Acolein in wine: Bacterial origin and analytical detection

by

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Thesis presented as partial fulfilment for the degree of Master of Science in Analytical Chemistry at the University of Stellenbosch

April 2010

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

22-11-2010

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SUMMARY

Wine quality is compromised by the presence of 3-hydroxypropionaldehyde (3-HPA) due to spontaneous conversion into acrolein under wine making conditions. Acrolein is highly toxic and is presence has been correlated with the development of bitterness in wine. Lactic acid bacterial strains isolated from South African red wine, *Lactobacillus pentosus* and *Lactobacillus brevis*, are implicated in accumulating 3-HPA during anaerobic glycerol fermentation. The environmental conditions leading to its accumulation are elucidated. In aqueous solution 3-HPA undergoes reversible dimerization and hydration, resulting in an equilibrium state between different derivatives. Interconversion between 3-HPA derivatives and acrolein is a complex and highly dynamic process driven by hydration and dehydration reactions. Acrolein is furthermore highly reactive and its steady-state concentration in complex systems very low. As a result analytical detection and quantification in solution is problematic. This study highlights the roles played by natural chemical derivatives and shows that the acrolein dimer can be used as a marker for indicating the presence of acrolein in wines. Solid-phase microextraction (SPME) coupled to gas chromatograph mass spectrometry (GC-MS) was validated as a technique for direct detection of the acrolein dimer in wine. The potential of a recently introduced sorptive extractive technique with a sample enrichment probe (SEP) was also investigated. The SPME technique simplifies the detection process and allows for rapid sampling of the acrolein marker, while SEP is more sensitive.

OPSOMMING

Die teenwoodigheid van 3-hidroksiepropioonaldehied (3-HPA) in wyn het ‘n negatiewe invloed op kwaliteit as gevolg van die moontlike omskakeling na akroleien tydens die wynmaak prosses. Akroleien is hoog toksies en is moontlik betrokke by die ontwikkeling van ‘n bitter komponent in wyn. Hierdie studie wys dat stamme van die melksuurbakteriële *Lactobacillus pentosus* en *Lactobacillus brevis*, geisoleer uit Suid-Afrikaanse wyn, 3-HPA tydens anaerobiese alkoholiese fermentasie kan opbou. Kondisies wat ontwikkeling beinvloed is bestudeer. 3-HPA ondergaan omkeerbare dimerisasie en hidrasie in oplossing en het ‘n ewewig tussen veskillende derivate tot gevolg. Omkakeling tussen 3-HPA derivate en akroleien is ‘n komplekse en hoogs dinamiese prosses wat gedryf word deur hidrasie en dehidrasie reaksies. Akroleien is verder hoogs reaktief en die ewewigskonsentrasie van hierdie aldehied in komplekse omgewings is laag. Analitiese waarneming en kwantifisering is gevolglik problematies. Hierdie studie lig die rol wat natuurlike chemise derivate speel duidelik uit en wys dat die akroleien dimeer as ‘n merker gebruik kan word om die teenwoodigeid van akoleien in wyn te staaf. Soliede-fase mikro-ekstraksie (SPME) gekoppel aan gas chromatografie massa spektroskopie (GC-MS) is gevalideer as ‘n tegniek vir die direkte waarneming van die akroleien dimeer in wyn. Die potensiaal van ‘n nuwe ekstraksie tegniek, gebasseer op ‘n peiler wat vir die monster verreik (SEP), was ook ondersoek. Die SPME tegniek is vinnig en vergemaklik analiese, terwyl SEP meer sensitief is.

BIOGRAPHICAL SKETCH

Rolene Bauer (née Kritzinger) was born in George, South Africa, on 24 August 1973. She obtained a BSc Agric degree, majoring in Microbiology, Biochemistry and Genetics, at Stellenbosch University. All postgraduate studies were conducted in the Department of Microbiology, Stellenbosch University. During this period she was employed as laboratory manager. She obtained a PhD in Microbiology, completed a degree course in Oenology at Stellenbosch University and is a Cape Wine Master. The study presented was conducted while employed as a Post Doctoral Fellow in Plant Biotechnology, Genetics Department, at the same University. Rolene is married to Florian F. Bauer, they have two children and reside in Stellenbosch.

ACKNOWLEDGEMENTS

Prof. Ben Burger, for his guidance and support

Prof. Andrew Crouch, for invaluable discussions

All my colleagues, for their advice, encouragement and friendship

My family, for their love and patience

PREFACE

This dissertation is presented as a compilation of manuscripts and each chapter is introduced separately. Chapter 2 is a review of literature applicable to the field of study, while chapters 3 and 4 present research results. The Appendix is added for additional information pertaining to this study. All manuscripts were submitted for publication in international peer reviewed journals and are written according to the style of the respective journal.

Chapter 1

General Introduction and Project Aims

Chapter 2

Rolene Bauer*, Donald A. Cowan, Andrew Crouch. Acrolein in wine: Importance of 3-hydroxypropionaldehyde and derivatives in production and detection. Submitted for publication: Journal of Agricultural and Food Chemistry.

Chapter 3

Rolene Bauer*, Maret du Toit and Jens Kossmann. Influence of environmental parameters on production of the acrolein precursor 3-hydroxypropionaldehyde by *Lactobacillus reuteri* DSMZ 20016 and its accumulation by wine lactobacilli. International Journal of Food Microbiology, in press.

Chapter 4

Rolene Bauer*, Andrew Crouch, Jens Kossmann, Fletcher Hiten and Ben V. Burger. Acolein dimer as marker for direct detection of acrolein in wine. Submitted for publication: Journal of Agricultural and Food Chemistry.

Chapter 5

Conclusions

Appendix I

Rolene Bauer*, Helène Nieuwoudt, Florian F. Bauer, Jens Kossmann, Klaus R. Koch and Kim H. Esbensen. Fourier transform infrared spectroscopy for grape and wine analysis. Analytical Chemistry, 80:1371–1379 (2008).

CONTENTS

Fourier Transform Infrared Spectroscopy for Grape and Wine Analysis

CHAPTER 1

General introduction and project aims

GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

Acrolein is an α,β-unsaturated carbonyl compound and is also known as 2-propenal or acrylaldehyde. Amongst the compounds in its class, acrolein is by far the strongest electrophile, shows the highest reactivity with nucleophiles, and is therefore a dangerous substance for the living cell (*1*). The compound is a pulmonary toxicant and an irritant of mucous membranes (*2*), and is considered by regulatory agencies to be one of the greatest non-cancer health risks of all organic pollutants. Acrolein has been detected in beverages and its presence correlated with the development of bitterness over time (*3*).

Due to complications with analytical detection of acrolein in solution few studies have evaluated its content in beverages. Levels of up to 2.8 mg/L have been reported in wine (*4, 5*) where glycerol is one of the most important byproducts of alcoholic hexose fermentation by yeasts. Acrolein may be non-enzymatically produced by a secession of H_2O from 3-hydroxypropionaldehyde (3-HPA), a product of bacterial glycerol fermentation. *In vivo*, a coenzyme B12-dependent glycerol dehydratase (EC 4.2.1.30) or its isoenzyme diol dehydratase (EC 4.2.1.29) converts glycerol into 3-HPA (*6, 7*). In the presence of glucose, 3-HPA may be reduced to 1,3-propanediol (1,3-PDO) by a NADH-linked dehydrogenase (1,3-PD oxidoreductase; EC 1.1.1.202). Several organisms are known to transform glycerol into 3-HPA and include the genera *Bacillus*, *Klebsiella*, *Citrobacter*, *Enterobacter*, *Clostridium* and *Lactobacillus* (*8*). Anaerobic utilization of glycerol does not, however, guarantee supply of the acrolein precursor. 3-HPA is normally an intracellular intermediate that does not accumulate, but is reduced to 1,3-PD which is excreted into the extracellular media. To date few lactic acid bacterial strains, all belonging to the genus *Lactobacillus,* have been shown to accumulate 3-HPA in the extracellular media (*9, 19, 11, 12, 13*). As these strains are members of species that not usually do occur in wine, the origin of acrolein in wine is disputed. Acrolein is after all a product of lipid peroxidation reactions that could be ubiquitously generated in biological systems (*14*).

Compounds such as acrolein that display poor chromatographic performance, high reactivity, high volatility or thermal instability, often need a derivatization step during the sample preparation procedure. Such methods are effective for quantification of certain aldehydes and ketones, but have not proved reliable for acrolein and other unsaturated carbonyls (*15*). Problems with derivative analysis include instability, long sample collection times, coelution of similar compounds, and ozone interferences. Although recent advances have been made in establishing derivatization methodology for measuring acrolein in ambient air (*16*), analysis from

liquid samples remains problematic. Being highly polar acrolein is furthermore soluble in water with which it reacts slowly and exothermically to form 3-HPA. Inter-conversion between acrolein and derivatives is a complex, highly dynamic and reversible process driven by hydration and dehydration reactions (*17*). 3-HPA and derivatives are likely to play a central role in the detection of acrolein in aqueous solutions such as wine.

1.2 PROJECT AIMS

1. The first aim of this study was to determine whether the presence of acrolein in wine may be linked to bacterial glycerol fermentation. The specific approaches of this project were as follows:
   i) Glycerol dehydratase-possessing wine lactobacilli were screened for the ability to accumulate the acrolein precursor, 3-HPA.
   ii) The influence of environmental conditions, relevant not only during winemaking but also for biotechnological synthesis, on 3-HPA production by lactobacilli were investigated.

3. The second aim of this study was to evaluate modern extraction techniques coupled to GC-MS as methodology for direct detection of acrolein in wine.

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CHAPTER 2

LITERATURE REVIEW

Acrolein in wine: Importance of 3-hydroxypropionaldehyde and derivatives in production and detection

LITERATURE REVIEW

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ABSTRACT

Lactic acid bacterial strains belonging to the genus *Lactobacillus* have been implicated in accumulating 3-hydroxypropionaldehyde (3-HPA) during anaerobic glycerol fermentation. In aqueous solution 3-HPA undergoes reversible dimerization and hydration, resulting in an equilibrium state between different derivatives. Wine quality is compromised by the presence of 3-HPA due to spontaneous conversion into acrolein under wine making conditions. Acrolein is highly toxic and has been implicated in the development of bitterness in wine. Interconversion between 3-HPA derivatives and acrolein is a complex and highly dynamic process driven by hydration and dehydration reactions. Acrolein is furthermore highly reactive and its steady-state concentration in complex systems very low. As a result analytical detection and quantification in solution is problematic. This paper reviews the biochemical and environmental conditions leading to accumulation of its precursor, 3-HPA. Recent advances in analytical detection are summarized and the roles played by natural chemical derivatives are highlighted.

1. Introduction

Acrolein is an α,β-unsaturated carbonyl compound and is also known as 2-propenal or acrylaldehyde. Amongst the compounds in its class, acrolein is by far the strongest electrophile, shows the highest reactivity with nucleophiles, and is therefore a dangerous substance for the living cell (*1*). The compound is a pulmonary toxicant and an irritant of mucous membranes (*2*), and is considered by regulatory agencies to be one of the greatest non-cancer health risks of all organic pollutants. Thresholds for acute effects of acrolein in humans, according to the International Program on Chemical Safety (IPCS), are summarized in Table 1 (*3*). Acrolein has furthermore been implicated in the development of bitterness in wine where it is non-enzymatically produced by a secession of H_2O from 3-hydroxypropionaldehyde (3-HPA), a product of bacterial glycerol fermentation (Fig. 1). *In vivo*, a coenzyme B12-dependent glycerol dehydratase (EC 4.2.1.30) or its isoenzyme diol dehydratase (EC 4.2.1.29) converts glycerol into 3-HPA (*4, 5*). In the presence of glucose, 3-HPA may be reduced to 1,3-propanediol (1,3-PDO) by a NADH-linked dehydrogenase (1,3-PD oxidoreductase; EC 1.1.1.202). Several organisms are known to transform glycerol into 3-HPA and include the genera *Bacillus*, *Klebsiella*, *Citrobacter*, *Enterobacter*,

Clostridium and *Lactobacillus* (*6*). 3-HPA is mostly an intracellular intermediate that does not accumulate, but is reduced to 1,3-PD. To date lactic acid bacterial strains belonging to the genus *Lactobacillus* were the only isolates shown to accumulate 3-HPA in the extracellular media (*7, 8, 9, 10, 11*).

3-HPA plays a central role, not only in the synthesis, but also the detection of acrolein in aqueous solutions and fermented products such as wine (pH 3 to 4), where glycerol is one of the most important byproducts of alcoholic hexose fermentation by yeasts. Acrolein is spontaneously formed by thermal intramolecular dehydration of 3-HPA and this transformation is enhanced by low pH and/or heat. Analytical detection in an alcoholic water solution is complicated due to near complete interaction with ethanol and water to form 3-Ethoxypropionaldehyde (3-EPA) and 3-HPA, respectively. As acrolein is highly reactive, its steady-state concentration in complex systems is not expected to be high. For these reasons, few studies have evaluated its content in beverages. Distillation has been employed to separate acrolein prior to its determination in wine (*12*) and levels of up to 2.8 mg/L have been reported (*13, 14*). Free 3-HPA is converted to acrolein during the process of distillation. The presence of acrolein was also reported in brandies (*15, 16*), rums and whiskies (*17, 18*), apple eau-de-vie (*19*), ciders (*20*) and beer (*21*).

This review highlights the importance of 3-HPA and derivates in the detection of acrolein in wine. The biochemical and environmental conditions leading to 3-HPA production are also reviewed.

2. Physical and chemical properties

At room temperature acrolein is a highly flammable, clear and colorless liquid with an intense acrid odor reminiscent of tomato fruit (R. Bauer, personal observation). The compound is highly volatile with conversion factors in air at 25°C and 101.3 kPa as follows: 1 mg/m^3 air = 0.44 ppm (*22*). It is very polar and highly soluble in water and many polar organic solvents including ethanol (*23*). Acrolein is the most reactive of the α,β-unsaturated aldehydes due to the conjugation of a carbonyl group with a vinyl group within its structure, conferring two reactive centers: one at the carbon-carbon double bond and the other at the aldehydic group. Typical reactions involving acrolein include Diels-Alder condensations, carbonyl and carbon-carbon double bond additions, oxidation, reduction, dimerization and polymerization. Commercial acrolein is at least 95.5% pure, containing water (up to 3.0% by weight) and other carbonyls (up to 1.5% by weight), mainly propanal and acetone. The pH of commercial acrolein is set with acetic acid between 5 and 6, providing stability by preventing aldol condensation. Hydroquinone is added as an inhibitor of vinyl polymerization (0.1-0.25% by weight). In the absence of an inhibitor, acrolein is subject to highly exothermic polymerization which is catalyzed by light and air at

room temperature to an insoluble, cross-linked solid. Inhibited, acrolein undergoes dimerization above 150°C. In the presence of strong bases or acids, polymerization occurs even with the inclusion of the inhibitor. Relevant physical and chemical data on acrolein are presented in Table 2.

3. Dynamics in aqueous solution

3.1. Acrolein

Acrolein does not contain hydrolysable groups, but reacts with water in a reversible hydration reaction to form 3-HPA. The hydration of acrolein is an equilibrium reaction that approximates first order kinetics with respect to acrolein (*24, 25, 26*). In dilute solution, whether in distilled water or when buffered between pH 5 to 9, the equilibrium constant is pH independent and approaches 12 at 20°C, indicating that approximately 92% of acrolein is in the hydrated form at equilibrium. This constant increases with a decrease in temperature, but also with a rise in initial acrolein concentration (*26*). Dimerization of 3-HPA probably displaces the equilibrium in favor of hydration.

The rate constant, on the other hand, is pH dependent and acrolein appears to be most stable between pH 5 and 6. The constant has been reported to increase with increasing acid concentrations (*24*), but also when the pH was raised from 5 to 9 (*25*). In dilute buffered solution the rate constant (0.015/hr at 21°C) translated to a half-life of 46 h at pH 7 compared to 38 h at pH 8.6. Data for the dependence of rate constant on temperature are not available in the literature, but when acidified solutions of acrolein were heated to 100°C, equilibrium with its hydrated form was reached in approximately 5 min (*24*). The authors have also shown that the rate constant is independent of the initial acrolein concentration.

In contrast to laboratory conditions, loss of acrolein (< 3 mg/L initial concentration) in field experiments was faster and decay continued to completion (*25*). In complex aquatic systems, processes other than hydration may contribute to acrolein dissipation; e.g., volatilization, adsorption, and absorption or uptake by organisms and sediments. Bearing in mind that acrolein moderately absorbs light within the solar spectrum at 315 nm (molar extinction coefficient of 26 L $mol^{-1}cm^{-1}$); the compound may even be photoreactive resulting in photolysis (*27*). The contribution of photolysis is, however, not well established (*28*).

3.2. The HPA system

Axelsson et al. (*29*) first reported a broad-spectrum antimicrobial action displayed by 3-HPA produced by a probiotic strain of *Lactobacillus reuteri.* This compound was patented in 1988 under the name reuterin and is used as a food preservative. In aqueous solution 3-HPA undergoes a reversible dimerization and hydration, resulting in equilibrium between three main forms, also referred to as the HPA system (*30*). As depicted in Figure 1, the forms include monomeric HPA (3-HPA), hydrated monomeric HPA (1,1,3-trihydroxypropane), and cyclic dimeric HPA (2-(2-hydroxyethyl)-4-hydroxy-1,3-dioxane). Few analytical results have been obtained in experimental conditions that were relevant to biological systems. ^{13}C NMR studies revealed that the HPA system is strongly influenced by concentration. Hydration of HPA in aqueous solution increased with dilution to the extent that the HPA dimeric and polymeric forms, predominant at concentrations above 1.2 M, were largely replaced by HPA hydrate when diluted to 0.03 M (Figure 2). The equilibrium state between the different HPA forms was reached within 15 min after dilution. HPA hydrate is likely to remain the dominant form at concentrations below 0.03 M, more relevant for biological systems, as seen by extrapolating the curve.

Figure 3 illustrates various oligomers of 3-HPA that may be present due to the addition of a hydroxyl group to an aldehyde group (*31*). As depicted in this figure, the HPA system and presence of oligomers appear to be pH-dependent. 3-HPA was however shown to be relatively stable in acidic solution at room temperature (*24*). ^{13}C NMR studies revealed little influence of moderate changes in pH (4.1 to 7.0) on the distribution of the three main forms in organic solution (*30*). Unspecified HPA derivatives formed under strong acidic conditions (12% DCl) or at pH 8.9. Clearly, the HPA system is not only highly dynamic, but complex and chemical characterization has proved difficult.

The effect of temperature on the composition of the HPA system has not been extensively studied. The system appears to be relatively stable at 4°C and HPA composition was not affected (*30*). At 20°C the production of acrolein was favored over time. Acrolein arises from the spontaneous intramolecular dehydration of 3-HPA and the process seems to be accelerated under acidic conditions and heat (*32*).

4. Acrolein in wine

4.1. Glycerol metabolism and bitterness

The development of unpleasant bitterness in certain wines has been recorded for decades and remains one of the least understood wine defects. A reduction of bitterness and astringency is generally anticipated as a wine ages due to oxidative polymerization and precipitation of the flavonoid phenols. Pasteur (*33*) first connected the development of bitterness in red wines with bacterial growth and a

concomitant loss of glycerol, while Voisenet (*12*) first correlated bitterness with the presence of acrolein. Acrolein is not a bitter compound, but appears to contribute to bitterness upon interaction with phenolic compounds such as tannins in wine (*34*), explaining why high phenolic red wines rather than white wines are associated with this problem.

Limited information is available on the content and origin of acrolein in wine. Concentrations as low as 10 ppm may cause a bitter taint (*35*). Wines that have undergone malolactic fermentation (MLF) have been reported to contain acrolein and were characterized by reduced glycerol content (*13, 14*).

4.2. Glycerol metabolism and 3-HPA yield

During alcoholic fermentation by yeast, glycerol is the major end product after ethanol and carbon dioxide and could serve as a carbon source for bacteria, especially when fermentable sugars have been exhausted. Microorganisms either produce glycerol from glucose, like yeast, or metabolize glycerol**.** Lactic acid bacteria (LAB) play an important role in malolactic fermentation of wine and may depend on glycerol to maintain viability when sugars are exhausted.

Anaerobic glycerol catabolism is not widespread among LAB and may occur via a reductive and/or an oxidative route (Figure 4). The reductive branch, common to all organisms involved, requires the presence of a functional coenzyme B_{12}-dependent glycerol/diol dehydratase that catalyse dehydration of glycerol into 3-HPA (*36*). Microorganisms that use glycerol as the sole carbon and energy source, more common amongst members of the genera *Citrobacter*, *Klebsiella* and *Enterobacter*, also involve the parallel oxidative biochemical pathway. This route is associated with carbon incorporation into cell mass and not only provides energy for anaerobic growth, but also reducing equivalents in the form of reduced nicotinamide adenine dinucleotide ($NADH_2$). Regeneration of the oxidized form (NAD^+) is achieved through the production of 1,3 PD, the end product of the reductive route, that serves as an electron sink. Yield of 1,3-PD per glycerol molecule is determined by the availability of $NADH_2$, which in turn is affected by the product distribution of the oxidative pathway. When simultaneously metabolized by both pathways, the yield of the reductive route accounts for about 50 to 66% of the glycerol consumed. This oxidative pathway is absent in heterofermentative LAB, since reducing equivalents and energy for growth are supplied by the co-fermentation of an additional substrate such as glucose or fructose (*37*). Co-metabolism of sugar and glycerol allow the generation of additional ATP from acetyl phosphate. The reductive route enables cells to recover the NAD^+ consumed during glycolysis (hexose catabolism), thereby maintaining the redox balance of the 6-phosphogluconate pathway.

