AMINO ACID ANALYSIS IN WINES BY LIQUID CHROMATOGRAPHY – UV AND FLUORESCENCE DETECTION WITHOUT SAMPLE ENRICHMENT

by

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Declaration

I the undersigned hereby declare that the work contained in this thesis 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

Summary

In this study, the analysis of amino acids using High Performance Liquid Chromatography (HPLC) with pre-column derivatisation was optimised. The derivatisation reagents include o-phthaldialdehyde (OPA), 9-fluorenylmethylchloroformate (FMOC) and iodoacetic acid (IDA). Detection was performed using UV and fluorescence in series. The developed method was utilised for the analysis and quantitation of amino acids in eighteen wines. The application of chemometric data evaluation was initiated.

Opsomming

Hierdie ondersoek behels die optimisering van die aminosuuranalise deur gebruik te maak van Hoë Druk Vloeistof Chromatografie (HDVC) in kombinasie met pre-kolom derivatisering. Die derivatisatiserings reagense sluit in o-phthaldialdehied (OPA), 9-fluorenielmetielchloroformaat (FMOC) en jodoasynsuur (IDA). Deteksie is gedoen deur gebruik gemaak van 'n ultraviolet (UV) en 'n fluorosensie detektor in serie. Die metode sodoende ontwikkel is gebruik vir die analise en kwantifisering van aminosure in agtien wyne. Die toepassing van chemometriese data evaluasie is ook geïnisieer.

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General introduction

The role of the amino acids in wine is a significant one. They are the main source of nitrogen for bacteria and yeast during fermentation. The rate and efficiency of fermentation can be monitored through amino acid analysis. The taste and flavour characteristics of the amino acids themselves and their interference in the fermentation process during the manufacturing of wine will strongly influence the organoleptic properties of the final product.

A reproducible, efficient separation and quantification technique of amino acids is therefore highly beneficial to enological studies.

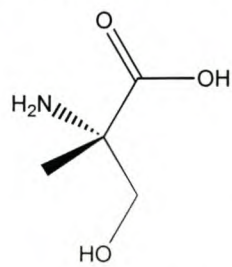
In this contribution, the relationship between the amino acid composition of wines and grapes they originate from is studied in order to allow us to relate them to the flavour, taste and mouth feel of wines in future work.

Today, many chromatographic techniques and methods exist for the analysis of amino acids in natural products, but none of these can determine the full spectrum of natural amino acids including the problematic ones like cysteine, proline and hydroxyproline. The main technique used is High Performance Liquid Chromatography (HPLC). Various derivatisation reactions have been studied to a great extent. The use of o-phthaldialdehyde (OPA) features prominently in the reagents listed. However, it has its own drawbacks in the fact that it does not react with proline, hydroxyproline or cysteine. Various additional reagents have been suggested to improve upon these disadvantages, to much success. In this thesis, a thorough study was performed to obtain a quick and efficient derivatisation and clean-up procedure involving the combination of OPA, 9-fluorenylmethylchloroformate (FMOC) and iodoacetic acid (IDA). This procedure was then followed by a quick and robust analysis by HPLC with UV and fluorescence detection in series. The possibility to couple the system to electrospray mass spectrometry was also investigated. First the characteristics of amino acids and their importance in wine are discussed before the different HPLC techniques and derivatisation strategies are covered.

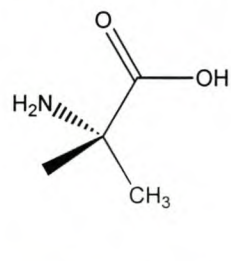
Chapter 1. Amino acids and their relevance in wine

1.1 General characteristics

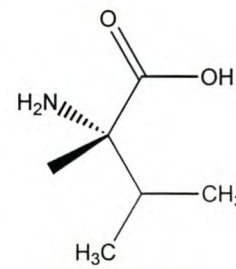
Amino acids are an important class of organic compounds that contain both the amino ($-NH_2$) and carboxyl (-COOH) groups [1] and, unlike the two other basic nutrients, sugar and fatty acids, amino acids contain nitrogen (about 16%, w/w) [2]. Amino acids serve as the building blocks of proteins. Known as the standard, or alpha, amino acids, they comprise alanine (ALA), arginine (ARG), asparagine (ASN), aspartic acid (ASP), cysteine (CYS), glutamic acid (GLU), glutamine (GLN), glycine (GLY), histidine (HIS), isoleucine (ILE), leucine (LEU), lysine (LYS), methionine (MET), phenylalanine (PHE), serine (SER), threonine (THR), tryptophan (TRP), tyrosine (TYR), valine (VAL) and proline (PRO).



