agric. 7:203-8 (1958).

128. Timberlake, C. F., and P. Bridle. Effect of substituents on the ionisation of flavylium salts and anthocyanins and their reactions with sulphur dioxide. Chem. Ind. 1965-6 (1966).

129. Timberlake, C. F., and P. Bridle. Interactions between anthocyanins, phenolic compounds, and acetaldehyde and their significance in red wines. Am. J. Enol. Vitic. 27:97-105 (1976).

130. Timberlake, C. F., and P. Bridle. Anthocyanins: colour augmentation with catechin and acetaldehyde. J. Sci. Food Agric. 28:539-44 (1977).

131. Timberlake, C. F., P. Bridle, M. G. Jackson, and L. Vallis. Correlations between quality and pigment parameters in young Beaujolais red wines. Ann. Nutr. Aliment. 32:1095-101 (1978).

132. Timberlake, C. F., P. Bridle, and S. S. Tanchev. Some unusual anthocyanins occurring naturally or as artifacts. Phytochemistry 10:165-9 (1971).

133. Tromp, A., and C. J. van Wyk. The influence of colour on the assessment of red wine quality. Proc. S. Afr. Soc. Enol. Vitic. 107-18 (1977).

134. Usseglio-Tomasset, L. Acquisitions récentes sur les phénomènes colloidaux dans les moûts et les vins. Ann. Technol. Agric. 27:261-74 (1978).

135. Van Buren, J. P., G. Hrazdina, and W. B. Robinson. Color of anthocyanin solutions expressed in lightness and chromaticity terms. Effect of pH and type of anthocyanin. J. Food Sci. 39:325-8 (1974).

136. Webb, A. D. Volatiles in grapes and wines. Proc. 5th Biennial Int. Codata Conf. 1976:101-8 (1977).

137. Weinges, K., and M. V. Piretti. Procyanidins. XIX. Isolierung des $C_{30}H_{26}O_{12}$-procyanidins B_1 aus Weintrauben. Justus Liebigs Ann. Chem. 748:218-20 (1971).

138. Weinges, K., and M. V. Piretti. Imbrunimento dei vini bianchi. II. Influenza dei polifenoli. Ann. Chim. (Rome). 62:46-53 (1972).

139. Weinges, K., H. Gorissen, and R. Lontie. Les procyanidines dimers naturelles. Ann. Physiol. Veg. 11:67-82 (1969).

140. Whiting, G. C., and R. A. Coggins. The role of quinate and shikimate in the metabolism of lactobacilli. Antonie van Leeuwenhoek J. Microbiol. Serol. 37:33-49 (1971).

141. Wildenradt, H. L., and V. L. Singleton. The production of aldehydes as a result of oxidation of polyphenolic compounds and its relation to wine aging. Am. J. Enol. Vitic. 25:119-26 (1974).

142. Williams, M., G. Hrazdina, M. M. Wilkinson, J. G. Sweeny, and G. A. Iacobucci. High-pressure liquid chromatographic separation of 3-glucosides, 3,5-diglucosides, 3-(6-O-*p*-coumaryl) glucosides and 3-(6-O-*p*-coumarylglucoside)-5-glucosides of anthocyanidins. J. Chromatogr. 155:384-98 (1978).

143. Williams, M., and G. Hrazdina. Anthocyanins as food colorants: effect of pH on the formation of anthocyanin-rutin complexes. J. Food Sci. 44:66-8 (1979).

144. Wulf, L. W., and C. W. Nagel. High-pressure liquid chromatographic separation of anthocyanins of *Vitis vinifera*. Am. J. Enol. Vitic. 29:42-9 (1978).

145. Wulf, L. W., and C. W. Nagel. Identification and changes of flavonoids in Merlot and Cabernet Sauvignon wines. J. Food Sci. 45:479-84 (1980).