Limited information is available regarding the organisms involved in accumulation of 3-HPA in wine (*10*). Krieling (*38*) reported glycerol dehydratase-possessing bacterial strains isolated from red wines. Strains represented species of

the genera *Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Pediococcus*. Anaerobic utilization of glycerol does not, however, guarantee supply of the acrolein precursor. 3-HPA is normally an intracellular intermediate that does not accumulate, but is reduced to 1,3-PD which is excreted into the extracellular media. Until recently members of *L. reuteri*, *L. coryniformis* (*7*) and *L. collinoides* (*8, 9*) were the only strains shown to accumulate 3-HPA in the fermentation medium. As these species do not usually occur in wine, the origin of acrolein in wine is disputed. Acrolein is after all also a product of lipid peroxidation reactions that could be ubiquitously generated in biological systems (*1*). Strains of *L. pentosus, L. brevis* (*10*) and *L. hilgardii* (*11*) isolated from wine were recently implicated with the ability to accumulate the acrolein precursor, 3-HPA. New questions may now be asked; e.g., to what extent do1,3-PDO producers leak 3-HPA into the fermentation media and how common is extracellular 3-HPA production in the microbial world?

4.3. Process conditions and 3-HPA yield

Product yield from glycerol as well as the fate of 3-HPA is not only dependent on the microorganisms and specialized enzymatic pathways involved, but also on the environmental conditions prevailing in wine.

4.3.1. Temperature and pH

Anaerobically cultured *L. reuteri* were reported to produce 3-HPA under physiological conditions of temperature and pH (*39*). Subsequently it was shown that there is no significant difference in 3-HPA production for temperatures between 15 and 37°C (*40*), while production was strongly favored at pH 6 (*10*). Although significant over a broad pH range, production was drastically reduced at pH values applicable to winemaking (pH 3-4).

4.3.2. Substrate availability

The presence of fructose, a residual sugar in wine, and glycerol is favorable for glycerol metabolism and accumulation of 3-HPA by heterofermentative LAB strains (*9*). 3-HPA production by *L. reuteri* was shown to increase with an increase in glycerol concentrations up to 300 mM (*10*), and glycerol dehydratase activity appeared to be inhibited by higher concentrations. When fructose was used as an electron acceptor to reoxidise NADH, the NAD^+/NADH ratio was increased (*9*). This ratio, rather than to the concentration of the nucleotides, is positively correlated to accumulation of 3-HPA (*12, 41*). The main sugar in fermenting grape juice is glucose. For resting *L. reuteri* cells, 1,3-PD was shown to be the major product of glycerol conversion when the molar ratio of glucose to glycerol is greater than 1.6 (*42*). A ratio less than 0.33 favored accumulation of 3-HPA, while no 1,3-PD was formed in the absence of glucose. The ratio between glucose and glycerol levels

therefore appears to be the determining factor regarding the product distribution of the reductive pathway. On the other hand, the presence of glucose, and also lactate, represses reduction of glycerol, probably through the disturbance of the redox balance in resting cells that impacts on the NADH-linked reduction of 3-HPA to 1,3-PD (*42*).

4.3.3. Cell concentration.

3-HPA seems to play a role in regulating its own production through quorum sensing, a mechanism that allows bacteria to sense and express target genes in relation to cell density (*10*). Production increases with an increase in cell concentration up to a threshold value. A sudden and severe drop in 3-HPA content at higher cell concentrations has been reported. The toxic effect of 3-HPA may be circumvented through regulation of its production. Sudden disappearance of 3-HPA from the external cell environment could be ascribed to the consequent enzymatic conversion of 3-HPA into 1,3-PDO and/or 3-hydroxypropionic acid.

5. Analytical detection

Numerous methods for the determination of aldehydes have been published (*43*), but fewer for acrolein. Methods developed for detection of acrolein are summarized in Table 3 (44-58). Spectrophotometric determination with 4-hexylresorcinol and a fluorometric method with m-aminophenol are common procedures. More recent practices involve gas chromatography (GC) and high performance liquid chromatography (HPLC). Compounds such as acrolein that display poor chromatographic performance, high reactivity, high volatility or thermal instability, often need derivatization during the sample preparation procedure. Sampling methods for acrolein and other airborne aldehydes in emissions (e.g. EPA method TO-11A) are generally based on carbonyl derivatizing agents such as 2,4-dinitrophenylhydrazine (DNPH), which produce hydrazones. Environmental samples and drinking-water are typically derivatized with *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine (PFBHA). These derivatives are desorbed with solvents and separated by HPLC followed by UV detection, or more recently identified with GC-MS. Detection limits reported for methods involving GC with electron capture detection and GC-MS with ion-selective monitoring were 3.5 and 16.4 µg/L, respectively (*59*). Such methods are effective for quantification of certain aldehydes and ketones, but have not proved reliable for acrolein and other unsaturated carbonyls (*43*). Problems with derivative analysis include instability, long sample collection times, coelution of similar compounds, and ozone interferences. Although recent advances have been made in establishing derivatization methodology for measuring acrolein in ambient air (*48*), analysis from liquid samples remains problematic.

General trends in the development of modern extraction techniques center on the use of adsorbents or absorbents for selective analyte extraction as an alternative to solvent extraction, and various systems have been developed for this purpose (*59*). Solid-phase microextraction (SPME) is generally preferred, since it is simple to use and the non-automated version requires neither the adaptation of the GC nor the need for additional expensive instrumentation (*60*). While traditional solid phase extraction (SPE) methods tend to be based on phases similar to that of liquid chromatography (LC), SPME phases tend to be similar to GC stationary phases. In this regard SPME is more versatile, since it can be employed for extracting solutes from both liquid and gaseous samples. The major disadvantage of SPME is the amount of available phase, which limits the mass of analyte extracted. Techniques such as stir bar sorptive extraction (SBSE) have been developed to deliver more sorptive stationary phase mass and surface area, and result in correspondingly higher sensitivity (*61*). In SBSE the analytes are enriched in a sorptive rubber sleeve on a magnetic stir bar allowing sampling from gas as well as liquid. Two other promising sample enrichment methods are the high-capacity sorption probe (HCSP) and solid-phase aroma-concentrate extraction (SPACE), developed by Pettersson et al. (*62*) and Ishikawa et al. (*63*), respectively. Since a very small volume of sorptive phase is used in SPME, thermal desorption of the enriched material takes place almost instantaneously. On the other hand, the large volumes of sorptive material employed with SBSE, HCSP and SPACE require cryofocusing of volatiles on the GC column after desorption from the fiber. The recent introduction of the high-capacity sample enrichment probe (SEP) overcomes this problem and allows analysis of volatiles from solid, aqueous and gaseous samples (*64, 65*). As with SPME, desorption and GC separation of the volatiles run almost concurrently, therefore no auxiliary thermal desorption and cryotrapping equipment are required. Another advantage of SEP analysis is the absence of ice formation in the column, a problem that could be encountered if small quantities of moisture are adsorbed during sampling and are subsequently cryotrapped on the column, resulting in the interruption of the carrier gas flow.

Few published studies have reported on the use of modern extraction techniques as alternatives to solvent extraction for direct analysis of acrolein (*56, 66*). Bauer et al. (*57*) recently reported on the suitability of SPME and SEP, in combination with headspace GC-MS, to measure a natural derivative, acrolein dimer, as a marker for detection of acrolein in complex matrixes such as wine.

6. Conclusions

Strains representing several LAB species, all belonging to the genus *Lactobacillus*, have been reported to be able to accumulate 3-HPA during anaerobic glycerol fermentation. This compound is patented under the name reuterin and

displays broad-spectrum antimicrobial activity. In aqueous solution, 3-HPA undergoes a reversible dimerization and hydration thus resulting in equilibrium between three main forms, also referred to as the HPA system. The HPA system is stable at 4°C, hence the use of reuterin in food stored at low temperatures such as dairy products. Spontaneous intramolecular dehydration of 3-HPA results in the formation of acrolein and the process appears to be accelerated under acidic conditions and heat. The formation of acrolein in food or upon indigestion may be an unappreciated risk that deserves further investigation. Temperatures as low as 20°C favor production of acrolein over time, emphasizing the importance of temperature control during the production and storage of high risk products such as wine that contain glycerol. Few studies have evaluated the content of acrolein in beverages and further work is required to fully elucidate the effect of process conditions on the formation in wine. Inter-conversion between acrolein and 3-HPA derivatives is a complex and highly dynamic process driven by hydration and dehydration reactions. Acrolein is furthermore highly reactive and its steady-state concentration in complex systems very low. As a result analytical detection and quantification in solution remains problematic.

Recent progress has, however, been made in determining the conditions required for bacterial production of 3-HPA. Based on these results, recommendations can be made with regard to minimizing its content in wine: 1) Inoculation with selected malolactic starter cultures (*67*) to reduce the risk of growth by 3-HPA producing strains. 2) Ensuring a wine pH between 3 and 4, since bacterial biomass production and 3-HPA accumulation is drastically reduced at low pH. 3) Maintaining reasonable concentrations of free SO_2. Unwanted bacterial growth is inhibited and acrolein forms stable disulfonate adducts in the presence of bisulfite anions. 4) Maintaining low temperatures in the cellar, wine storage facilities and during transport.

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Table 1. Thresholds for the acute effects of acrolein in humans[a]

Effect	Concentration*		Time
	($mg/m^{3)}$	(ppm)	(min)
Odour perception	0.07	0.03	-
Eye irritation	0.10	0.04	5
Nasal irritation	0.30	0.13	10
Increased eye blinking	0.30	0.13	30
Decreased respiratory rate	0.70	0.31	40
Lacrimation	1.00	0.44	5
Extreme irritation of mucosal membranes	2.00	0.88	0.3

[a] Adapted from IPCS Health and Safety Guide No. 67 (3)

* Conversion factor: 1 mg/m^3 air = 0.44 ppm at 25°C and 101.3 kPa (760 mmHg)

Table 2. Physical and chemical properties of acrolein[a]

Property	**Data**
Chemical name	2-Propenal
Alternate names	acrolein, acrylaldehyde
CAS Number	107-02-8
Structural formula	CH2=CHCHO
Molecular weight	56.06
Boiling point (°C at 101.3 kPa)	52.1 to 53.5
Melting point	-86.95°C
Vapour pressure (kPa at 20°C)	29.3 to 36.5
Water solubility (g/L at 20°C)	206-270
Organic solvent solubility	miscible
Henry's law constant (dimensionless at 25°C)	7.8 to 180
Log K_{ow}	-1.1 to 1.02
Log K_{oc}	-0.210 to 2.43
Relative density (20°C)	0.8427 to 0.8442
Relative vapour density	1.94
Vapour pressure (20°C)	29.3 kPa
Log *n*-octanol-water partition coefficient	0.9
Odour perception threshold	0.07 mg/m^3
Odour recognition threshold	O.48 mg/m^3
Explosive limits of vapor and air	2.8% to 31%

[a] Adapted from IPCS Health and Safety Guide No. 67 (*3*)

Table 3. Methods for determination of acrolein

Sample matrix	Sample preparation	Assay	LOD	Reference
Exhaust gas	Derivitize with O-benzyl-hydroxylamine; brominates, reduce and extract with diethyl ether	GC-ECD	Not Reported	*44*
Exhaust gas	Derivatize with DNPH impregnated filters; toluene extraction	GC-FID	0.05 mg/m^3	*45*
Air	Draw air through sodium bisulfite containing cartridge; react with 4-hexylresorcinol in an alcoholic TCA solvent with $HgCl_2$ as catalyst to form a colored complex	Colorimetry	22.9 µg/m^3	*46*
Air	Adsorb on sorbent coated with 2-(hydroxymethyl) piperidine; desorp with toluene	GC-NSD	6.1 µg/m^3	*47*
Air	Collect in sodium bisulfite mist chamber; liberate carbonyls from bisulfite addicts; derivitize with PFBHA; solvent extraction	GC-MS	0.012 µg/m^3	*48*
Moist air	Collect in DNPH-impregnated adsorbent tubes in presence of CaCl; acetonitrile extraction	HPLC-UV	0.01 mg/ m^3	*49*
Biological samples	Derivitize with DNPH; extract with chloroform HCl solvent; dry with nitrogen; dissolve in methanol	HPLC-UV	1 ng	*50*
Liquid and solid wastes	Purge with inert gas; trap with suitable adsorbent; desorp as vapour onto GC column	GC-FID	Matrix dependent	*51*
Water	Derivitize with *O*-methoxylamine; brominates, reduce and extract with diethyl ether	GC-ECD	0.4 µg/L	*52*
Water	Derivitize with PFBHA	GC-MS	Not Reported	*53*
Aqueous solution	Derivitize with PFBHA	MIMS-EIMS	10µg/L	*54*
Cider and Calvados	Derivatize with MBTH	GC-NPD	0.6 - 60 µg/L	*55*
Urine	SPME	GC-MS	60 µg/L	*56*
Wine	SPME/SEP; detection of acrolein dimer, a natural derivative	GC-MS		*57*

LOD, limit of detection; DNPH, 2,4-dinitrophenylhydrazine; TCA, trichloroacetic acid; PFBHA, *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine, MBTH, 3-methylbenzothiazolone hydrazine; ECD, electron capture detection; FID, flame ionization detection; GC, gas chromatography; HPLC, high-performance liquid chromatography; UV, ultraviolet; MIMS, membrane introduction mass spectrometry, EIMS; electron impact mass spectrometry; MS, mass spectrometry; NSD, nitrogen selective detection; SPME, Solid-phase microextraction; SEP, sample enrichment probe

Figure 1. Glycerol catabolism and acrolein production. Enzyme 1: coenzyme dependent glycerol dehydratase; Enzyme 2: 1,3-propanediol oxidoreductase; $\rightleftharpoons$ equilibrium reactions.

Figure 2. Concentration-dependent distribution of the main forms of the HPA system in D_2O at 20 °C measured by quantitative ^{13}C NMR (adapted from *30*).

Figure 3. Chemical structures of the HPA system and oligomers that may be present in acidic and basic environments (adapted from *31*).

Figure 4. Anaerobic glycerol fermentation. The reactions up to pyruvate are common to all organisms involved. Pyruvate utilization differs amongst microorganisms.

Figure 1

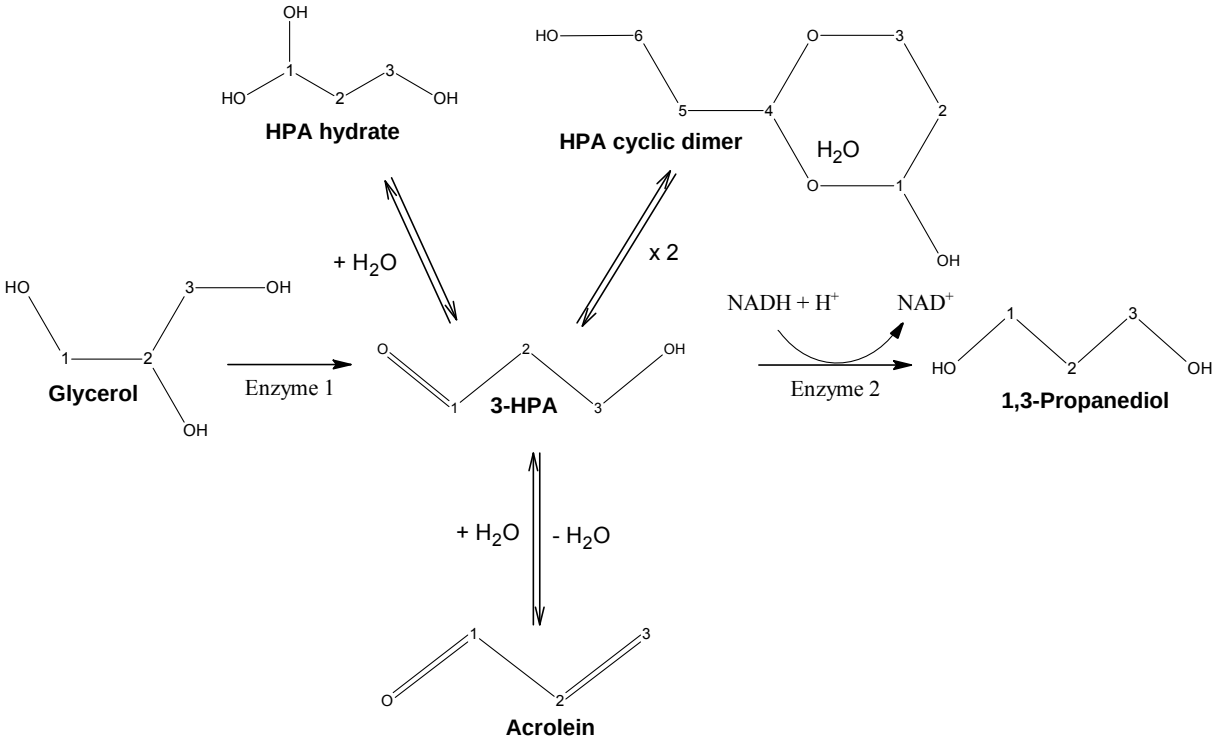

HPA hydrate
HPA cyclic dimer
H_2O
+ H_2O
x 2
Glycerol
Enzyme 1
3-HPA
NADH + H^+
NAD^+
Enzyme 2
1,3-Propanediol
+ H_2O
- H_2O
Acrolein

Figure 2

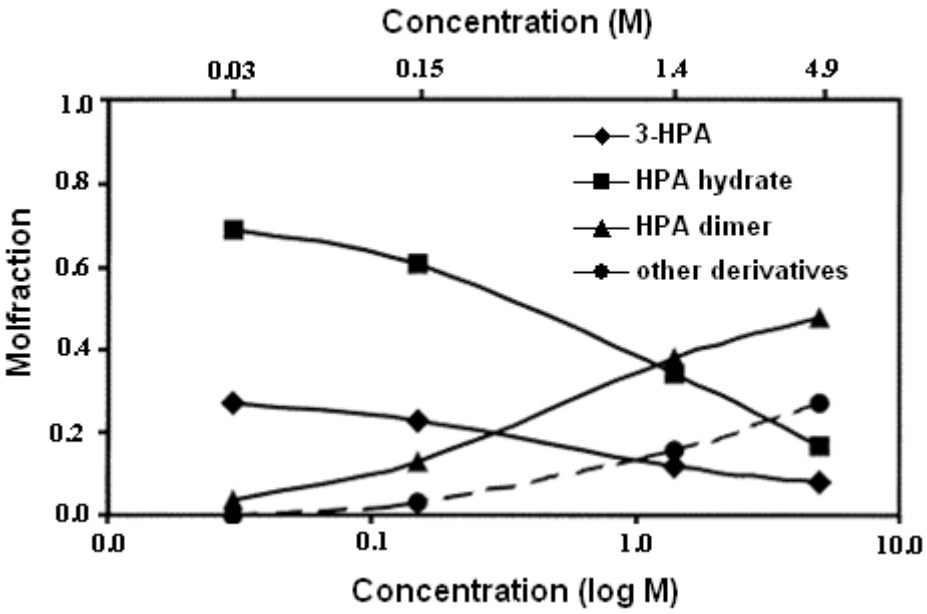

Concentration (M)
0.03
0.15
1.4
4.9
1.0
0.8
0.6
0.4
0.2
0.0
Molfraction
3-HPA
HPA hydrate
HPA dimer
other derivatives
0.0
0.1
1.0
10.0
Concentration (log M)