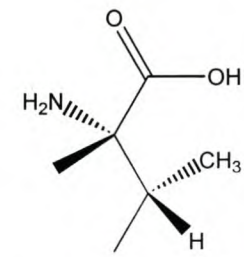
Serine (SER)



Alanine (ALA)

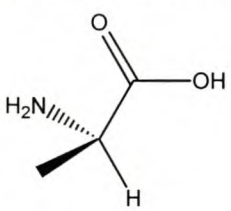


Valine (VAL)

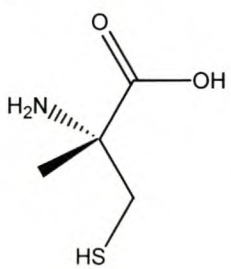


Threonine (THR)

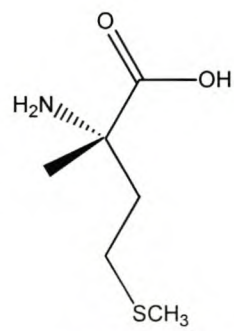
The amino and carboxyl groups are both attached to a single carbon atom, which is called the alpha carbon atom. Attached to the carbon atom is a variable group R. It is in the R group that the molecules of the 20 amino acids differ from one another. In the simplest of the acids, glycine, the R consists of a single hydrogen atom. Other amino acids have more complex R groups that contain carbon as well as hydrogen and may include oxygen, nitrogen or sulfur.



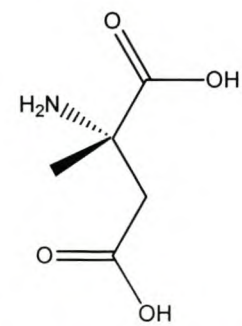
Glycine (GLY)



Cysteine (CYS)

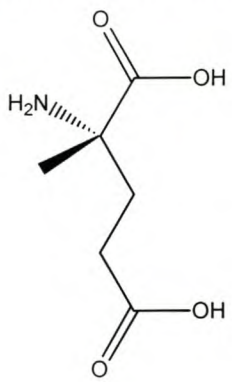


Methionine (MET)

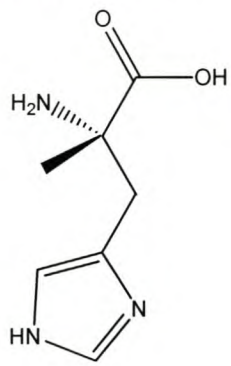


Aspartic Acid (ASP)

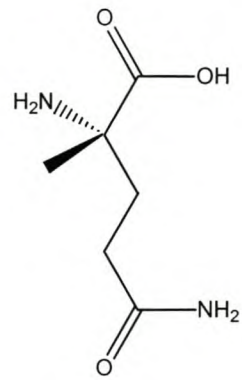
When a living cell makes proteins, the carboxyl group of one amino acid is linked to the amino group of another to form a peptide bond. The carboxyl group of the second amino acid is similarly linked to the amino group of a third, and so on, until a long chain is produced. This chainlike molecule, which may vary from 50 to several hundred amino acid subunits, is called a polypeptide. A protein may be formed of a single polypeptide chain or it may consist of several such chains held together by weak molecular bonds. Each protein is formed according to a precise set of instructions contained within the genetic material of the cell. These instructions determine which of the 20 standard amino acids are to be incorporated into the protein, and in what sequence. The side groups of the amino acid subunits determine the final shape of the protein and its chemical properties. An extraordinary variety of proteins can be produced from the same 20 subunits. Proteins are needed by every living organism, and next to water, make up the largest portion of our body weight since it is contained in muscles, organs, hair, etc [2]. The standard amino acids serve as raw materials for the manufacturing of many other cellular products, including hormones, and pigments. In addition, several of these amino acids are key intermediates in cellular metabolism.