Figure 3

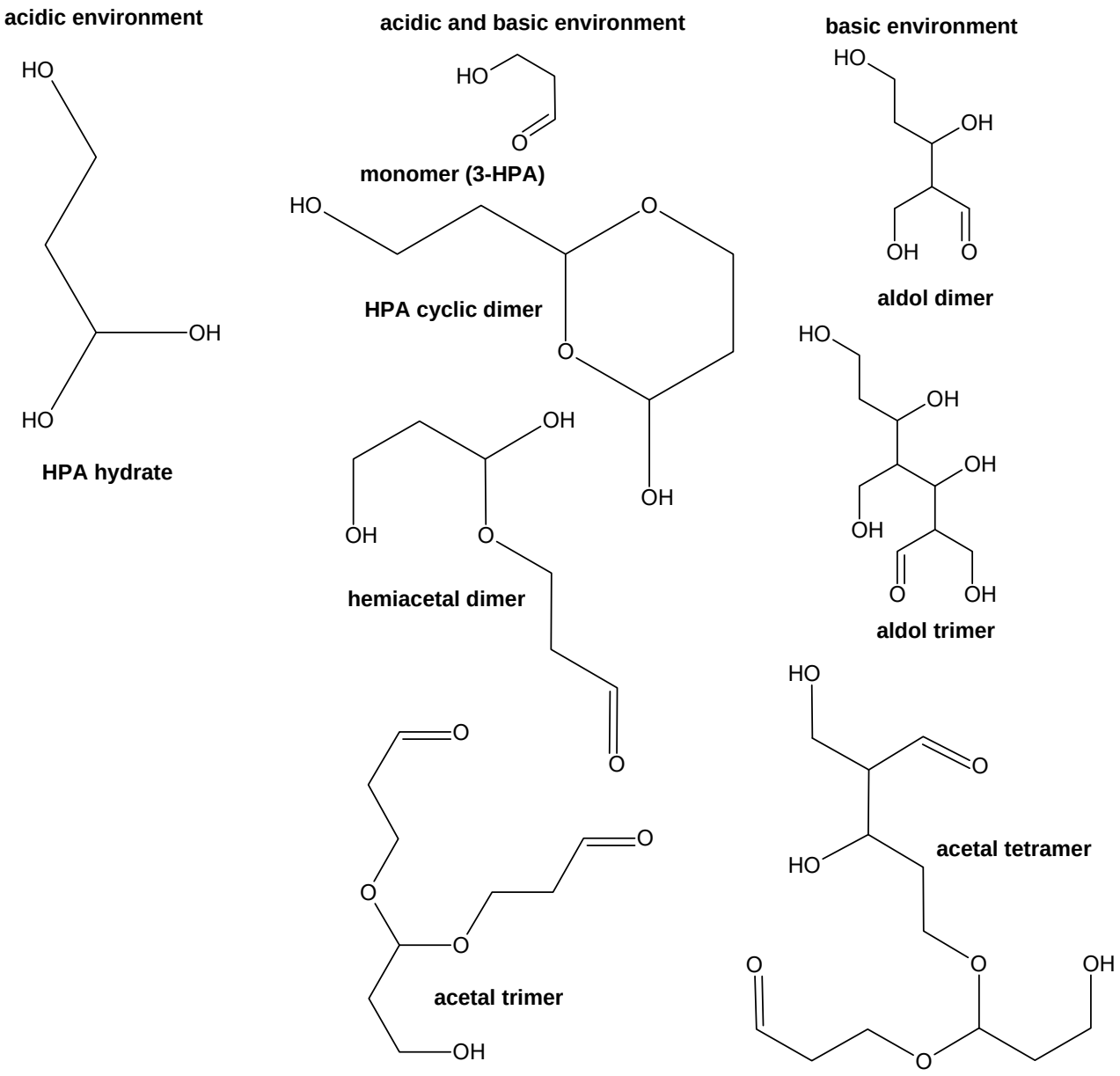
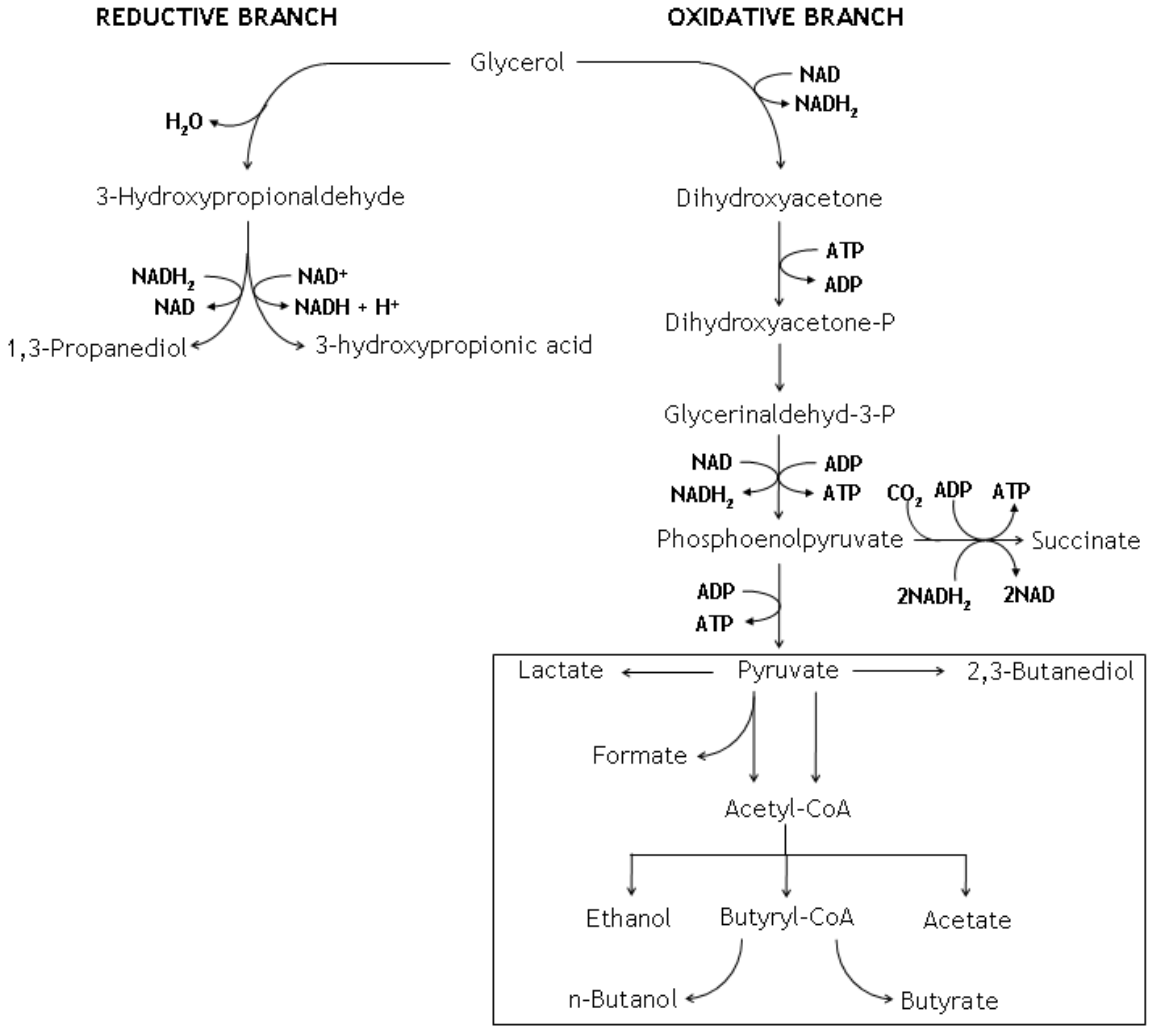

acidic environment
HO
OH
HO
HPA hydrate
acidic and basic environment
HO
O
monomer (3-HPA)
HO
O
HPA cyclic dimer
O
OH
OH
OH
O
hemiacetal dimer
O
O
O
O
O
acetal trimer
OH
basic environment
HO
OH
OH
O
aldol dimer
HO
OH
OH
OH
O
OH
aldol trimer
HO
O
HO
acetal tetramer
O
O
OH
O

Figure 4


REDUCTIVE BRANCH
OXIDATIVE BRANCH
Glycerol
H2O
NAD
NADH2
3-Hydroxypropionaldehyde
Dihydroxyacetone
NADH2
NAD
NAD+
NADH + H+
1,3-Propanediol
3-hydroxypropionic acid
ATP
ADP
Dihydroxyacetone-P
Glycerinaldehyd-3-P
NAD
NADH2
ADP
ATP
CO2
ADP
ATP
Phosphoenolpyruvate
Succinate
2NADH2
2NAD
ADP
ATP
Lactate
Pyruvate
2,3-Butanediol
Formate
Acetyl-CoA
Ethanol
Butyryl-CoA
Acetate
n-Butanol
Butyrate

CHAPTER 3

RESEARCH RESULTS

Influence of environmental parameters on production of the acrolein precursor 3-hydroxypropionaldehyde by *Lactobacillus reuteri* DSMZ 20016 and its accumulation by wine lactobacilli

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ABSTRACT

Wine Lactic acid bacteria belonging to the genus *Lactobacillus* are known to convert glycerol into 3-hydroxypropionaldehyde (3-HPA) during anaerobic glycerol fermentation. Wine quality can be gravely compromised by the accumulation of 3-HPA, due to its spontaneous conversion to acrolein under wine making conditions. Acrolein is not only a dangerous substance for the living cell, but has been implicated in the development of unpleasant bitterness in beverages. This study evaluates the effect of individual environmental parameters on 3-HPA production by *Lactobacillus reuteri* DSMZ 20016, which only proved possible under conditions that allow accumulation well below the threshold concentration affecting cell viability. 3-HPA production was optimal at pH 6 and in the presence of 300 mM glycerol. Production increased with an increase in cell concentration up to an OD_{600} of 50, whereas higher cell concentrations inhibited accumulation. Data presented in this study suggest that 3-HPA plays a role in regulating its own production through quorum sensing. Glycerol dehydratase possessing bacterial strains isolated from South African red wine, *L. pentosus* and *L. brevis*, tested positive for 3-HPA accumulation. 3-HPA is normally intracellularly reduced to 1,3-propanediol. This is the first study demonstrating the ability of wine lactobacilli to accumulate 3-HPA in the fermentation media. Recommendations are made on preventing the formation of acrolein and its precursor 3-HPA in wine.

1. Introduction

Axelsson et al. (1987) first reported broad-spectrum antimicrobial action of 3-hydroxypropionaldehyde (3-HPA) produced by *Lactobacillus reuteri.* This compound was protected by patent in 1988 under the name reuterin and is proposed to be largely responsible for the probiotic effects of *L. reuteri* (Talarico et al., 1988). Probiotics are live microbial feed supplements which when administered in adequate amounts confer a health benefit on the host (WHO, 2002). Hence, *L. reuteri* is applied as a probiotic in the health care of humans and animals, while the food industry has started industrial applications with *L. reuteri* to enhance the quality and value of milk products and is using reuterin as a food preservative. Lactic acid

bacteria (LAB) belonging to the genus *Lactobacillus* are known to convert glycerol into 3-HPA by a coenzyme B12-dependent glycerol dehydratase (EC 4.2.1.30) or its isoenzyme diol dehydratase (EC 4.2.1.29) (Smiley and Sobolov, 1962; Toraya et al., 1976). 3-HPA is normally an intracellular intermediate that does not accumulate, but is reduced to 1,3-propanediol (1,3-PDO) by a NADH-linked dehydrogenase (1,3-PD oxidoreductase; EC 1.1.1.202). Only a few lactobacilli strains have been identified with the ability to accumulate 3-HPA extracellularly (Garai-Ibabe et al., 2008; Martin et al., 2005; Pasteris and Strasser de Saad, 2009; Sauvageot et al., 2000). 3-HPA is a valuable compound that serves as a precursor for chemicals such as acrolein, acrylic acid, 1,3-PDO, and polymers (Vollenweider and Lacroix, 2004). Production is generally achieved through traditional chemistry from petrochemical resources as a dehydration product of glycerol. Due to increasing public demand for environmentally friendly products, recent studies aimed at optimizing bacterial 3-HPA production (Doleyres et al., 2005; Zamudio-Jaramillo et al., 2008).

3-HPA is a precursor to acrolein which is spontaneously produced through the secession of H_2O under conditions of low acidity and/or heat. Acrolein, being a highly reactive α,β-unsaturated aldehyde, is a pulmonary toxicant and an irritant of mucous membranes that is considered by regulatory agencies to be one of the greatest non-cancer health risks (ORL-RBT LD50 = 7 mg kg^{-1}; ORL-RAT LD50 = 46 mg kg^{-1})(Esterbauer et al., 1991; Seaman et al., 2006). Acrolein is very polar and highly soluble in water and organic solvents, such as ethanol. Since analytical detection of acrolein in alcoholic water solution is complicated, few studies evaluated its content in beverages (Bauer et al., unpublished results). Its presence has however been reported in fermented foodstuffs, such as beer, wine and their distillation products, where glycerol is one of the most important by-products of alcoholic fermentation by yeasts. Levels of up to 2.8 mg/L have been reported in wine (Dittrich et al., 1980; Sponholtz, 1982).

Wine quality can be compromised by the presence of 3-HPA, due its spontaneous conversion to acrolein under wine making conditions (Bauer et al., unpublished review). Acrolein is not only highly toxic, but may result in the development of bitterness upon combination with polyphenols in wine (Rentschler and Tanner, 1951). Ethanol increases the intensity of the bitter taste as well as the duration of the bitter sensation (Noble 1994). For wines destined for distillation to make e.g. brandies the concern is greater, since acrolein is easily produced from 3-HPA when exposed to heat. Wine quality is also jeopardized through the disappearance of glycerol, a principal component of wine (Bauer et al., 2008) that plays an important role in sensorial evaluation (Nieuwoudt et al., 2002).

This study evaluates the influence of environmental conditions, relevant not only during winemaking but also for biotechnological synthesis, on 3-HPA production by *Lactobacillus reuteri* DSMZ 20016. Glycerol dehydratase-possessing wine lactobacilli were screened for the ability to accumulate this compound.

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2. Materials and methods

2.1. Strains and growth conditions

LAB strains were cultured in de Man, Rogosa, and Sharpe (MRS) medium (Biolab, Merck, South Africa; De Man et al., 1960) at 30 °C without agitation. Environmental conditions leading to 3-HPA production were investigated using *L. reuteri* strain DSMZ 20016. Glycerol dehydratase-possessing lactobacilli (25 strains), identified with species-specific PCR primers as members of the species *L. plantarum*, *L pentosus*, *L. hilgardii*, *L. paracasei* and *L. brevis* (Krieling, 2003), were screened for the accumulation of 3-HPA. Krieling (2003) isolated these strains during commercial red wine fermentations (alcoholic and malolactic) and the presence of the glycerol dehydratase gene was confirmed with the GD1 and GD2 PCR primers (Claisse and Lonvaud-Funel, 2001). 3-HPA production was determined for washed cells cultured in MRS broth until late-exponential phase and in the presence of 20 mM glycerol.

2.2. Bacterial 3-HPA production from glycerol

L. reuteri cells, cultured without agitation in MRS for 24 hrs until late-exponential phase (OD_{600} = 2 to 3) and in the presence of 20 mM Glycerol, were harvested by centrifugation (3,000 x *g* for 5 min at 20 °C) and washed with potassium phosphate buffer (0.1 M, pH 6.0). For determining pH dependence of 3-HPA accumulation, washed cells were resuspended to an OD_{600} = 10 (*ca* 1 x 10^9 cfu/ml) in either 0.1 M potassium phosphate buffer (pH 6 to 8) or 0.1 M Na-acetate buffer (pH 3 to 5). Cellular suspensions (OD_{600} = 10) were supplemented with a fixed concentration of glycerol, periodically sampled, and analyzed for bioconversion to 3-HPA at 20°C. The effects of initial biomass concentration (OD_{600} values ranging from 10 to 70) and glycerol concentration (100 to 400 mM) on the accumulation of 3-HPA were studied independently. Cell suspensions prepared with wine lactobacilli (OD_{600} = 10) were analyzed at pH 6 and with 300 mM glycerol.

Samples collected for 3-HPA quantification were centrifuged (15,000 x *g* for 5 min at 4 °C), the supernatants sterile-filtered (0.22 µm), and stored at -20 °C until measurement. The initial rate of accumulation was estimated from the slope of the first few data points. Reported data are means for triplicate experiments and analyses. Dilution and plating of samples for viable cell enumeration were carried out immediately after sampling as described in Bauer et al., (2009). Cell enumeration was performed in duplicate by plate count on MRS agar.

2.3. Quantification of 3-HPA

The assay for 3-HPA content was based on a colorimetric method by Cohen and Altshuller (1961) developed for acrolein. 3-HPA was first dehydrated to acrolein, which in turn reacts with 4-hexylresorcinol, in the presence of $HgCl_2$ as catalyst, to form a colored complex that absorbs light at 605 nm. In short, 0.5 ml saturated ***trichloroacetic acid (TCA)***, 0.012 ml of a 4-hexylresorcinol solution (50% w/v in ethanol) and 0.02 ml of an $HgCl_2$ solution (3% w/v in ethanol) were mixed with 0.5 ml of sample. The mixture was incubated at 60 °C for 15 min, allowed to cool down at 20 °C for an additional 15 min and the absorbance immediately recorded at 605 nm. Samples were diluted to assure absorbance readings below 0.85. Since 3-HPA is not commercially available, acrolein was used to standardize the assays (1 mol of 3-HPA dehydrates to 1 mol of acrolein). The standard curve was prepared in the 0.02 to 0.50 mM range (R^2 = 0.999) with a relative standard deviation (n = 10) of 2.1 %.

3. Results and Discussion

3.1. Environmental conditions affecting 3-HPA accumulation

3-HPA at a certain threshold concentration is toxic for the producing strain, while cell viability was shown to be crucial for glycerol biotransformation (Vollenweider and Lacroix, 2004). As a consequence, little is known about the process conditions affecting 3-HPA accumulation. Preliminary results revealed that *L. reuteri* cell viability was not significantly affected ($P<0.025$) at concentrations below 35 mM over a period of at least 2 hrs (data not shown). This study evaluates the effect of individual environmental parameters on bacterial production, rather than aiming for optimal 3-HPA yield (Doleyres et al., 2005; Zamudio-Jaramillo et al., 2008). This proved only possible under conditions that allow 3-HPA accumulation below the threshold concentration affecting cell viability.

A two-step process, consisting of biomass production followed by glycerol biotransformation to 3-HPA by resting cells, was employed (Doleyres et al., 2005). Reproducibility of biotransformation was ensured by buffering cell suspensions, rather than preparing suspensions in water. Glucose was omitted to prevent 3-HPA reduction to 1,3-PDO (Lüthi-Peng et al., 2002). Temperature was maintained at 20 °C, as no significant difference in 3-HPA production was observed for temperatures between 15 and 37 °C (Doleyres et al., 2005).

The effect of pH on 3-HPA accumulation was studied in the presence of 300 mM glycerol. *L. reuteri* cells were resuspended to an OD_{600} = 10 (*ca* 1 x 10^9 cfu/ml) in the appropriate buffer (pH range 3 to 8). Although evident over the entire range,

production was strongly favored at pH 6 (Fig. 1). Considering also the measured increase in 3-HPA over a period of 90 min, all further studies were determined at pH 6.

The effect of glycerol concentrations, ranging from 100 mM to 400 mM, on 3-HPA accumulation is depicted in Fig. 2. The initial rate of production increased with a raise in glycerol concentration up to the highest concentration tested. Highest 3-HPA yield was however achieved with 300 mM, approaching a maximum value after 120 min compared to 90 min with 400 mM. Glycerol dehydratase activity appears to be inhibited by high concentrations of substrate, as suggested previously (Kajiura et al. 2001; Knietsch et al. 2003; Talarico and Dobrogosz 1990; Toraya et al., 1976).

The effect of biomass concentrations on 3-HPA accumulation (Fig. 3) was determined with cell suspensions ranging in OD_{600} between 10 and 70 (*ca.* $1x10^9$ to $7x10^9$ cfu/ml). Yield was restricted by limiting the glycerol content to 50 mM. At all cell densities tested, viability was not significantly affected ($P<0.025$) over a period of at least 2 hrs. The rate of 3-HPA production increased with an increase in cell density. At the highest mass tested (OD_{600} = 70), production peaked after about 50 minutes followed by a sudden and severe drop in 3-HPA content. These data suggest that 3-HPA plays a role in regulating its own production, possibly to circumvent its toxic effect at high concentrations. Regulation of microbial metabolic pathways is known to be coupled to cell density in many bacteria (Whitehead et al., 2001). Chung et al. (1989) observed that 3-HPA production was increased in the presence of heterologous cells, suggesting that *L. reuteri* may possess a mechanism for sensing the number of cells and respond to this stimulus by producing 3-HPA. This phenomenon is known as quorum sensing, a mechanism that allow bacteria to sense and express target genes in relation to cell density (Anderson et al., 2001).

The sudden drop in 3-HPA concentration is surprising. Disappearance of 3-HPA from the external cell environment was also evident when 3-HPA was produced at very high concentrations (Doleyres et al., 2005; Zamudio-Jaramillo et al., 2008) and was ascribed to cell death, dimerization or binding to cellular matter. With this study, maximum 3-HPA yield was low and cells therefore remained viable, while the analytical method employed ensures dehydration and quantification of 3-HPA and its hydration products (Bauer et al., unpublished review). Binding of 3-HPA to cellular matter (Sung et al., 2003) is expected to happen more gradually and should also be evident at lower cell densities. A possible explanation may be the enzymatic reduction of 3-HPA into 1,3-PDO, a pathway that is tightly regulated (Lüthi-Peng et al., 2002) and normally occurs intracellularly. Alternatively, 3HPA may be used as a substrate to generate 3-hydroxypropionic acid (Garai-Ibabe et al., 2008).

3.2. Accumulation of 3-HPA by wine lactobacilli

Glycerol dehydratase-possessing *Lactobacillus* strains isolated from wine (Krieling, 2003) were screened for 3-HPA accumulation by employing the

methodology and conditions established in this paper. Albeit at lower concentrations, two strains tested positive for 3-HPA secretion namely, *L. pentosus* (strain 46) and *L. brevis* (strain 89)(Fig. 4). This is the first report on 3-HPA accumulation by members of these species.

Several organisms are known to transform glycerol into 3-HPA and include the genera *Bacillus*, *Klebsiella*, *Citrobacter*, *Enterobacter*, *Clostridium* and *Lactobacillus* (Bauer et al., unpublished review). 3-HPA is however by and large an intracellular intermediate that does not accumulate, but is reduced to 1,3-PDO which is secreted into the extracellular media. Lactic acid bacterial strains belonging to the genus *Lactobacillus* are the only isolates shown to accumulate 3-HPA extracellularly and include members of *L. reuteri* (Talarico et al., 1988), *L. coryniformis* (Martin et al., 2005) and *L. collinoides* (Garai-Ibabe et al., 2008; Sauvageot et al., 2000). The presences of these organisms are however not anticipated in wine, which raised the question on the origin of acrolein in wine. Acrolein is after all not just a by-product of anaerobic glycerol fermentation, but is also a product of lipid peroxidation reactions that could be ubiquitously generated in biological systems (Uchida et al., 1998). Recently Pasteris and Strasser de Saad (2009) implicated another wine isolate, *L. hilgardii* (strain X_1B), with the ability to accumulate 3-HPA. Having now identified a few wine lactobacilli with the ability to biotransform glycerol and accumulate the acrolein precursor 3-HPA, new questions may be asked. How common is extracellular 3-HPA accumulation in the microbial world? To what extent do 1,3-PDO producers leak 3-HPA into the fermentation media?

The fate of 3-HPA is dependent on the microorganisms and specialized enzymatic pathways involved, as well as the environmental conditions prevailing in wine. Product yield under winemaking conditions needs to be established and further work is required to fully elucidate the presence of acrolein in wine. Based on the limited knowledge available, a few recommendations are made to minimizing its presence. 1) Most importantly, prevent the growth of unwanted LAB strains in wine. Winemaking normally involves two fermentation processes: an alcoholic fermentation conducted by yeast, and malolactic fermentation (MLF) performed by LAB. Bauer and Dicks (2004) recently reviewed methodology for the control and/or prevention of MLF in wine. While encouraged in cool viticulture regions, uncontrolled MLF, especially in wines with a high pH typical of warmer viticulture regions, may cause spoilage or result in the accumulation of 3-HPA. Inoculation with selected LAB starter cultures reduces the risk of growth by unwanted strains. 2) Ensure a wine pH between 3 and 4. Bacterial biomass production and 3-HPA accumulation is drastically reduced at low pH. 3) Maintain reasonable concentrations of free SO_2. Not only is unwanted bacterial growth inhibited, but acrolein was shown to form stable disulfonate adducts in the presence of bisulfite anions (Seaman et al., 2006). 4) Maintain low temperatures in the cellar, wine storage facilities and during transport. Although 3-HPA is relatively stable at 4 °C, incubation at 20 °C favored production of acrolein over time (Vollenweider et al., 2003).

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Figure 1
The effect of pH on 3-HPA accumulation by resting cells of *L. reuteri* DSMZ 20016 (OD_{600} = 10) with 300 mM of glycerol. Data are presented as an average of three experimental repeats with values not varying by more than 5%. Error bars were omitted as they complicate the figure.

Figure 2
The effect of glycerol concentration on 3-HPA accumulation by resting cells of *L. reuteri* DSMZ 20016 (OD_{600} = 10) at pH6. Data are presented as an average of three experimental repeats with values not varying by more than 5%. Error bars were omitted as they complicate the figure.

Figure 3
The effect of cell concentration on 3-HPA accumulation by resting cells of *L. reuteri* DSMZ 20016 at pH6 and with 50 mM glycerol. Data are presented as an average of three experimental repeats with values not varying by more than 5%. Error bars were omitted as they complicate the figure.

Figure 4
3-HPA accumulation by resting cells of wine lactobacilli (OD_{600} =10) at pH6 and with 300 mM glycerol. Error bars represent the RSD of the data (n=3).

Figure 1

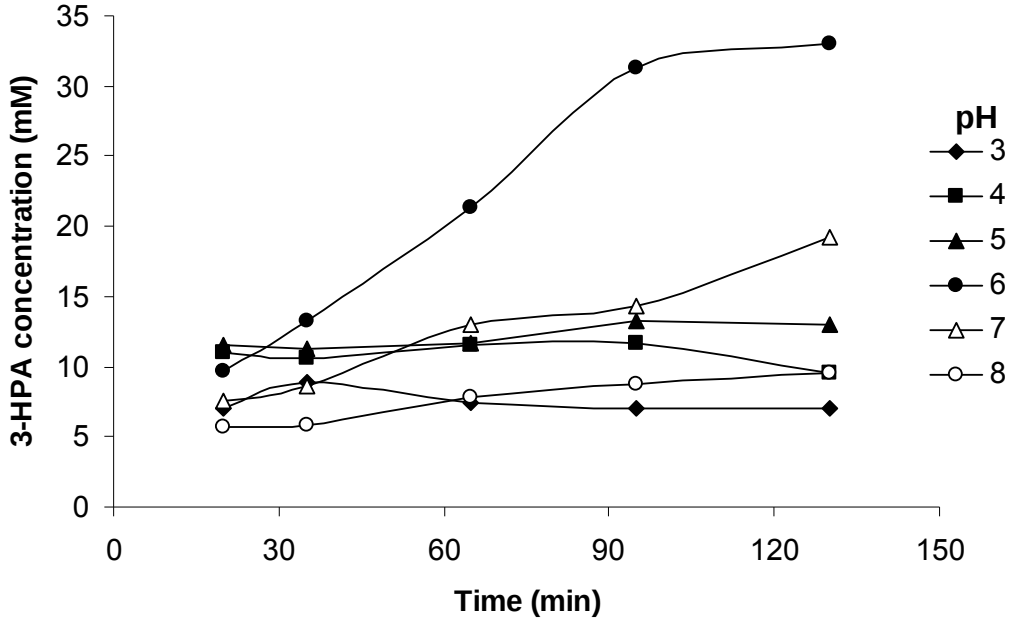

3-HPA concentration (mM)
35
30
25
20
15
10
5
0
0
30
60
90
120
150
Time (min)
pH
3
4
5
6
7
8

Figure 2

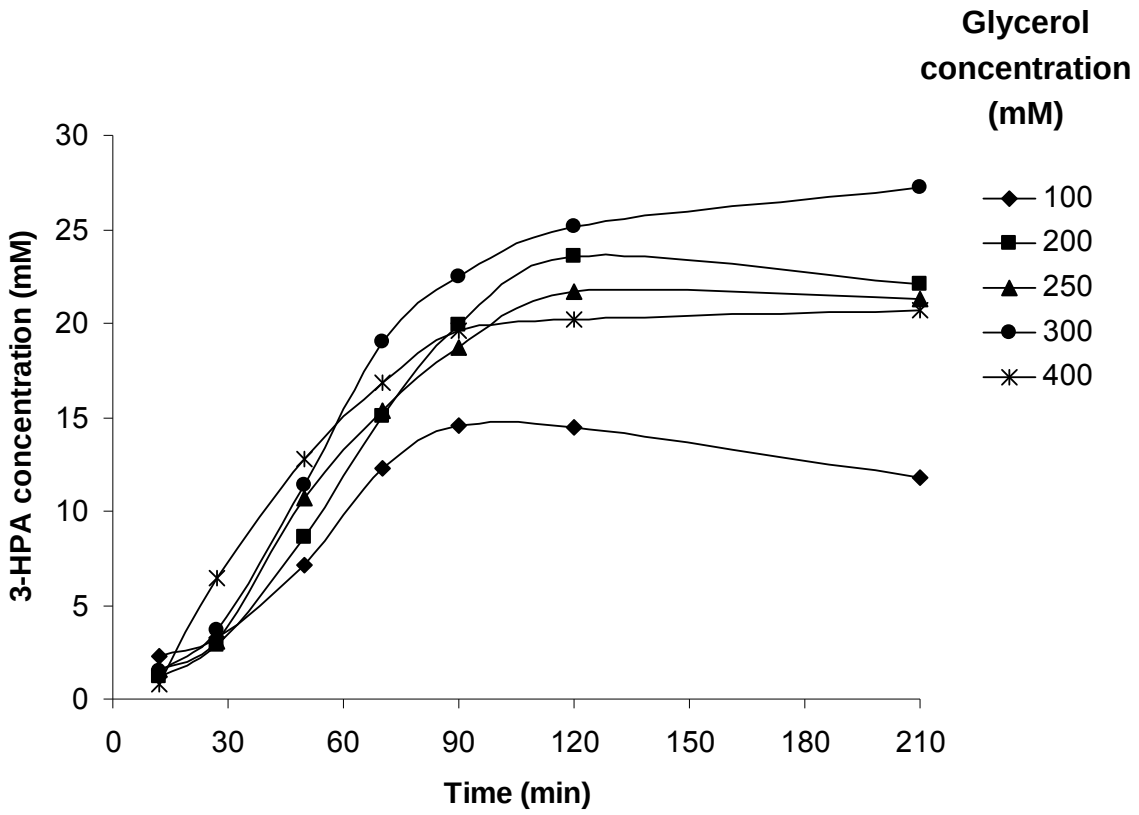

Glycerol concentration (mM)
100
200
250
300
400
3-HPA concentration (mM)
0
5
10
15
20
25
30
0
30
60
90
120
150
180
210
Time (min)

Figure 3

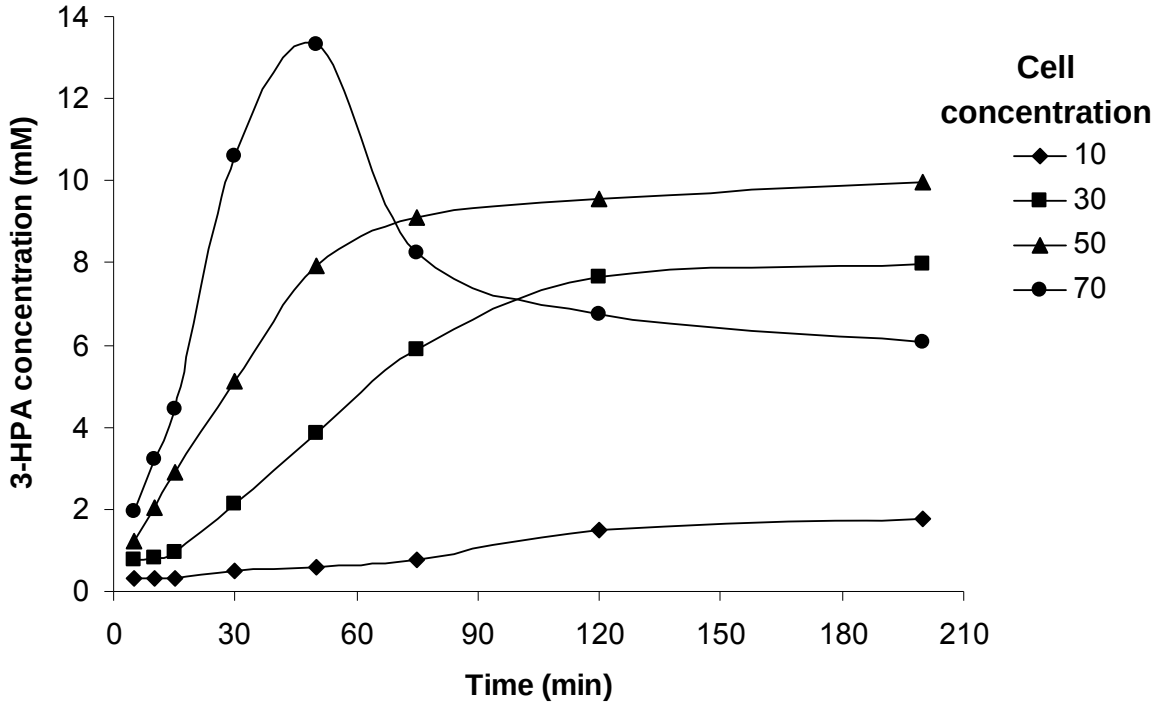
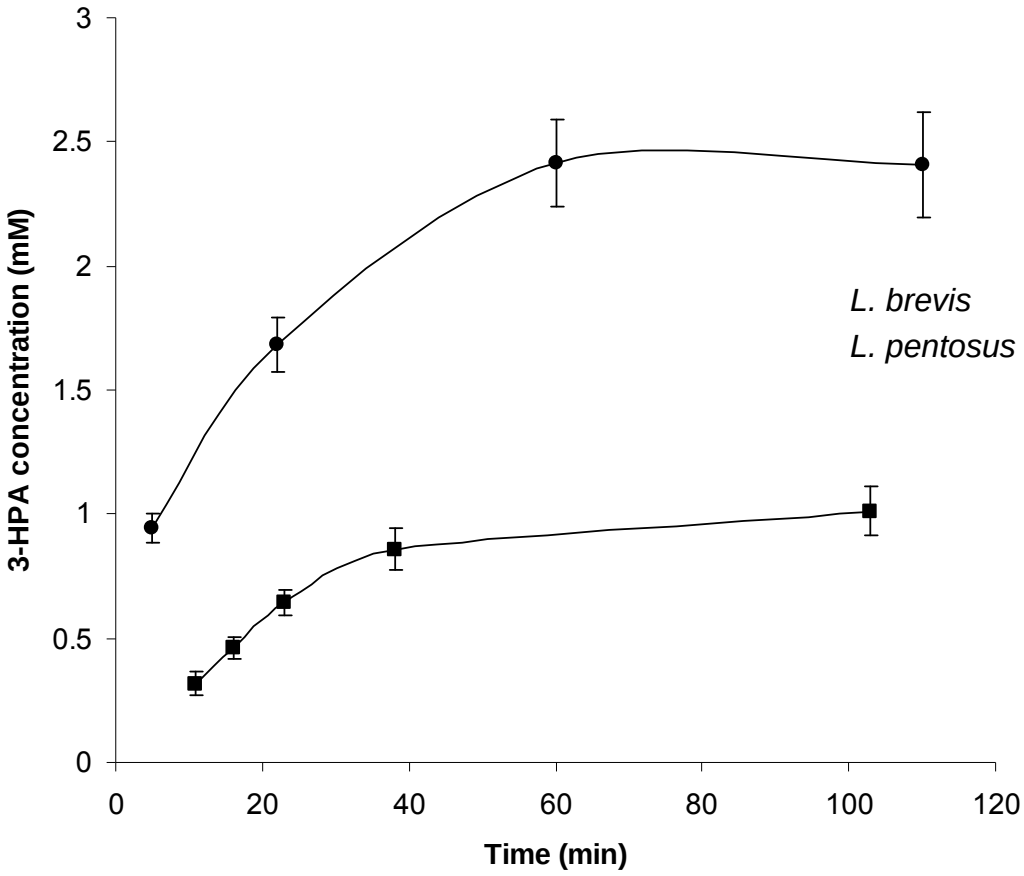

3-HPA concentration (mM)
14
12
10
8
6
4
2
0
0
30
60
90
120
150
180
210
Time (min)
Cell concentration
10
30
50
70

Figure 4


3-HPA concentration (mM)
Time (min)
L. brevis
L. pentosus
0
0.5
1
1.5
2
2.5
3
0
20
40
60
80
100
120

CHAPTER 4

RESEARCH RESULTS

Acrolein dimer as marker for direct detection of acrolein in wine

Acrolein dimer as marker for direct detection of acrolein in wine

ABSTRACT

Some lactic acid bacterial strains isolated from wine have been shown to accumulate 3-hydroxypropionaldehyde (3-HPA) during anaerobic glycerol fermentation. Wine quality can be gravely compromised by the presence of 3-HPA, due to spontaneous conversion into acrolein under wine making conditions. Acrolein is very toxic and its presence in beverages has been correlated with the development of unpleasant bitterness. Interconversion between 3-HPA derivatives and acrolein in aqueous solution is a complex and dynamic process driven by hydration and dehydration reactions. Acrolein is furthermore highly reactive and its steady-state concentration is low in complex systems. Analytical detection and quantification in aqueous solution are problematic as the compound quickly dissipates. Here we report on the detection of an acrolein dimer, a natural derivative formed under conditions prevailing in wine, and investigate its potential as a possible marker for acrolein detection. Solid-phase microextraction (SPME) coupled to gas chromatograph mass spectrometry (GC-MS) was validated as a technique for direct detection of the acrolein dimer. The potential of a recently introduced sorptive extractive technique with a sample enrichment probe (SEP) was also investigated. The SPME technique simplifies the detection process and allows for rapid sampling of the acrolein marker, while SEP is more sensitive.

Introduction

Acrolein, also known as 2-propenal or acrylaldehyde, is an α,β-unsaturated carbonyl compound. In its class acrolein displays the highest reactivity with nucleophiles (*1*), making it a dangerous substance for the living cell even when present in minute concentrations. Acrolein, a pulmonary toxicant and an irritant of mucous membranes (*2*), is considered by regulatory agencies to be one of the greatest non-cancer health risks of all organic pollutants. Acrolein has furthermore been implicated in the development of bitterness upon combination with polyphenols in wine (*3*). Under conditions prevalent during winemaking acrolein is non-enzymatically produced by an elimination of H_2O from 3-hydroxypropionaldehyde (3-HPA), a product of bacterial glycerol fermentation. Lactic acid bacteria (LAB) isolated from wine and belonging to the genus *Lactobacillus,* have been implicated in accumulating 3-HPA (*4, 5*).

Bauer *et al*. (*6*) reviewed methodologies for determining acrolein and these are generally based on carbonyl derivitizing agents combined with solvent extraction. Compounds such as acrolein, that display poor chromatographic performance, high reactivity, high volatility, and thermal instability, generally require derivatization during sample preparation. While advances have been made in establishing appropriate methodology for measuring acrolein in ambient air (*7*), the analyses of liquid samples are more problematic. Problems with derivative analysis include instability, co-elution of other compounds, long sample collection times, and ozone interference. Not only is analytical detection complicated, but acrolein interacts with ethanol and water and, due to its high reactivity, has a low steady-state concentration in complex systems. For these reasons, few studies are known to have evaluated its content in beverages. Distillation has been employed to separate acrolein prior to its determination in wine and levels of up to 2.8 mg/L have been reported (*8, 9*).

Trends in the development of modern analytical extraction techniques center on the use of adsorbents or sorbents for selective analyte extraction as alternatives to solvent extraction. Various systems have been developed for this purpose (*10*) of which solid-phase microextraction (SPME) is generally preferred due to its versatility and ease of use **(***11***).** In SPME a very small volume of sorptive phase is utilized and thermal desorption of the enriched material takes place almost immediately. The amount of available phase conversely limits the mass of analyte extracted. Promising sample enrichment methods such as stir bar sorptive extraction (SBSE), high-capacity sorption probe (HCSP) and solid-phase aroma-concentrate extraction (SPACE), have been developed to deliver more sorptive stationary phase mass and surface area, and relates to correspondingly higher sensitivity (*12, 13, 14*). The major disadvantage of the latter techniques relates to the large volumes of sorptive material employed, which necessitates cryofocusing of volatiles on the GC column after desorption from the fibre. Introduction of the high-capacity sample enrichment probe (SEP) circumvents this problem (*15*). As in SPME, desorption and GC separation of the volatiles run almost concurrently (*16*) and no auxiliary thermal desorption and cryotrapping equipment are therefore required.

Few published studies reported on the use of modern extraction techniques as alternatives to derivitization for direct detection of acrolein in aqueous solution (*17, 18*). These studies were, however, only successful if analysis was performed after a short contact time of the sample with acrolein. We report on the determination of the acrolein dimer (3,4-dihydro-2H-pyran-2-carbaldehyde), a natural derivative that might be used as a marker for acrolein detection. The paper evaluates SPME, and reports on the relative new sorptive extractive technique SEP, coupled to GC-MS as methodology for direct detection of the acrolein dimer in complex matrixes such as wine. Parameters affecting sorption of the acrolein dimer to the SPME fiber are discussed. Linearity, detection limits and precision of the procedure are evaluated.

2. Experimental

2.1. Headspace SPME extraction and GC-TOFMS detection

Samples were diluted 1:4 with water and 5 ml were introduced into 20 mL vials containing 2.4 g of sodium chloride (analytical grade). Vials were closed with silicon/Teflon-faced septa and installed in a PAL Combi autosampler (CTC Analytics, Zwingen, Switzerland). A SPME polyacrylate 85 µm fiber (Supelco, Bellefonte, PA, USA), was conditioned prior to analysis according to the instructions of the manufacturer. The fiber needle was automatically inserted through the septum of the vial and the fiber exposed to the headspace for 15 min at 40 °C, unless indicated otherwise, while being agitated at 250 rpm (agitator cycle: 5 sec on, 2 sec off).

An Agilent 6890N gas chromatograph (Agilent Technologies, Palo Alto, California, USA) was coupled to a Waters GCT Premier time-of flight mass spectrometer (Micromass, Manchester, UK). Waters MassLynx software (version 4.1) was used to operate the GC-TOFMS system. Helium was used as a carrier gas at a constant flow rate of 1 ml/min. Extracted analytes were desorbed into the GC injection port operated in the splitless mode at 220 °C for 10 min to ensure complete desorption and to avoid carry-over between samples. Volatile components were separated on an open-tubular fused silica capillary column (30 m x 0.25 mm ID) coated with 0.25 µm 5% Phenyl-substituted polydimethylsiloxane (J&W Scientific, Folsom, CA, USA). The temperature program after injection was as follows: 8 min isothermal at 37 °C, and ramped at 20 °C/min to 280 °C. The temperatures of the line-of-sight interface and ion-source were set at 250 °C and 180 °C, respectively. Detection was performed in scan mode with from *m/z* 35 to 225 at 4.0 scans per second using perfluoro-tri-n-butylamine as reference.