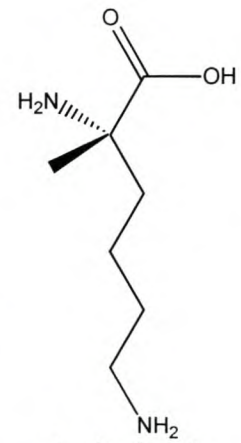
Glutamic Acid (GLU)



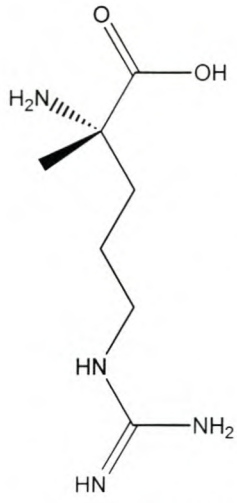
Histidine (HIS)



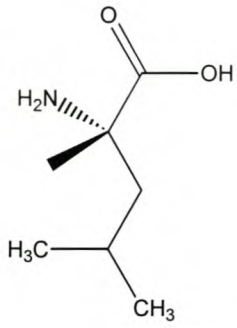
Glutamine (GLN)



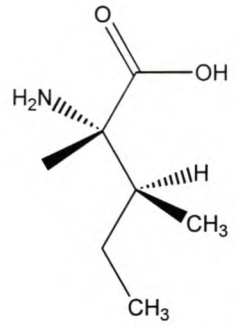
Lysine (LYS)



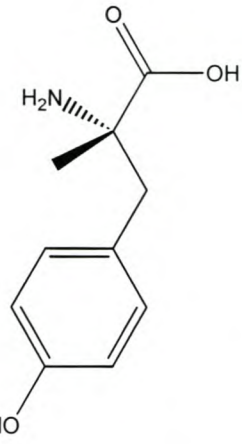
Arginine (ARG)



Leucine (LEU)

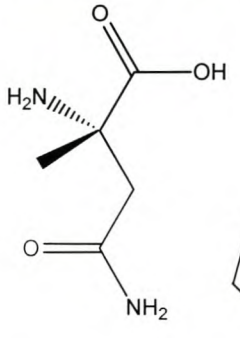


Isoleucine (ILE)

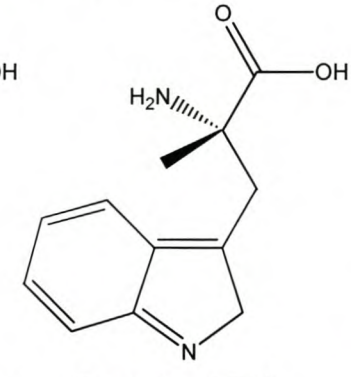


Tyrosine (TYR)

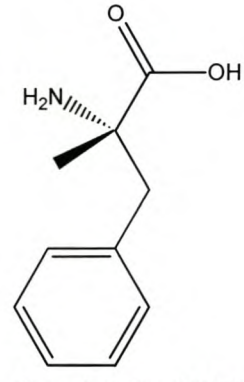
Most plants and microorganisms are able to make all the amino acids they require for normal growth. Animals, however, must obtain some of the standard amino acids from their diet in order to survive; these particular amino acids are called essential. Essential amino acids for humans include lysine, tryptophan, valine, histidine, leucine, isoleucine, phenylalanine, threonine, methionine and arginine. They are found in adequate amounts in protein-rich foods from animal sources or in carefully chosen combinations of plant proteins. Amino acids, except glycine, are naturally present in the L form [2].



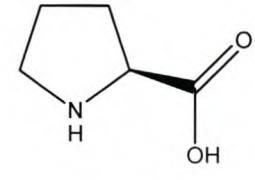
Asparagine (ASN)



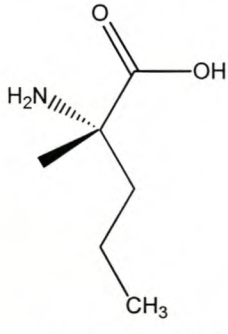
Tryptophan (TRP)



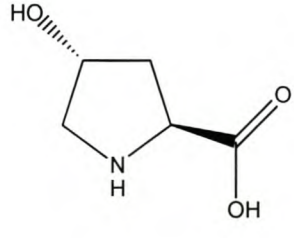
Phenylalanine (PHE)



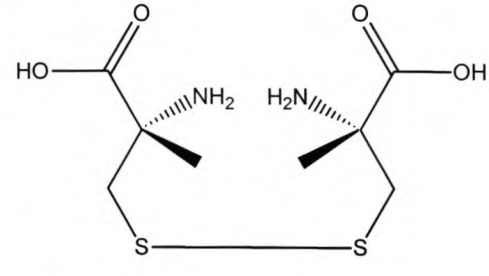
Proline (PRO)



Norvaline (NOR)



Hydroxyproline (HYP)



Cystine (CY2)

In addition to the 20 standard amino acids forming proteins, more than 150 other amino acids have been found in nature, including some that have the carboxyl and amino groups attached to separate carbon atoms (β-amino acids). These amino acids are generally formed by modification of the side chain after the peptide chain has been formed. These unusually structured amino acids are most often found in fungi and higher plants.