2.2. Headspace SEP extraction and GC-MS detection

Instead of the stainless steel rods used by Burger *et al*. (*16*) to manufacture SEPs, polyimide-coated fused silica tubing was used as supports (stems) for the PDMS rubber tubing in this study. Thus, lengths of the fused silica tubing (130 mm x 0.7 mm O.D.)(Polymicro, Phoenix, USA) were sealed off on both sides using an oxy-methane burner. Using ethanol as lubricating agent, the PDMS sheaths were gently slipped over the tips of the stems and positioned with one end of a sheath about 1 mm from one end of each of them. Undiluted wine samples (50 ml) for headspace SEP analysis were placed in glass vials (100-ml volume) containing 12 g of sodium chloride and a glass-encapsulated stir bar. Vials were sealed with a phenolic cap adapted with a stainless-steel insert (Burger et al., 2006). SEPs, preconditioned as described by Burger et al. (2006) and with the GC septum and septum cap already in place, were introduced into the headspace of samples via the metal inserts. The GC septum caps were tightened and samples stirred at 40 °C for 30 min.

The organic volatiles trapped in the sorptive phase of the SEP, were desorbed in the split/splitless GC-injector port of a Carlo Erba QMD 1000 GC-MS instrument operated in the splitless mode at 220°C, and were separated on a glass open-tubular column (40 m x 0.3 mm I.D.) coated with 0.37µm PS 089 (5% Phenyl-substituted polydimethylsiloxane, DB5 equivalent) using helium as carrier gas at a linear flow velocity of 32.25 cm/s, which was temperature programmed as follows: 25°C isothermal for 5 min, ramped from 25°C to 60°C at 2°C/min and then from 60°C to 280°C at 20°C/min. The temperatures of the line-of-sight interface and ion-source were set at 250 and 180°C, respectively. Lowresolution electron impact mass spectra (LR-EIMS) data were acquired at 70 eV at 1 scan per second over the mass range m/z 40 to 120.

3. Results and discussion

3.1 Detection of acrolein dimer

Analytical detection and quantification of acrolein in solution was problematic as the compound could not be detected after 3 hrs of contact time with the sample (data not shown). Prior to analyzing the acrolein dimer, several other natural derivatives were considered for quantification, including 3-HPA (**Table 1**). None of these gave sufficient signal-to-noise ratios for quantification purposes. Identification of target peaks from the total ion chromatogram (TIC) was simplified through the use of extracted ion chromatograms (EICs) (*19, 20*). Peak areas of ions 112.05 and 83.05 were used for quantification of the acrolein dimer (**Figure 1**). TOF GC-MS permits high scan rates, provides enough data points for peak integration and quantitative analyses, and allows for accurate mass measurements. The measured molecular ion for the acrolein dimer (**Figure 2A**) was 1 mDa removed from its calculated monoisotopic mass. This together with the good library match (**Figure 2B**) confirms the identity of the acrolein dimer.

3.2 Headspace SPME method optimization

Operational conditions for the headspace SPME method were studied in a model wine solution (12% ethanol, 0.5 g/L tartaric acid, pH = 3) spiked with 5 mM of acrolein. The temperature of the incubator during extraction is referred to as extraction temperature. An increase in extraction temperature from 30 to 60 °C increased the yield for the acrolein dimer more than 3 fold (**Figure 3A**). Higher temperatures were not evaluated. For practical convenience all further analysis were performed at 40°C. Extraction time refers to the duration of exposure of the fiber to the headspace of the sample. Chromatographic response increased approximately 1.5 fold with an increase in extraction time from 5 to 15 min. (**Figure 3B**). The effect of ionic strength on extraction efficiency was tested by the addition of

sodium chloride to the diluted wine sample. Concentrations close to saturation point yielded a 10 fold increase in extraction efficiency (**Figure 3C**). Salting-out reduces sample matrix effects and therefore improves method accuracy, while the equilibrium time in the headspace vial is shortened (*21*). Maximum sensitivity was achieved with the split/splitless injection port in splitless mode. Chromatographic response was enhanced more than 15 fold (**Figure 3D**).

3.3 Headspace SPME method validation

After establishing the optimal experimental conditions for determining the acrolein dimer in a model wine solution, validation of the headspace SPME-GC-TOFMS was carried out using wine as matrix. Different wine samples were first spiked with acrolein (5 mM) and the dimer measured after 10-12 hrs of contact. Although headspace analysis minimizes interferences resulting from organic compounds in complex matrixes, quantification was shown to be highly dependent on the composition of individual samples (**Figure 4**). Two blended wines (red and white, respectively) displayed similar chromatographic response and were chosen for validation of the established methodology.

Quantitative linearity of the method was evaluated using a series of standard wine solutions spiked with acrolein (**Figure 5**). Lines of best fit, depicting the relationship between peak response for the acrolein dimer and the acrolein concentration added, were determined by linear least-squares regression. The results are summarized in **Table 2.** The calibration curves were linear over the tested concentration range with correlation coefficients (R) close to 0.99. The limits of detection (LOD) were obtained by the equation LOD = KS_Y/m, where S_Y and m denote the estimated standard deviation of the intercept and slope of the calibration line, respectively, with a 98% confidence level (K = 3.3) (*22, 23*). The slopes of analytical curves for acrolein dimer differed considerably between the two wines, again an indication of the strong matrix effect. Given that the dimer is a natural derivative, its true concentration in the experimental wines is furthermore not known. For the same reason the accuracy of the method could not be determined. Regression analysis and parameters for LOD and LOQ may therefore vary for different wines depending on e.g. pH and phenolic content. The data obtained with this methodology should be used taking these limitations into consideration.

Precision or repeatability, expressed as relative standard deviation (RSD) of the peak areas, was evaluated by 5 replicate analysis of wine samples spiked with 5 mM acrolein. Intra-day precision was satisfactory; being below 10%. When analyses were repeated 7 days later, RSD values were slightly higher (up to 12.5%), but still well within the acceptable range using SPME **(***24*).

3.4 Headspace SEP GC-MS methodology

The installation of a SEP in the injector of gas chromatographs with snap-on injector caps is so awkward that highly volatile compounds are lost before the installation can be completed. Application of SEP would require modification of the GC-MSTOF instrumentation. The probe was therefore inserted into the injector port of a conventional quadrupole GC-MS instrument. Due to the inherent complexity of biological samples, relative low concentration of the acrolein dimer, and the broadness with which highly volatile compounds are eluted from thin-film capillary columns in the SEP technique, data reduction processing was largely complicated by the co-elution of the dimer and isobutyl acetate (data not shown). This complicating factor was circumvented in analyses on the TOFMS instrument by selecting the ions m/z 112.05 and 83.05, which is not possible with a quadrupole instrument with unit resolution. The possibility to circumnavigate this problem by using a thick-film capillary column from which the dimer will elute as a sharper peak, or a column coated with a different stationary phase was not investigated in the present study. However, if only the non-overlapping part of the dimer's peak is used for quantification, it is clear that the SEP technique is about two orders of magnitude more sensitive than SPME. As for its monomer (*6*), conventional GC-MS analysis does not provide sufficient chromatographic resolution for the separation of the acrolein dimer when extracted from complex mixtures.

3.5 Stability of the acrolein dimer in wine

Few studies evaluated the content of acrolein in beverages due to complications experienced during analytical detection. Instability of acrolein in aqueous solution presents the biggest hurdle. Acrolein quickly dissipated from the wine samples and could only be detected within the first 3 hrs after its addition (results not shown). Acrolein content in cider was also shown to diminish rapidly during the first hours of contact (*25*). The acrolein dimer, on the other hand, was still detectable after 28 days in red wine (**Figure 6**). Acrolein is easily polymerized and undergoes Diels-Alder reaction with itself yielding the acrolein dimer. Factors initiating polymerization include the presence of acids (including sulfur dioxide), oxidizing agents, or exposure to light and heat. Acrolein and its dimer are highly toxic and have a very reactive free aldehyde group (*26*). Being highly polar acrolein is furthermore soluble in water and ethanol with which it reacts slowly and exothermically to give 3-HPA and 3-ethoxypropionaldehyde (3-EPA), respectively. Inter-conversion between acrolein and derivatives is a complex, highly dynamic and reversible process driven by hydration and dehydration reactions (*6*). Further studies on the evolution and transformation of acrolein and its derivatives in wine should be pursued and other compounds produced from this aldehyde characterized.

4. Conclusions

Analytical detection and quantification of acrolein in solution remains problematic. This study shows that the acrolein dimer can be used as a marker for indicating the presence of acrolein in wines. Due to the instability of the compound, the analytical approach aimed to reduce sample preparation steps by applying sorptive sample extraction techniques and direct thermal desorption. GC-MS analysis using a quadrupole mass spectrometer did not provide sufficient chromatographic resolution for separation of the target analyte from interfering compounds. TOFMS allowed for qualitative and quantitative measurements of the acrolein dimer. The technique provided excellent mass accuracy (around 1 mD), and extracted ion chromatograms with a very narrow mass window could be used. This enabled elimination of background ions, which dramatically improved selectivity in a complex matrix such as wine. Sensitivity of the analytical approach, however, requires improvement. Compared to SPME, more sorptive stationary phase and a larger surface area are available with the SEP technique. Hurdles may be overcome by combining SEP with TOFMS, a route that is currently under investigation. Acrolein and its dimer are dangerous substances for the living cell and a study is required to fully elucidate the effect of process conditions on the formation of these compounds in wine. This work lays the foundation for such studies.

References

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Table 1. Natural derivatives of acrolein.

Alternate name	*Chemical name (IUPAC*)*	*CAS number*	*Chemical formula*	*MW (g/mol)*
Acrolein	prop-2-enal*	107-02-8	C_3H_4O	56.06
Acrolein dimer	3,4-dihydro-2H-pyran-2-carbaldehyde*	77890-80-3	$C_6H_8O_2$	112.13
3-HPA	3-hydroxypropanal*	2134-29-4	$C_3H_6O_2$	74.08
HPA hydrate	1,1,3-trihydroxypropane	-	$C_3H_8O_3$	92.09
HPA cyclic dimer	2-(2-hydroxyethyl)-4-hydroxy-1,3-dioxane	-	$C_6H_{12}O_4$	148.16
HPA aldol trimer	-	-	$C_9H_{18}O_6$	222.24
HPA acetal tetramer	-	-	$C_{12}H_{22}O_7$	278.30

597-31-9

Table 2. Linear regression parameters of acrolein dimer. Red and white wine samples were spiked with acrolein and analyzed with SPME over time.

Sample	Range (mM)	Slope	Intercept	m	S_Y	Data points	Correlation coefficient	LOD (mM)	LOQ (mM)	Precision (%RSD)
Intra-day										
White wine	0.5 - 20	2666	-531	68	641	6	0.9974	0.35	1.06	9.4
Red wine	0.5 - 20	2050	-1043	99	934	6	0.9907	0.35	1.06	8.5
After 7 days										
White wine	0.5 - 20	1044	-610	39	363	6	0.9946	0.35	1.07	12.5
Red wine	0.5 - 20	1320	-718	78	733	6	0.9862	0.32	1.06	9.7

m, Standard deviation of the slope; S_Y, Standard deviation of the intercept; Limit of detection (LOD) = $3.3S_Y/m$; Limit of quantitation (LOQ) = $10S_Y/m$; Relative standard deviation (RSD) was measured at 5 mM (n = 5)

Figure 1. Detection of acrolein dimer by headspace GC-TOFMS. Total ion chromatogram (TIC) with acrolein dimer peak eluting at 7.11 min; **B**, Extracted ion chromatogram for ion at *m/z* 112.05 (monoisotopic mass of acrolein dimer).

Figure 2. Identification of the acrolein dimer. **A**, Extracted mass spectrum; **B**, The spectrum aligned to the library mass spectrum of the acrolein dimer ($C_6H_8O_2$) with a reverse match of 824 using NIST MS Search 2 program and the NIST05 library.

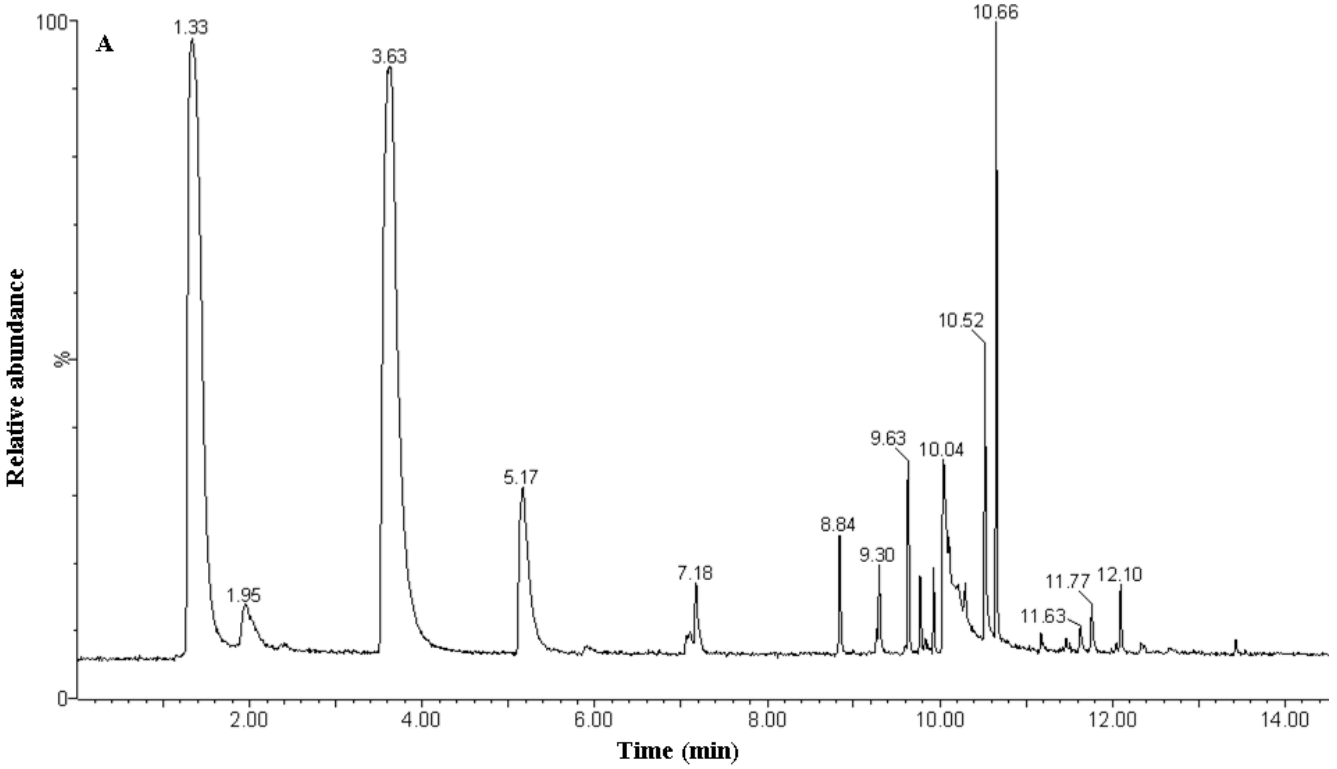
Figure 3. Comparison of the operational conditions for the HS-SPME method. **A**, extraction temperature; **B**, extraction time, **C**, ionic strength (NaCl); **D**, split/splitless GC injector mode (splitting ratio of 1:5). Peak areas are normalized and error bars represent the RSD of the data (n=3).

Figure 4. Matrix effect in wine analyzed by headspace SPME-GC-TOFMS. Wines samples were spiked with 5 mM acrolein and analyzed within 10 to 12 hrs at 4°C. Error bars represent the RSD of the data (n=3).

Figure 5. Calibration curves for acrolein dimer in wine (red and white) spiked with different concentrations of acrolein and analyzed by headspace SPME-GC-TOFMS over time.

Figure 6. Stability of the acrolein dimer. A red wine sample was spiked with 5 mM acrolein, stored at 4°C, and analyzed over time by headspace GC-TOFMS.

Figure 1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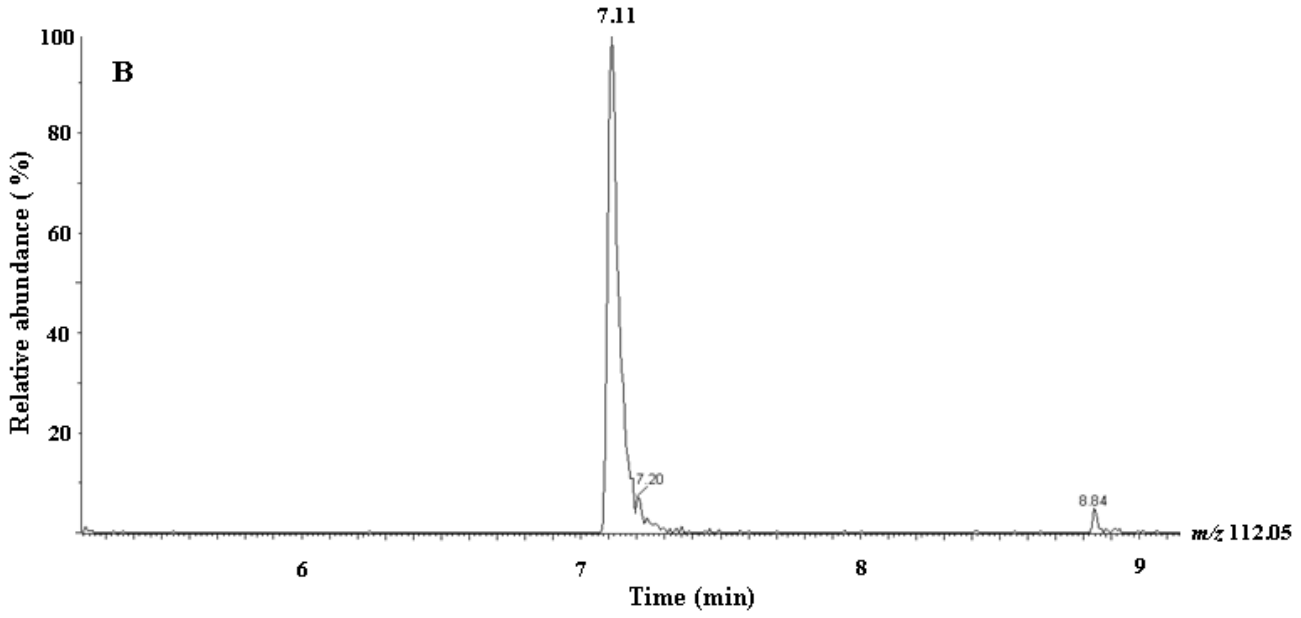
14.00
Time (min)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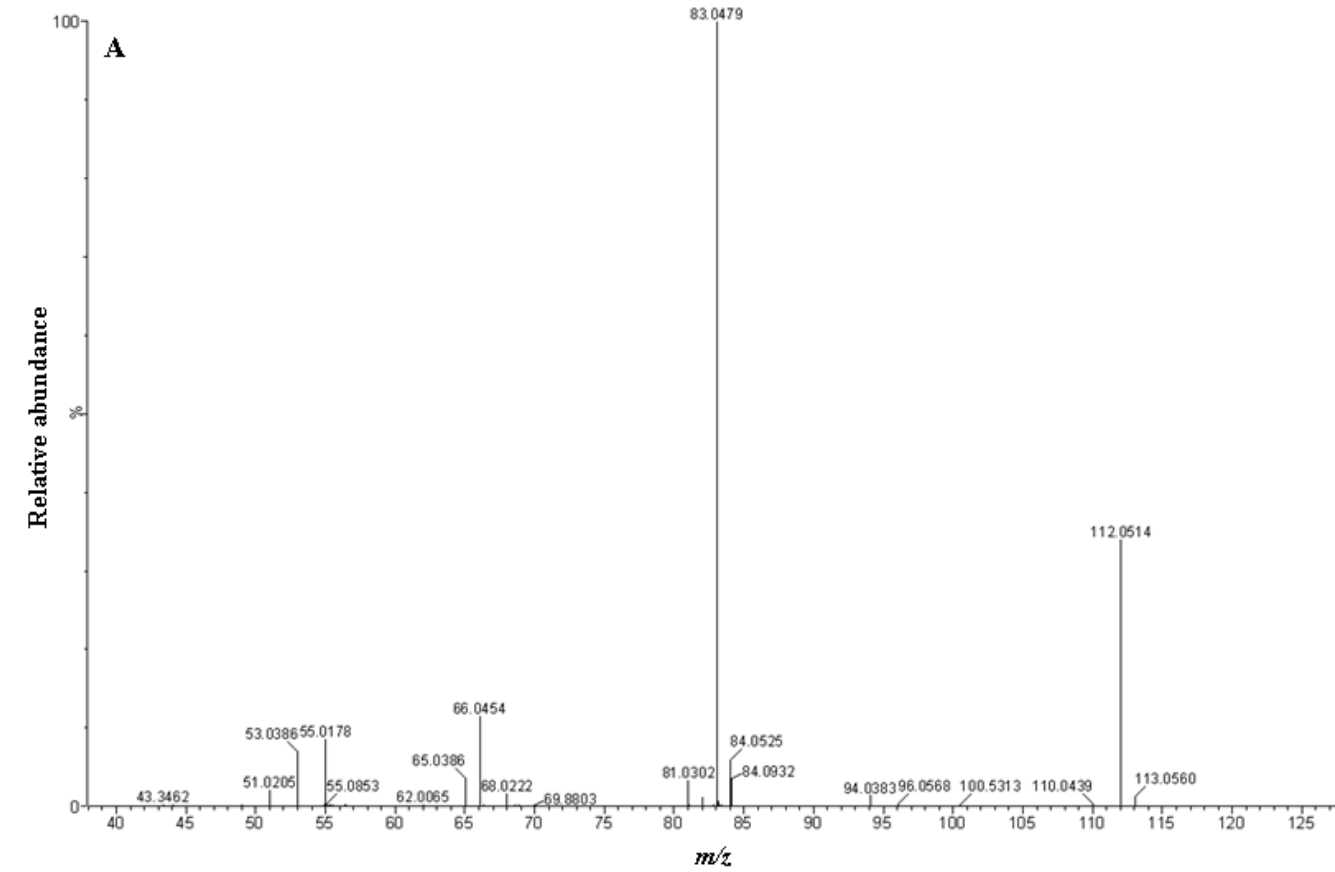
9
Time (min)

Figure 2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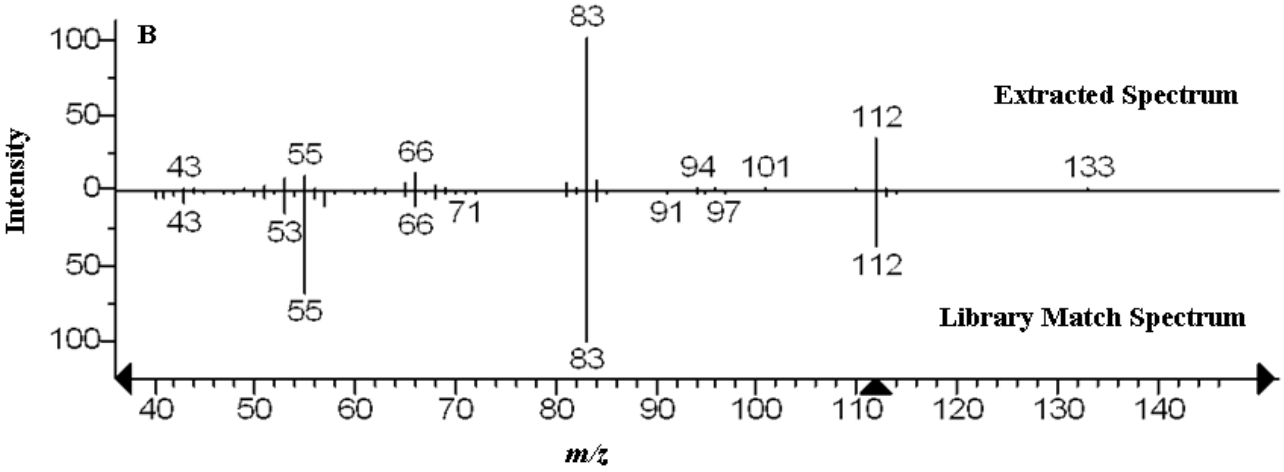
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Extracted Spectrum
Library Match Spectrum
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66
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101
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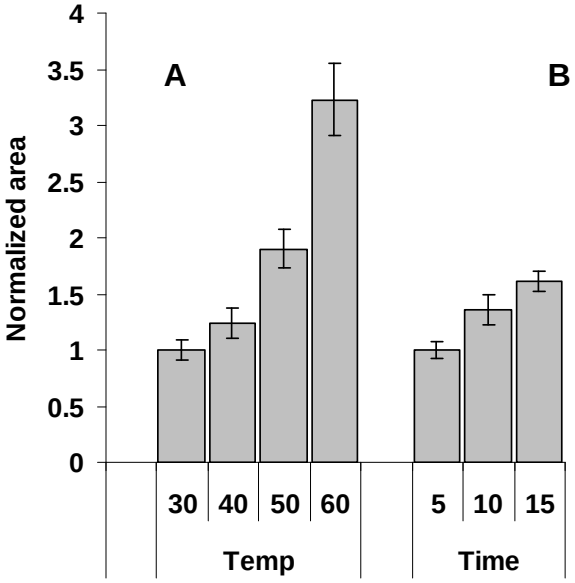
71
91
97
53

Figure 3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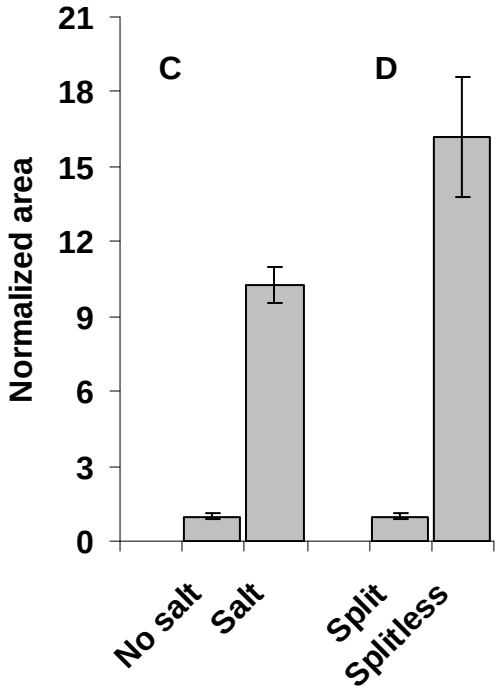
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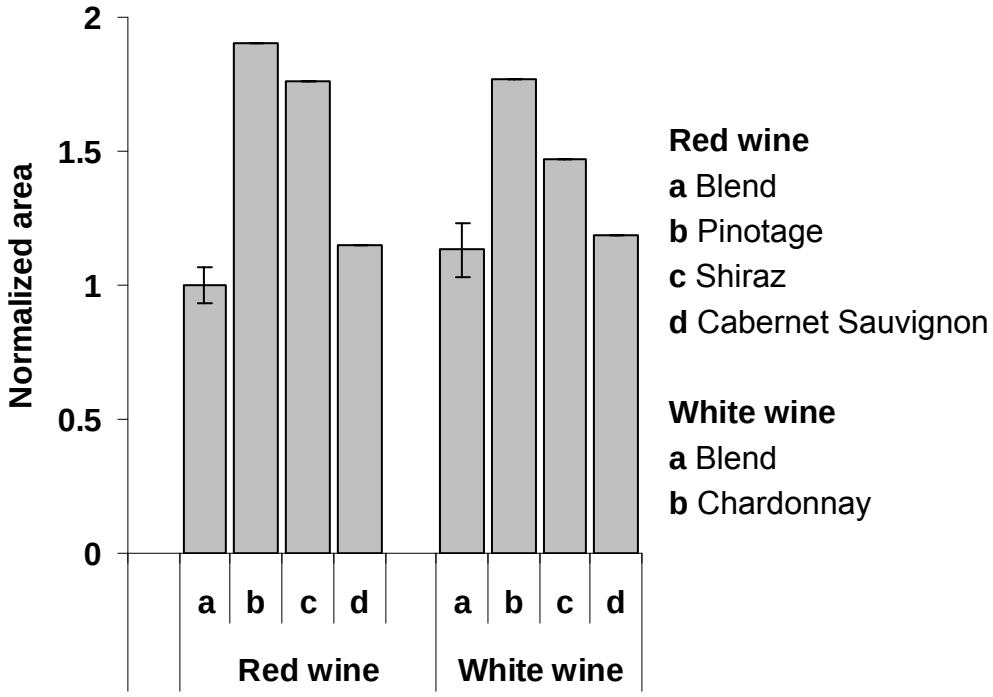
Figure 4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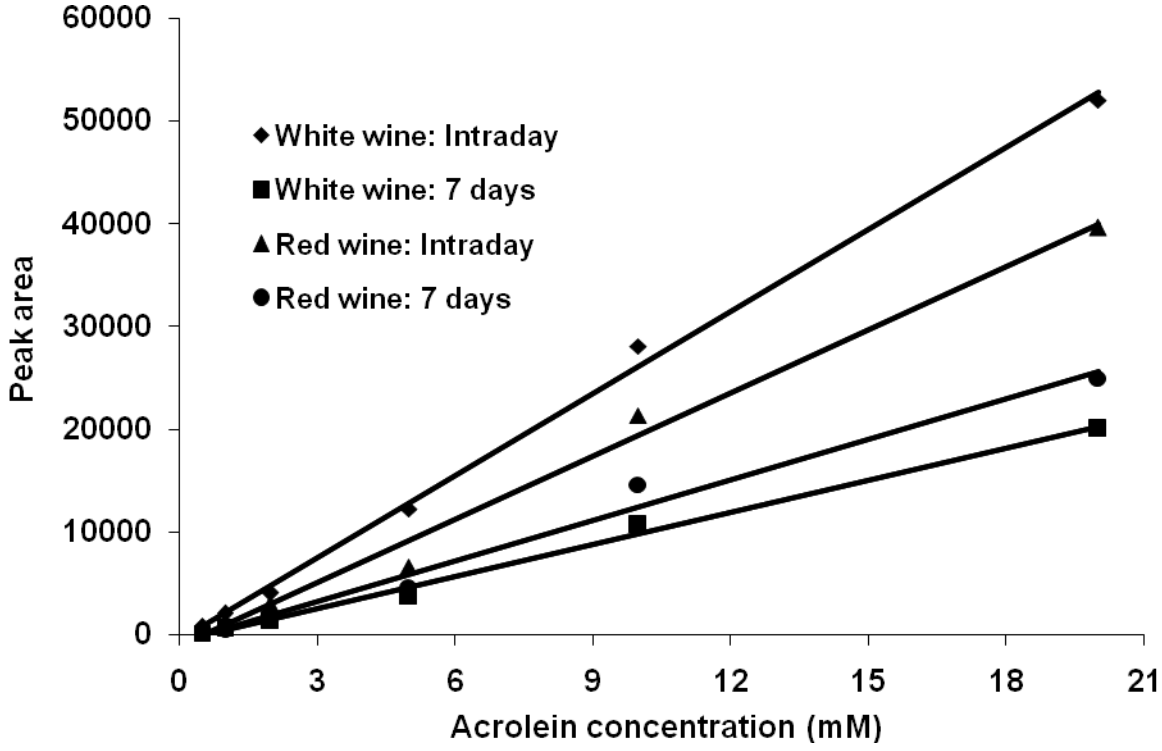
c Shiraz
d Cabernet Sauvignon
White wine
a Blend
b Chardonnay

Figure 5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Peak area
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White wine: 7 days
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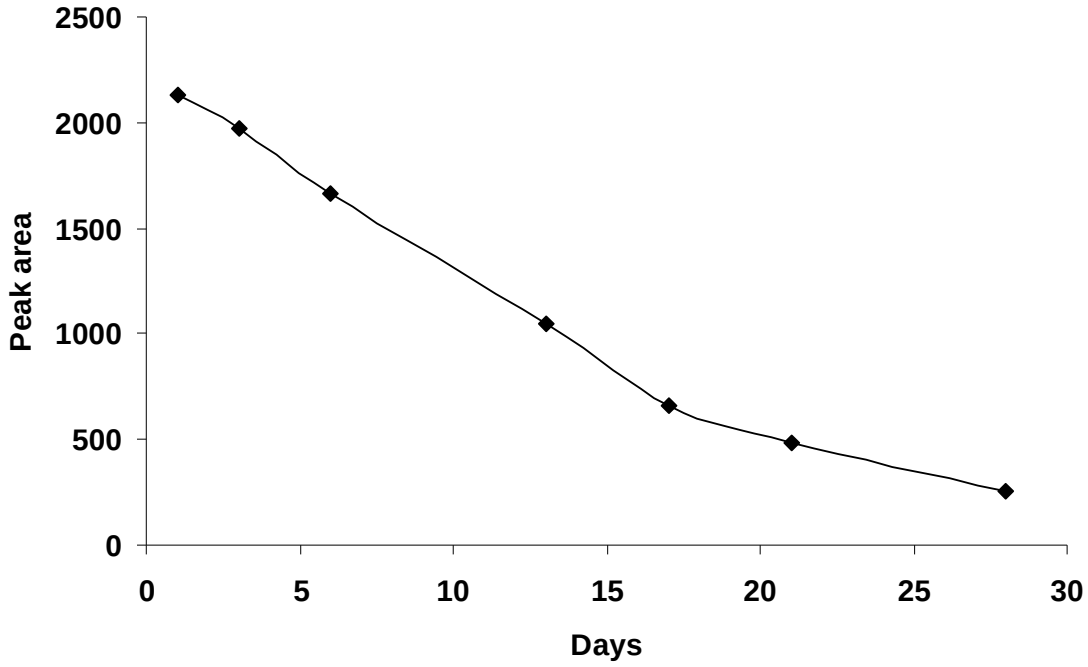
18
21
Acrolein concentration (mM)

Figure 6



CHAPTER 5

General Conclusions

GENERAL CONCLUSIONS

Wine quality can be gravely compromised by the presence of 3-HPA, due to its spontaneous conversion to acrolein under wine making conditions. Acrolein is not only a dangerous substance for the living cell, but has been implicated in the development of unpleasant bitterness in beverages. This is the first study demonstrating the ability of wine lactobacilli to accumulate 3-hydroxypropionaldehyde (3-HPA) in fermentation media. The effect of individual environmental parameters on bacterial 3-HPA production is evaluated. Based on this study recommendations are made to minimizing the presence of the acrolein precursor and the formation of acrolein in wine.

Analytical detection and quantification of acrolein in solution is problematic. In the initial stage of the project Fourier Transform Infrared Spectroscopy in combination with Chemometrics was explored as a detection technique (Appendix I), but proved unsuccessful due to the relative low concentrations present in wine. We subsequently showed that the acrolein dimer can be used as a marker for direct detection of acrolein in wines. Due to the instability of the compound, the analytical approach was aimed at minimizing sample preparation steps by applying sorptive sample extraction techniques and direct thermal desorption. Techniques investigated include the recently introduced sample enrichment probe (SEP) and solid-phase microextraction (SPME). Although SEP is more sensitive, the SPME technique is automated which simplifies the detection process and allows for rapid sampling. GC-MS analysis using a quadrupole mass spectrometer did not provide sufficient chromatographic resolution for separation of the target analyte from interfering compounds. TOFMS allowed for qualitative and quantitative measurements of the acrolein dimer. The technique provided excellent mass accuracy (around 1 mD), and extracted ion chromatograms with a very narrow mass window could be used. This enabled elimination of background ions, which dramatically improved selectivity in a complex matrix such as wine. SPME coupled to gas chromatograph mass spectrometry (GC-MS) was effectively validated as a technique for direct detection of the acrolein dimer in wine. Sensitivity of the analytical approach, however, requires improvement. More sorptive stationary phase and a larger surface area are available with the SEP technique compared to SPME. Hurdles may be overcome by combining SEP with TOFMS, a route that is currently under investigation. Acrolein and its dimer are dangerous substances for the living cell and a study is required to fully elucidate the effect of process conditions on the formation of these compounds in wine. This work lays the foundation for such studies.

APPENDIX I

Fourier Transform Infrared Spectroscopy for Grape and Wine Analysis

Assignment submitted in partial requirement for the Cape Wine Master diploma

APPENDIX I

Fourier Transform Infrared Spectroscopy for Grape and Wine Analysis

ABSTRACT

Fourier transform infrared (FT-IR) spectroscopy is a non-destructive analytical technique that provides structural information on molecular features of a large range of compounds. The main advantages offered by FT-IR spectroscopy are speed, high degree of automation, medium resolution and cost effectiveness. Recent improvements in FT-IR instrumentation together with advances in fiber optics and chemometrics (multivariate data analysis) have provided an analytical tool that is suitable for routine qualitative analysis and process control in many industries. Although widely applied in the food industry, acceptance of FT-IR technology in the grape and wine industry has been relatively slow and mainly restricted to large wineries. Attitudes towards developments in analytical chemistry are changing rapidly and the influence of both consumers and regulatory bodies are starting to affect the industry. Winemaking demands constant product monitoring and requires process control from the start of grape ripening up to the bottled product. Direct spectroscopic measurement is well suited in this context since it allows for rapid and simultaneous analysis of several compounds (e.g. alcohol, sugars, acidity, glycerol, phenolic compounds, and sulfur dioxide) with minimal sample preparation and reagent consumption. Commercially available FT-IR instrumentation with versatile and innovative software, designed specifically for grape and wine analysis, has recently received much attention. This review evaluates the status and usefulness of application of FT-IR technology and chemometrics in grape and wine analysis.

CONTENTS

INFRARED TECHNIQUES

FT-IR spectroscopy is based on the principle that functional groups within a sample will vibrate upon exposure to infrared (IR) radiation. Spectroscopic techniques, particularly in the near-IR (NIR) and mid-IR (MIR) wavelength regions of the electromagnetic spectrum, have come of age through the development of chemometrics, advanced multivariate statistical methods and almost unlimited computing power. The distinction between NIR and MIR techniques is diminishing and the methods are rather seen as complementary, with each spectral region providing unique advantages. For a thorough presentation of the theory and concepts of FT-IR and spectroscopy see (*1*, *2*).

Mid-Infrared. Most compounds with covalent bonds absorb specific frequencies of electromagnetic radiation in the infrared (IR) region of the electromagnetic spectrum.

The IR region lies at wavelengths between those associated with visible light (400 to 800 nm) and those associated with microwaves (>1mm). Only asymmetric bonds with a dipole moment that changes as a function of time are capable of absorbing IR radiation and absorptions characteristic of each type of bond (e.g. N-H, C-H, O-H, C-O, C=O, C-C, C=C) are found in specific small sections of the vibrational IR region. In terms of wavelengths the vibrational IR region ranges from about 2500 nm to 25000 nm, corresponding to wavenumbers of 4000 to 400 cm^{-1} (μm = $1/cm^{-1}$ x 10000). The unit 'wave-number' is the conventional unit used in IR spectroscopy, since it is directly proportional to the energy of radiation. The simplest types of vibrational motion in a molecular bond are the stretching and bending modes which give rise to 'fundamental' absorptions in which vibrational transitions in molecules are induced resulting in transitions from the ground state to the lowest energy excited state. Although fundamental absorptions or vibrations are visualized as strong bands or peaks, an IR spectrum is complicated by the presence of weak overtone-, combination- and difference bands. Overtones result from molecules excited from the ground state to higher energy states and which correspond to multiples of the frequency of the fundamental absorption. Combinational bands result from frequencies that are vibrationally coupled, while difference bands result from the difference between two interacting bands. No two molecules of different structure have exactly the same IR absorption pattern, thus the IR spectrum of a pure sample can be described as a molecular fingerprint characteristic of any chemical or biochemical substance.

Near-Infrared. The NIR spectral region lies between the visible and infrared regions of the electromagnetic spectrum corresponding to the 14000 – 4000 cm^{-1} range. While the MIR part of the spectrum contains many sharp peaks and is very information rich, the NIR spectra consist of overtones and combination bands of the fundamental molecular absorptions. The predominant NIR spectral vibration feature is C-H associated stretching as both C-H stretch combination bands and C-H overtones occur four times between 14500 and 3300 cm^{-1}. The closer one approaches the fundamental region the more detailed is the vibrational information. The main advantages offered by NIR spectroscopy compared to other vibrational spectroscopic techniques, are low absorbtivity and low reflectivity. Due to low absorbtivity, NIR energy passes easily through samples without rapid attenuation. As a result sample pathlengths can be much longer than for MIR analysis (varying from 1 mm to more than 10 cm), permitting analysis of most samples without need for sample pretreatment. Low reflectivity allows NIR energy to penetrate beneath the surface of even visually opaque samples. By contrast, the high reflectivity of UV or visible light wavelengths precludes analysis of most opaque samples and intact grape berries.

Even though advanced infrared technology has been welcomed enthusiastically (*3*) and has led to an almost explosive application flurry regarding quantitative analysis there is a critical success factor: accuracy. The reliability of the analytical results is

directly proportional to the degree that the analytical volumes employed represent the lots from which they were sampled truthfully: loads, drums, tanks, vats, reactors etc. Since sampling rates of $1:10^6$ are typical and far from trivial, representative sampling is critical.

MULTIVARIATE STATISTICAL TECHNIQUES

Traditionally, much of the modeling of wine analytical data has been conducted in a univariate manner, typically by examining the response of a single isolated variable on the overall matrix and ignoring the effects of chemical or physical interactions between the large numbers of constituents present in the sample. With the introduction of modern analytical instrumentation capable of multivariate responses, food quality control is now often based on indirect, full-spectrum measurements of the chemical and physical properties of a sample. As a result, extremely large data sets are generated where essential information may not be readily evident. Data analytical techniques that deal with univariate methods, such as the analysis of variance (ANOVA), is of limited use in complex data analysis. Multivariate analysis, chemometrics, developed from the 1970s, provides means of quantifying constituents that are involved in complex matrix interactions without eliminating matrix interferences. The use of chemometrics for calibration, validation and comparison of main components of wine as determined by IR is indispensable, hence a short discussion of important concepts.

Qualitative and quantitative IR spectroscopic methods typically require application of multivariate calibration algorithms to model the spectral response to chemical or physical properties of the samples used for calibration. Measured frequencies often have to be pre-processed through a series of mathematical procedures, including various forms of scaling, corrections and Fourier transformation (FT), to yield an absorbance spectrum in a most suitable form. Vibrational bands (peaks) are generally overlapping and may often appear non-specific and poorly resolved. Correlation of the spectrum to actual concentrations of the relevant components in the sample matrix is achieved through a calibration process that involves multivariate statistical procedures of many kinds. The approaches and methodology in chemometrics, including the latest advances, are described in more detail in several books and reviews (*4, 5, 6, 7*). The section below aims at summarizing multivariate data analysis methods that are typically used in combination with modern IR spectrometers.

Methods based on Multiple Linear Regression (MLR) relate variations in the selected response (Y-variable) to the variations of several linearly independent predictors (X-variables) with the purpose of predicting the dependent response variable (*5*). In the obtained regression model, the regression coefficient expresses the link between variation in the predictors and variation in the response. The attending squared multiple correlation coefficient R^2 is used as a goodness-of-fit

measure in the sense that the closer the value is to one, the better is the prediction for a given validation data set. Both R^2 and the Root Mean Squared Error of Prediction (RMSEP) are often used to validate a model (*5*). RMSEP is a measurement of the average difference between predicted and reference response values at the validation or prediction stage and is expressed in the same units as the original response values. While RMSEP can be interpreted as the average prediction error over the entire calibration concentration range, the root mean square of calibration (RMSEC) is a measure of the average modeling error only. The standard error of cross-validation (RMSECV) is another statistical indicator commonly used to evaluate the performance of calibration models (*5*), but this approach should only be used when one does not have access to a specific validation data set ('test set'). Cross-validation is a method where successive groups of samples from the calibration data set are kept out of calibration to be used for prediction, until all samples have been kept out at once, again only advisable in the absence of a test set (7). Bias, a term often referred to in validation, is the systematic difference between predicted and measured values. The ratio of standard deviation of the calibration data to the standard error of prediction (or cross validation), the so-called RPD value, is still another popular indicator of the predictive ability of a calibration model and it has been proposed that this value can be used to evaluate the suitability of the concentration range against the precision of a model for quantification (*8*). A RPD value of less than three suggest that the calibration model is unsuitable for quantification, a value between 3 and 5 indicates its suitability for screening, while a value greater than 5 suggest that the model is suitable for quantification. The RPD measure must not be used in isolation if it is based on cross-validation, since it is then only concerned with modeling statistics.

Partial Least Squares Regression (PLSR) is the most commonly used regression technique in the field of chemometrics (*5, 7*). The technique is based on so-called bilinear projection in the sense that two sets of variables [X,Y] are linked to each other by means of linear projection models in both X and Y. Instead of e.g. applying MLR to the full set of regressors, the information carried by the original X-variables is projected onto a smaller set of latent, uncorrelated variables called PLS-components. Since the Y-data are actively used in estimation of these latent variables in the X-space, the first PLS-components will always be the most relevant for predicting Y-variables. PLS-components point out main associations between X- and Y-variables internally while optimizing the effective interrelationships between the X-data and Y-data for optimal prediction characteristics. In multivariate calibration, a biased regression technique such as PLSR is preferred to classical MLR, since MLR gives rise to high variance when regressors are highly correlated (colinearity). Another weakness associated with MLR is the assumption that errors only exist for the Y-variable. PLSR can establish if a new test sample is similar enough to those used in training. If not, the model should not be used for prediction, which limits the error of

applying a regression model to an outlier sample (i.e. a sample whose chemical structure is not commensurable with those of the calibration set).

Principal Component Analysis (PCA) is the most often used bilinear modeling method which gives an interpretable overview of the main information in one, single multidimensional data table, X (*7*). The information carried by the original X-variables is projected onto a smaller set of 'latent' variables called principal components (PCs). Unlike PLS-components, PCs are calculated without taking information in the Y into account (there is no Y-data for a PCA). The principal components model, in decreasing order, as much of the variation in the data as possible and since PCs are orthogonal to one another, each PC can be interpreted independently. The so-called scores are used to study the inter-object relationships in terms of similarities as well as differences, while complementary loadings are also much used to detect and interpret interrelationships between different variables. When PCA is carried out on X-variables, the resultant PCs can be used as X-predictors in a variant of MLR known as Principal Component Regression (PCR), in which some of the MLR problems have been eliminated.

Disjoint PCA modeling by Soft Independent Modeling of Class Analogy (SIMCA) focuses on classifying object so as to be assigned to disjoint data classes based on the similarities between members of the same data class (a natural group, cluster of objects). A new sample will be rejected if it is not similar enough to other members of the class (*7*). SIMCA classification also enables identification and interpretation of outlier samples or samples that are poorly predicted by calibration models.

A more comprehensive discussion of the essential chemometric background for NIR and MIR multivariate data analysis and modeling is quite beyond the limits of this overview. Today a realm of excellent chemometric textbooks and reviews exists. In addition to those mentioned above, see also (*9*).

INSTRUMENTATION

In conventional IR spectroscopy, a beam of electromagnetic radiation is projected through a sample which, depending on the nature of the molecular bonds formed by all the IR active molecules within the sample, will absorb a quantifiable amount of IR radiation at specific frequencies. The energy absorbed is measured at different wavelengths. Results are presented in an IR spectrum where absorption intensity is typically plotted against either wavenumber or wavelength (*1*). Two types of infrared spectrophotometers are commonly in use, dispersive and the more modern Fourier transform (FT) instrumentation. While a dispersive instrument measures spectral points sequentially and records a spectrum in the desired frequency domain, the design of the optical pathway of FT instrumentation produces a complex time-dependent response, called an interferogram, which is essentially the variation of the absorbed IR intensity as a function of time (*1*). The conventional frequency domain IR spectrum is obtained by mathematically performing the Fourier transform of the

interferogram. Compared to the dispersive type, the FT-IR technique is characterized by far greater speed and produces spectra with better signal-to-noise ratio, which can conveniently be digitally manipulated. Today FT-IR spectrophotometers form the basis of IR-related studies, particularly in relatively new application fields such as wine analysis.

Spectral acquisition with multicomponent analytical FT-MIR instrumentation, in particular the WineScan FT120 (FOSS Electric, Denmark) has recently received much attention due to impressive performance in terms of accuracy, precision, and speed of analysis (*10, 11, 12, 13*). WineScan instrumentation is equipped with a DTGS pyroelectric IR detector and incorporates innovative calibration software based on PCA and PLSR that has been designed specifically for grape and wine analysis. Measurements are carried out in liquid flow-through cells equipped with two CaF_2 windows mounted slightly non-parallel to minimize internal reflections. Each window has a 10 mm diameter circle with 2 mm thickness, providing an optical aperture of 88.54 mm^2 and a cell volume of 3.276 µl. A polyethylene terephtalate spacer provides a 37 µm optical path-length. The interferogram for the sample in the cell is recorded using an optical resolution of 14 cm^{-1} x 2000 cm^{-1}, and averaging on the basis of 12 scans. The instrument is equipped with a HeNe laser (632 nm) and a silicium detector to track the position of the moving mirror in the interferometer. This laser not only determines the resolution but also the speed of the interferometer. The regression algorithms used for quantitative applications incorporates absorptions at selected wavenumbers or groups of wavenumbers (also referred to as filters) to generate a linear equation that best fits reference values in a data set. The most prominent wavenumbers at which the correlation between the measured absorbance and the corresponding reference values for the analyte of interest is the highest, are selected by the software of the instrument. Although the whole spectral range (929 to 5011 cm^{-1}) is stored for each calibration sample, filter selection is restricted to the following ranges: 965-1582, 1698-2006 and 2701-2971 cm^{-1}. The regions 1582-1692 and 2971-3627 cm^{-1} contain strong water absorption bands preventing energy passing through the cell, while the region from 3627 cm^{-1} onwards is eliminated because it contains very little useful information. It is possible to access the commercial algorithms and change critical parameters such as slope, intercept, filters and regression coefficients to improve the accuracy of prediction.

Commercial wine samples can be scanned without sample preparation, however a filtration step is recommended in order to remove particulate matter. Samples containing CO_2 need to be degassed.

NIR instrumentation used by the wine industry include Diode array spectrometers (25000-9100 cm^{-1}) and FOSS NIRSystems 6500 (25000 to 4000 cm^{-1}) devices. Spectrophotometers with a limited wavelength range are preferred, since they are less expensive and more rugged than traditional scanning monochromator instruments capable of scanning the full vis and NIR wavelength range.

CALIBRATION AND METHOD VALIDATION

Calibration is a crucial stage of data analysis where a model is fitted to the available data in such a way that it describes the data as faithfully as possible, while validation establishes the accuracy and precision and thus how well a model will perform for future samples taken from the same population as the calibration samples (*7*). When several chemical components are to be determined, calibration requires selection of the most relevant spectral wavelengths first, followed by informed use of multivariate calibration, typically PLSR, to optimize the calibration equations for each of the different parameters.

An independent set of samples is required to validate the accuracy, precision and the robustness of the calibration model. In regression, validation allows for an estimation of the average predicted error in future predictions. The outcome of the validation stage is generally expressed by a validation variance, e.g. $(RMSEP)^2$. Everything else equal, the lowest RMSEP signifies the best prediction model performance if validated on a test set. Cross-validation, based on the training data set alone, invariably leads to an estimate of the prediction performance which is over-optimistic and thus often unrealistic (7).

Few published studies have focused on validation of FT-IR analytical methodology for wines by comparing the FT-MIR prediction of wine components with standard reference methods (*10, 14, 15, 13*). These studies typically relied on spectral acquisition with WineScan FT120 and the MIR signals were calibrated by PLSR mathematics. The model developed by Patz *et al*. (*13*) allowed rapid screening of wine and must simultaneously for components and physical parameters such as alcohol, relative density, extract, sugar-free extract, refraction, conductivity, glycerol, total phenols, reducing sugars, fructose, glucose, sucrose, pH, total acidity, individual organic acids, and total SO_2 (Table1). Both R^2 statistics and RMSEP values were used to evaluate the calibration model.

Calibration introduces the requirement for quantitative classification in the spectra, because it assigns a response value, and also for qualitatative classification by distinguishing between adequate or deviating (outlier) samples (*16*). Such classifications arise from the presence of two different structures in a data set. One is related to the carrying over of systematic information from X to Y, the response, and it is identified by PLSR when calibrating. The other structure is internal to the X-space, and can for example be identified by using cluster analysis. This internal structure is not solely related to the response structure, even though both are based on the spectral data. In the stage of building and validating a PLSR model, it is possible to identify within these structures samples that represent outlier spectral data (X) and/or samples that present abnormalities in the response (Y). When using an established model on new spectra for prediction, it is however only possible to detect samples which present spectral abnormalities. In other words, information is not only on the spectra, but also in the relationships between the spectra and the

response. This underlines the necessary understanding that the applicability of a PLSR model can only be guaranteed by ensuring that the samples used in the training set are representative of the calibration scenario and also of the situation pertaining to the future prediction application of the model. Esbensen (*7*) deals in depth with these critical modeling-validation issues. Clearly, the evaluation of calibration models based on regression statistics alone often presents an overoptimistic view of the predictive abilities of the model. Ideally a calibration model should not only allow for the identification and interpretation of outliers in the sample set, but should also allow for the identification of poorly predicted samples, and sample types for which the model would not be suitable (*12*). What may appear as outlying samples in a first, limited context, may actually serve well as samples to be included in an augmented training data set. The key issue for a calibration data set is its representivity with respect to the matrix to be analyses and to future concentration ranges and data structure, not only the modeling characteristics.

Since wine samples present high variability due to origin, vintage, cultivar, viticulture, viniculture and wine style, apparent outliers may indeed on closer inspection turn out to be legitimate representatives of the data. Nieuwoudt *et al*. (*12*) evaluated PCA as a model for classification of outlier samples on spectra acquired with WineScan FT120. The authors showed that PCA, in combination with the SIMCA approach, can be used as a tool to differentiate between outliers and samples that are poorly predicted by calibration models. The necessary minimal understanding of the nature of any particular data structure (i.e. in the form of assigning status to the objects as training set, validation set, outliers, etc.) is very much a problem-dependent matter. In general it is dangerous to rely on automated algorithmic approaches (‘blind-folded’ calibration and validation). Proper multivariate calibration is not a matter that can be carried out by anyone new to this kind of analytical methodology, but needs serious study and experience (*5, 7*).

Table 1. Validation of FT-IR spectroscopy for German wine analysis (adapted from *13*)

Parameter	Range	Mean	R^2	RMSEP
Alcohol (vol%)	7.4-14.0	11.4	0.9819	0.16
Alcohol (g/L)	58.7-110.7	90	0.9753	1.4
Sugars (g/L)				
Before conversion	1.5-220.8	23.1	0.9978	1.7
After conversion	1.5-234.7	23.8	0.9983	1.3
Glucose	0.2-63.5	6.5	0.9957	0.7
Fructose	0.0-165.7	14.9	0.9983	1.1
Organic acids (g/L)				
Total acid	3.72-14.10	6.14	0.9734	0.25
pH	2.49-3.99	3.37	0.8344	0.12
Tartaric acid	0.80-3.30	2	0.4228	0.47
Malic acid	0.0-6.60	2.3	0.8110	0.63
Volatile acid	0.14-1.41	0.44	0.7680	0.09
Citric acid	0.0-2.30	0.32	0.4875	0.26
Glycerol (g/L)	5.20-27.80	7.85	0.9831	0.47
Phenolic compounds				
Total phenol (mg/L)	134-2260	570	0.9594	126
SO^2 (mg/L)				
Total (Tanner-Brunner)	32-588	120	0.7029	41
Total (automated photometry)	7-415	86	0.8431	33
Free	0-58	20	0.1196	12

CHALLENGES

FT-MIR spectroscopy is a rapid method for screening must and wine (*13*). The major advantages of the method are its simplicity and its capacity to produce enormous amounts of information within a very short time. With respect to process control, rapid analyses allows for the possibility to make quick decisions on how a

wine or must should be further treated. Fermentation processes can be sufficiently supervised allowing disturbances to be quickly addressed. IR absorption technology can potentially be extended to allow wine identification or classification in terms of varietal origin and geographical region of origin (*17*).

FT-IR should be considered as an addition to conventional analytical techniques rather than a replacement. There are four main challenges associated with wine analysis by FT-IR. Firstly, the similarity between IR absorption profiles because wine samples are chemically very similar and so are many of the wine components that are routinely measured. The second challenge relates to the presence of interferences, which is mainly due to the dominating absorption of ethanol and water in the mid-IR region. Two wavenumber regions, 2970-3620 cm^{-1} and 1550-1710 cm^{-1}, contribute to considerable noise in spectra and are associated with the absorbance of water (*12*). Other compounds that may be present in high concentrations, such as reducing sugar and organic acids, also interfere with certain analysis (*18*). Interferences are compensated for by multiple-wavenumber measurements and by subtraction of the water signal. The third challenge relates to the presence of outlier samples which differ in some respect from the samples that made up the training set. Due to the high degree of variation in wine samples, calibration requires a large number of samples with important dissimilarities among them. The stage of fermentation and the maturation period have been identified as major sources of variation (*12*). The fourth challenge relates to concentration limitations in the sense that FT-IR spectroscopy is not generally suitable for determination of components present below 0.2 g/L (*13*). The application of FTIR methodology is also limited for parameters such as sulfide or chloride ions and other inorganic species that do not absorb in the IR region (*18*).

The advantages and disadvantage of FT-MIR with multicomponent analytical instrumentation such as WineScan is summarized in Table 2. Full potential for quality and production control with FT-IR spectroscopy has not been realized. The following section gives an overview of studies evaluating FT-IR, in terms of both NIR and MIR, as a method to measure important parameters in wine.

Table 2. Advantages and disadvantages of FT-MIR spectroscopy (adapted from *13*)

Advantages	Disadvantages
Speed (< 90 sec per sample)	Relative high investment cost
Good reproducibility, precision and accuracy	Require calibration with large number of samples
Low maintenance and running cost	Calibration model can be influenced by adulteration
Easy to handle	Concentration limitations (LOD = 0.2 g/L)
High degree of automation	
Limited sample pre-treatment	
Non destructive	
Process control from grapes to finished wines	
Low environmental impact due to low chemical consumption	

GRAPE AND WINE COMPOSITION

Alcohol Content. In wine the total alcohol content comprises primarily out of ethanol. Ethanol is mainly produced by *Saccharomyces cerevisiae* during alcoholic fermentation and is one of the main components of natural wine (6.5 to 16% v/v) and distillation products. Another source of ethanol in certain styles of wines is through fortification with gape-derived spirits, usually distilled at approximately 96% v/v ethanol. A major contaminant in distilled spirits is methanol, which originates from microbial activity in the raw material used for distillation (*19*). Both ethanol and methanol are therefore routinely monitored during distillation. Fortification is not the only source of methanol in wine. Methanol may occur in trace amounts in natural wine, presumably resulting from enzymatic activity during winemaking (*20*). In the 1980's the European wine industry was troubled by several cases of adulteration of wine with methanol (*21*). Clearly, there is a need for fast and reliable methods for determination of both ethanol and methanol content in wine and wine-based spirits. Total alcohol is traditionally measured by densitometry with the assumption that the major component is ethanol. Methanol is commonly measured with gas chromatography (GC), a relatively complex and slow procedure that requires an experienced analyst. Due to the advantages offered by IR spectroscopy, this methodology has been used with success in the determination of alcohol in beer and

other alcoholic beverages (*22, 21*). Although application of NIR in finished wine has been limited due to the lack of success in obtaining reliable calibrations, determination of alcohol with NIR is relatively simple. The most common use of NIR in wine analysis has indeed been for the determination of ethanol (*23, 24, 25*). The OH bond in ethanol produces a near-infrared band that is easily distinguished from the OH band of water. Ethanol is furthermore easily distinguished from methanol in the near-infrared region (*21, 26*). Grape spirits, being a relatively simple matrix, presented even less complication in NIR calibration (*27*). PLSR models allowed almost perfect correlation between reference values and NIR spectra of grape spirits for both total alcohol and methanol (Table 3).

Not only NIR, but also MIR has been used with success to quantify the total alcohol content of wine. In a complex matrix such as wine, MIR spectral data is generally easier to interpret than corresponding NIR spectral regions. The results presented by Patz *et al.* (*13*) were comparable with official EC wine methods (EEC No. 2676/90) in terms of both repeatability and reproducibility (Table 1).

Table 3. NIR spectroscopy calibration statistics

Parameter	Sample Type	Range	R^2	RMSEP or RMSECV*	RPD	Reference
Alcohol						
Total alcohol (% v/v)	Grape Spirits	93.7-96.5	0.960	0.08		(27)
Methanol (g/L)	Grape Spirits	0.02-188.8	0.998	0.06		(27)
Acidity						
pH	Red grape homogenates	3.66-4.26	0.72	0.07*	1.4	(31)
pH	Intact red grapes		0.50	0.08*	1.4	
Phenolic compounds						
Color (mg/g)	Red grape homogenates	0.38-1.72	0.90	0.10*	4.2	(31)
Color (mg/g)	Intact red grapes		0.50	0.14*	1.9	
M3G (mg/L)	Red wine ferments	± 183	0.81-0.94	17.5-31.5*	2.5-4.3	(35)
Pigmented polymers (mg/L)	Red wine ferments	± 21	0.82-0.94	3.2-26.8*	2.1-5.8	(35)
Tannins as catechin (mg/L)	Red wine ferments	± 318	0.7-0.97	49.1-118*	1.8-3.0	(35)

Total Soluble Solids (TSS). The soluble solids in unfermented musts and wines are mainly sugars (90%), in particular glucose and fructose. The TSS content, traditionally measured as either ºBrix or ºBalling with a refractometer, provides an indication of grape maturity and signals the optimal time for harvesting. Both NIR and MIR show potential to be used as alternatives to refractometry. MLR combined with PCA displayed a linear correlation between NIR spectra and sugar content of must (*28*). Comparatively, MIR displayed superior predictive capability (*29*) and WineScan instrumentation in particular has been applied with success in the analysis of wine (*13*). Over a broad concentration range almost perfect correlation was obtained between reference values and PLSR modulated spectra representing total sugar, fructose and glucose (Table 1). Calibration of total sugars in dry wines (RS<5g/L) may be complicated by the presence of high concentrations of organic acids having similar IR absorption bands (C=O, C-O and OH). Better recovery is expected in sweeter wines where the sugar to organic acid ratio is higher (18). Prediction was nevertheless compromised at above 20 g/L total sugar (*13*). Sucrose absorbs strongly in the MIR wavelength range and although this sugar is not expected to be present in 'still' wines, recently bottled sparkling wines may contain measurable amounts of unfermented sucrose. Due to the structural similarity between sucrose and its monosaccharides, glucose and fructose, separation of the respective spectral responses is complicated.

Acidity. Grape and grape juice quality is usually assessed by measuring not only TSS, but also acidity (*30*). The main organic acids in wine are tartrate (*ca* 1.5-4 g/L), malate (*ca* 0-4.0 g/L), lactate (*ca* 0.1-3.0 g/L), acetate (>0.2 g/L measured as volatile acidity), citrate (*ca* 0-0.5 g/L) and, in some cases, succinate (0-2 g/l) so that the 'acidity' is generally measured in terms of both total titratable acid and the pH of the wine. A diode array spectroscopic instrument (25000 – 9000 cm^{-1}) has been evaluated as an alternative method to allow for rapid prediction of pH in red grapes (*31*). These authors compared the visible (25000 – 14000 cm^{-1}), NIR (14000 – 9000 cm^{-1}) and visible+NIR (25000 – 9000 cm^{-1}) regions by applying PLSR statistics. The possibility of extending NIR technology to intact berry samples was also explored in this study. NIR radiation easily passes through berry and liquid samples and may potentially permit the analysis of whole grape berries. Calibration statistics obtained in the NIR region were however less than satisfactory (Table 3). On the other hand, good results were obtained when analyzing homogenized grapes in the visible region (R^2_{val} = 0.90; RMSECV = 0.04% v/v). The values obtained correspond well to the predictive capability of expensive scanning monochromotor instruments (*32*). Comparative studies between NIR and MIR in the analysis of grape homogenates suggest that MIR has superior predictive capability for pH (*29*).

Using finished German wines of different styles as samples, FT-MIR WineScan spectra displayed linear correlation with both total acidity (R^2 = 0.97; RMSEP = 0.25% v/v) and pH (R^2 = 0.83; RMSEP = 0.12% v/v) as given by PLSR modulation

(*13*). For individual organic acids, reasonable correlations were obtained for malate (R^2 = 0.81; RSEMP = 0.63% v/v) and volatile acidity (R^2 = 0.77; RSEMP = 0.09% v/v). The calibration model did not allow for quantification of tartrate and citrate. On the other hand, close to 100% recovery has been obtained for tartrate when wine samples were spiked with different organic acids (*33, 34*). Samples used for calibration in the cited recovery studies did not include late harvest wines, unlike the German study where wine samples covered a broad range of sugar concentrations. Organic acids and sugars have a carbonyl group (C=O) in common and also share C-O and OH IR bands, unlike for ethanol and glycerol. As a result, sugar may interfere with the quantification of organic acids. Analysis of individual organic acids may furthermore be complicated by spectroscopic interferences arising from similarities between IR spectra of organic acids themselves, especially if these analyte concentrations are present close to the limit of detection. Interferences can be reduced by careful choice of absorbance filters. Vibrational bands of most relevance in calibration for individual organic acids appear to be as follow: (i) C=O stretch of carboxylic acid (±1720 cm^{-1}) for total acidity, tartrate and malate; (ii) OH bend of the carboxylic acid (± 1400 cm^{-1}) for citrate; (iii) CH_3 bend (± 1375cm^{-1}) for volatile acidity (*34*). The characteristic C=O stretch is of lesser importance when calibrating for organic acids present in low concentrations (e.g. lactate, citrate and volatile acidity) due to the presence of interfering compounds (e.g. sugars) displaying absorption bands in the same IR region.

Glycerol. Glycerol is quantitatively a major component of wine (2-15 g/L), and its determination at various stages of the winemaking process provides information related to quality control. Patz *et al.* (*13*) used mid-IR in combination with PLSR to quantify glycerol from samples representing a wide range of German wine styles (RMSEP = 0.47 g/L). Nieuwoudt *et al*. (*12*) developed a PLS-based glycerol calibration model for South African wine (reducing sugar content < 30 g/L, alcohol content > 8% v/v) based on FT-IR spectra gathered with a WineScan instrument. The model provided satisfactory predicative ability (RMSEP = 0.4 g/L) and was suitable for quantification purposes (RPD = 5.6). Similarly, a calibration model for late harvest wines (RS 31-147 g/L, alcohol > 11.6% v/v) was established with a prediction error RMSECV = 0.65 g/L. The majority of variation (> 85%) in glycerol content of wines of various styles was correlated to absorbance filters 1229-929 cm^{-1} which incorporates the C-O stretch bands of glycerol. PCA cluster analysis suggested subdivision of samples according to wine styles in terms of alcohol and sugar content to obtain calibration models that display acceptable prediction accuracies. Furthermore, young wines and wines in different stages of fermentation displayed great variation when compared with bottled wine and should therefore be carefully monitored in terms of prediction accuracies.

Phenolic Compounds. Phenolic compounds contribute to both color and the organoleptic properties of red wine, in particular mouth-feel (*30*). Color in red wine mainly originates from grape-derived anthocyanins, such as malvidine-3-glucoside (M3G), which are converted into pigmented polymers during fermentation and ageing. Condensed flavonoid tannins, such as catechin and epicatechin, are mainly responsible for astringency. Non-volatile phenolic acids are precursors of volatile phenols, which contribute to various organoleptic important aromas in wine. The structure of wine phenolics varies significantly when wine ages in the tank or in the barrel and in the bottle. Established methods for determination of wine phenolics are generally based on colorimetric or chromatographic techniques, in particular HPLC. These techniques require sample preparation and chemical manipulations which make them time consuming, laborious and costly. Furthermore, chromatographic analyses are still limited to simple and short polymeric molecules. Visible+NIR spectroscopy (25000 – 4000 cm^{-1}) showed promise for simultaneous determination of various phenolic compounds (M3G, pigmented polymers and tannin) in fermenting must and wine samples representing different red varieties and vintages (*35*). Specificity of cross evaluated PLS calibrations was not optimal (R^2 > 0.7; RPD 1.8 to 5.8) due to inconsistencies arising from many color changes that occur during fermentation (Table 3). Color changes are governed by different oxidation, condensation, polymerization and precipitation reactions. PCA revealed the spectral regions which displayed highest correlations and these were the visible 540 nm region, which is dominated by color pigments, and the region around 2200-2300 nm. As expected, it was also the visible region that allowed best prediction of color (R^2_{val} = 0.92; RMSECV = 0.07 mg/g) in homogenized grape samples (*31*). The region 2200-2300 nm relates to the presence of polyphenolic compounds involving tannins (*36*). Although NIR allows for better prediction of color than MIR, best prediction was obtained when combining the respective spectral regions (*26, 29*). FT-IR spectroscopy may potentially be extended to qualitatively monitor fermentations in terms of its phenolic content and, if successful, should allow better process control during maturation of the finished wine (*26*).

Sulfur Dioxide. Sulfite (< 1g/L) is routinely added to grape must and wine to restrict the growth of indigenous yeast and bacteria and to act as an antioxidant (*37, 28*). At wine pH (pH 3 to 4) sulfite predominates as 'free SO_2', consisting mainly of bisulfate anion (HSO_3^{1-}) and a small proportion of molecular SO_2 ($SO_2 \cdot H_2O$) and sulfite anion (SO_3^{2-}) present in equilibrium. Certain yeast strains produce significant amounts of SO_2 during alcoholic fermentation. Free SO_2 (especially HSO_3^{1-}) bind with carbonyl compounds in wine to form what is known as 'bound SO_2' with weak antimicrobial properties. Both 'bound' and 'free' forms are routinely measured during all stages of the winemaking process, due to the fluctuating equilibrium existing between free and bound SO_2, and the difference in properties displayed by these forms. Due to associated health risks, only the total amount of SO_2 in finished wines

is strictly regulated. Free SO_2 is however generally maintained at above 30 mg/L throughout the winemaking process to prevent microbial spoilage. Correct SO_2 usage is clearly one of the most important practices employed in winemaking and could benefit from fast, reliable quantification methods. While little useful information is obtained for sulfides from the infrared spectrum, single-bonded (S-O) compounds display several strong bands in the region 1000-650 cm^{-1} and double-bonded sulfur-containing compounds (S=O) display several characteristic stretches within the region 1375-1050 cm^{-1} (*1*). FT-IR analysis of sulfate in wine has been studied after the addition of sulfuric acid (*18*). At the pH of wine, sulfuric acid is entirely dissociated to sulfate ions, the absorption bands (S=O) at 1150-1050 cm^{-1} were particularly useful since this IR region is different than the regions employed for most other organic compounds. As a result, recovery of sulfate ions present in low concentrations (< 1g/L) were better than for other compounds at the same concentration levels, e.g. total sugars in dry wines. Mounting consumer demands for safe alternatives to chemical preservatives such as SO_2 has led to an interest in different solutions (*39, 40, 41, 42*) and a resulting decrease in the quantities added to must and wine (often below 0.1 g/L). SO_2 concentrations in finished wines may therefore be present at levels close to the limit of detection of FT-MIR (0.2 g/L). It was therefore not unexpected to find that WineScan data validated against standard references methods only allowed for the qualitative screening of total SO_2 (R^2 = 0.70) and not its quantification (13). Furthermore, reference results for free SO_2 (R^2 = 0.12) displayed no correlation to spectral data. Low performance was most probably due to SO_2 concentrations in these wines being close to (total SO_2 < 0.6 g/L) or below (free SO_2 < 0.1 g/L) the limit of detection of FT-MIR (Table 1).

SENSORY PROPERTIES

Aroma and flavor are the main determinants of grape and wine quality. Volatile compounds are responsible for aroma, while flavor refers to the total organoleptic experience resulting from both volatile and non-volatile components. Up to date most studies have focused on the contribution of volatile compounds to wine aroma (*43*). These compounds originate from the grape berry, the metabolism of yeast and bacteria, oak wood extraction, and chemical reactions upon processing and storage. Traditionally, volatile compounds are analyzed by gas chromatography combined with mass spectrometry (GC-MS), a technique that is not only expensive but also time consuming. GC-MS have furthermore limited success in terms of prediction of sensory characteristics due to lack of knowledge regarding aroma composition of wines. Recent developments in MS based electronic nose (MS-eNose) instruments allow headspace characterization of different food and beverage products (*44*). Chemometrics combined with analytical techniques such as IR and MS-eNose make

it possible to obtain a fingerprint of a sample matrix in terms of its volatile components (*45, 46*). Still, such an approach does not readily link a particular characteristic such as a sensory attribute to one or more individual compound. While aromatic compounds are often present in concentrations below the limit of detection of FT-IR, many non-volatile compounds related to wine flavor are present at higher concentrations and offer possibilities for objective quality grading by FT-IR. Organic compounds such as ethanol and phenolics, particularly anthocyanin pigments, were shown to be correlated to wine quality (*26*). Non-volatile compounds can also play a more indirect role in wine by reacting with volatiles, either chemically, physically or in a perceptual manner, to alter flavor and aroma (*47*). Furthermore, grape precursors such as glycoconjugates give rise to volatile components during winemaking (*8*). FT-MIR has been successfully applied to measure individual glycoconjugates in Muscadet grapes (*48*). Glycoconjugates significantly contribute to the characteristic aroma and flavor of a given grape variety. Quantization of such precursors could therefore be a useful index for winemakers to determine not only grape quality, but also optimal maturity of grapes and varietal origin.

IDENTIFICATION OF MICROBIAL STRAINS

Various studies have shown that FT-IR spectra can be used to identify and differentiate microorganisms down to the level of subspecies, strain or even serotype (*49, 50, 51, 52*). The technique produces a complex fingerprint of intact microbial cells with spectra that reflect the total biochemical composition of the microorganism, e.g. membrane and cell wall components, proteins and nucleic acids. The main bacterial contaminants in wine are acetic acid bacteria and lactic acid bacteria (LAB). LAB identified in wine represent the genera *Lactobacillus*, *Leuconostoc*, *Oenococcus*, and *Pediococcus* (*37*). Of these, only *Lactobacillus* species have been subjected to differentiation by FT-IR spectroscopy (*53, 54*). Although not applied to wine samples, the technique proved to be rapid, reliable and reproducible and, in conjunction with multivariate techniques, can potentially be used for rapid screening of bacterial contamination in wine. This methodology may be extended to yeast typing at strain level and has already been successfully applied to differentiate between mixed populations of food-borne yeast strains including *Saccharomyces cerevisiae* (*55, 56, 57*).

CONCLUSIONS AND FUTURE PROSPECTS

FT-IR is exploited in very diverse fields including biomedical diagnostics, environmental monitoring, (bio)chemical analysis, food processing and biotechnology. Application of analytical MIR and NIR spectroscopy in grape and wine analysis lag behind other application fields, but considering the quantitative

increase and qualitative broadening of application within the grape and wine industry, as seen from publications and conference proceedings (Table 4) from the last 5 years, this is rapidly changing. IR-spectroscopy combined with multivariate calibration methods allows for simultaneous identification and quantification of several analytes in wine without prior separation or sample preparation. Generally MIR spectral data is analytically easier to interpret than corresponding information obtained in the UV-visible and NIR spectral regions. Based on calibration statistics, MIR appears to be superior for at least pH, alcohol and TSS, while NIR and UV-visible regions display better prediction for color. FT-IR technology could potentially be extended to allow rapid analysis of intact grapes while on the vine with inexpensive hand-held instruments (*58*).

Hyphenating FT-IR with analytical separation tools, particularly capillary electrophoresis (CE), will most certainly allow for many new developments. Dynamic flow separation systems is expected to reduce complications linked to interferences during calibration. The separation ability of CE is extremely high and only micro-volumes of sample are required. FT-IR is fully compatible with the narrow capillaries employed in CE. The winemaking process demands constant product monitoring and will undoubtedly benefit immensely from on-line analysis. Recent developments with on-line hyphenated flow cells aim at simultaneously fulfilling the specific demands posed by both the spectrometer and CE instrumentation (*59*). Such technologies promise to separate and identify a wide range of analytes in picogram quantities in the near future.

Wine is a complex medium and several different analytical methods for quantification of diverse classes of chemical components in one sample are frequently required. Time and cost restrictions necessitate that a maximum of reliable analytical results can be obtained, always subjected to large numbers of samples. It should be emphasized in this overview that for reliable analysis, it is essential to implement a representative sampling scheme. Suffice here to mention but a few key overviews, which deals specifically with the Theory of Sampling (TOS) in relation to on-line analysis and its optimal relationship to the entire process- or processing context (*60, 61, 62, 63, 64*).

Table 4. Recent conference proceedings

This table serves as a source of reference, providing access to research results that have not been published in peered reviewed Journals.

1999

Gishen, M., Dambergs, R.G. Kambouris, A., Kwiatkowski, M., Cynkar, W.U., Høj, P.B., and Francis, I.L. Application of near infrared spectroscopy for quality assessment of grapes, wine and spirits. The 9th International Near Infrared Spectroscopy Conference, Verona, Italy.

2001

Dambergs, R.G., Kambouris, A., Schumacher, N., Francis, I.L., Esler, M.B., and Gishen, M. Wine quality grading by near infrared spectroscopy. The 10th International Near Infrared Spectroscopy Conference, Kuonguji, Korea.

Esler, M.B., Gishen,M., Francis, I.L., Dambergs, R.G., Kambouris, A., Cynkar, W.U., and Boehm, D.R. Effects of variety and region on near infrared reflectance spectroscopic analysis of quality parameters in red wine grapes. The 10th International Near Infrared Spectroscopy Conference, Kuonguji, Korea.

Gishen, M., Iland, P.G., Dambergs, R.G., Esler, M.B., Francis, I.L., Kambouris, A., Johnstone, R.S., and Høj, P.B. Objective measures of grape and wine quality. The 11th Australian Wine Industry Technical Conference, Adelaide, Australia.

2002

Cozollino, D., Gishen, M., Dambergs, R.G., Cynkar, W., Kwiatkowski, M., Herderick, M., and Høj, P. The application of near infrared spectroscopy for monitoring changes during red wine fermentation. The 11th International Near Infrared Spectroscopy Conference, Cordoba, Spain.

Cozollino, D., Gishen, M., Dambergs, R.G., Cynkar, W., and Høj, P. Determination of colour and pH in whole and homogenized grape samples using a diode array spectrophotometer. The 11th International Near Infrared Spectroscopy Conference, Cordoba, Spain.

Cozzolino, D., Gishen, M., Dambergs, R.G., Cynkar, W., and Høj, P. Standardisation and calibration transfer between spectrophotometers for red grape homogenates. The 11th International Near Infrared Spectroscopy Conference, Cordoba, Spain.

Dambergs, R.G., Cozzolino, D., Cynkar, W., Esler, M., Janik, L., Francis, I.L., Høj, P., and Gishen, M. Strategies to minimize matrix-related error with NIRS analysis of wine grape quality parameters. The 11th International Near Infrared Spectroscopy Conference, Cordoba, Spain.

Manley, M., De Bruyn, N., and Downey, G. Classification of three-year old, unblended South African brandy with Near Infrared Spectroscopy. The 11th International Near Infrared Spectroscopy Conference, Cordoba, Spain.

2004

Cozzolino, D., Smyth, H.E., Dambergs, R.G. and Gishen, M. Multivariate analysis (chemometrics): a novel approach in modern interdisciplinary sciences. The 12th Australian Wine Industry Technical Conference, Melbourne, Australia.

Cozzolino, D., Cynkar, W.U., Janik, L., Dambergs, R.G., Francis, I.L. and Gishen, M.. Measurement of colour, total soluble solids and pH in whole red grapes using visible and near infrared spectroscopy. The 12th Australian Wine Industry Technical Conference, Melbourne, Australia.

Dambergs, R.G., Stummer, B., Zanker, T., Cozzolino, D., Gishen, M and Scott, E. Near infrared spectroscopy as a tool for detection of powdery mildew in homogenized grapes. The 12th Australian Wine Industry Technical Conference, Melbourne, Australia.

Dambergs, R.G., Kambouris, B., Cynkar, W.U., Janik, I.L., Cozzolino, D., Henschke, P.A., and Gishen, M. A comparison of near infrared and mid-infrared spectroscopy for the analysis of yeast assimilable nitrogen in grape juice. The 12th Australian Wine Industry Technical Conference, Melbourne, Australia.

Janik, L.J., Cynkar, W.U., Cozzolino, D., Gishen, M., and Dambergs, R.G. The potential of attenuated total reflectance mid-infrared spectroscopy for grape compositional analysis. The 12th Australian Wine Industry Technical Conference, Melbourne, Australia.

Nieuwoudt, H.H., Prior, B.A., Pretorius, I.S., Manley, M., and Bauer, F.F. The application of Fourier-transform infrared spectroscopy and multivariate data

analysis for the design of calibration sets for glycerol in wine. The 12th Australian Wine Industry Technical Conference, Melbourne, Australia.

2006

Lochner, E., Nieuwoudt, H.H., and Lambrechts, M.G. The evaluation of Fourier transform infrared (FT-IR) spectroscopy for the determination of alcohol concentration in cream based liqueurs. The 3rd International Viticultural and Oenology Conference, Somerset West, South Africa.

Lochner, E., Silcher, T., Nieuwoudt, H.H., and Lambrechts, M.G. The evaluation of Fourier transform infrared (FT-IR) spectroscopy for the determining chemical constituents of fortified wines. The 3rd International Viticultural and Oenology Conference, Somerset West, South Africa.

Lochner, E., Nieuwoudt, H.H., and Lambrechts, M.G. Rapid analysis of alcoholic beverages using Fourier transform infrared (FT-IR) spectroscopy. The 3rd International Viticultural and Oenology Conference, Somerset West, South Africa.

Louw, L., Nieuwoudt, H., Lambrechts, M., Swanepoel, M., Naes, T., and van Rensburg, P. Chemical profiling of South African young wines using Fourier transform infrared spectroscopy. The 3rd International Viticultural and Oenology Conference, Somerset West, South Africa.

Malherbe, S., du Toit, M., Bauer, F.F., and Nieuwoudt, H.H. A simple and effective strategy for the selection of calibration samples for FT-IR spectroscopy. The 3rd International Viticultural and Oenology Conference, Somerset West, South Africa.

Malherbe, S., du Toit, M., Bauer, F.F and Nieuwoudt, H.H. Problem fermentations: An industrial case study investigating the discrimination possibilities of FT-IR spectroscopy. The 3rd International Viticultural and Oenology Conference, Somerset West, South Africa.

Nieuwoudt, H.H., Werth,C., Lochner, E., Stander, A., Groenewald, A., Noble, S., Magerman, C., and Swanepoel, M. Evaluation of Fourier transform infrared spectroscopy for routine wine analysis in South-Africa. The 3rd International Viticultural and Oenology Conference, Somerset West, South Africa.

De Groot, A., Osborne, C.D., Nieuwoudt, H.H., du Toit, M., and Van Rensburg, P. Using FTIR-ATR as a rapid tool in discrimination of yeast and bacteria. The 3rd

International Viticultural and Oenology Conference, Somerset West, South Africa.

Kleintjies, T.V., Lambrechts, M., Nieuwoudt, H.H. and Lochner, E. Development of Fourier transform near infrared (FT-IR) spectroscopy calibration models for the determination of alcohol, density, obscuration and colour in spirit products. The 3rd International Viticultural and Oenology Conference, Somerset West, South Africa.

ACKNOWLEDGEMENTS

I have consulted several specialists in the field of FT-IR and chemometrics and am grateful for their generosity and invaluable discussions: Dr. Hélène Nieuwoudt and Prof. Florian F. Bauer from the Institute for Wine Biotechnology (Department of Viticulture and Oenology, Stellenbosch University), Prof. Jens Kossmann from the Institute for Plant Biotechnology (Department of Genetics, Stellenbosch University), Prof. Klaus R. Koch from the Department of Analytical Chemistry (Stellenbosch University) and Prof. Kim H. Esbensen from the ACABS research group (Aalborg University Esbjerg , Denmark). They have read and approved the manuscript. This manuscript has furthermore been accepted for publication (March 2008) in an international peer reviewed journal, 'Analytical Chemistry' (Impact Factor = 6.2).

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