1.2 Amino acids in wines

The total amount of nitrogen within a wine or the pulp and skin of the crushed grapes before fermentation, commonly referred to as the must, includes both organic and inorganic forms (mineral nitrogen). The organic forms of nitrogen include: amino acids, oligopeptides and polypeptides, proteins, bio amines, nucleic nitrogen, amino sugar nitrogen and pyrazines. Of these, amino acids are the most prevalent form of nitrogen in musts and wines by weight [3].

Grape juice is converted to wine via fermentation. Grapes contain yeasts, mould and bacteria. When the juice is placed into a container and kept at the optimum temperature, naturally present or commercially available yeast (added by the winemaker) acts on the sugar in the grape juice and converts it to alcohol and carbon dioxide [1].

The overall process of fermentation is to convert glucose sugar ($C_6H_{12}O_6$) to ethanol (CH_3CH_2OH) and carbon dioxide gas (CO_2). The reactions within the yeast to make this happen are very complex but the overall process is as follows [4]:

$$C_6H_{12}O_6 \xrightarrow{\text{Yeast}} CH_3CH_2OH + CO_2$$

Glucose — Alcohol — Carbon dioxide

A solution of sugar will remain unchanged for an indefinite period of time. To induce fermentation, a portion of some nitrogenous body, itself undergoing decomposition, must be present. Yeast consists of vegetable egg-shaped cells that increase during its

action as fermentation agent. In wine making there can be sufficient natural yeast in the grape to cause fermentation without the use of yeast [4], however, it is common practice to add yeast during fermentation of the wine, leading to faster and better control over fermentation.

The amino acids have several important functions in the wine production. They are the main nitrogen source for yeast during fermentation of carbohydrates as well as bacterial nutrients during malolactic fermentation. They are flavour precursors and unfortunately also precursors of undesirable by-products [5,6,7].

Amino acids in wine originate from different sources. They can stem from the grape variety itself, although original amino acids are claimed to be completely metabolised during fermentation. Other factors besides the grape variety are the production area, cultivation methods, yeast strain and conditions of fermentation [6]. Some amino acids are released by live yeast at the end of fermentation, or excreted by proteolysis during the autolysis of dead yeast. They may even be produced by enzymatic degradation of grape proteins [5,6]. In studies concerning the autolysis of yeast in champagnes [8] it was shown that the concentration of amino acids increases the longer the champagne is in contact with the yeast. It was also found that this generally improves the organoleptic quality of the champagne, specifically with regards to "aromatic" quality. Therefore it can be assumed that the increase in amino acid concentration directly affects the aroma and thus the quality of the champagne.

Yeasts cannot assimilate proline in the absence of oxygen, which means that when fermentation is complete, the relative amounts of proline are often higher than those of the other amino acids. Proline can therefore be used as a marker for ripeness. It has been observed that there is a steady increase of amino acid content in the grapes as they ripen. A total nitrogen assay of grape must has shown that the total amount of nitrogen in the grape may vary from year to year. This can be attributed to variations in grape ripeness [3].

A deficiency in assimilable nitrogen can lead to poor yeast growth, stuck fermentations and the production of hydrogen sulfide. Nitrogen content is one of the limiting factors of yeast growth and sugar attenuation. Addition of assimilable

nitrogen has been shown to increase the rate of fermentation and to reduce the time required for fermentation completion by increasing sugar usage and therefore ethanol formation [9]. Molecular methods have been used to monitor the changes in the yeast population in a continuous wine fermentation [10].

Because of the polyfunctional nature of amino acids they can easily undergo reactions even under mild conditions. For example, amino acids can easily react with α-dicarbonyl containing compounds like acetoin, acetol, glyoxal, methylglyoxal, diacetyl and pentane-2,3-dione [11]. These dicarbonyl compounds can be formed in wine through the Maillard reaction which can take place during bottling of the wine (sometimes at high temperatures) or during self keeping of the wine because of increases in temperature favourable for the reaction to take place. Although all amino acids are known to react with these α-dicarbonyl compounds, sensory perceptible outcomes are observed only for a few of them and in particular cysteine. The thiol function that it carries on its side chain is also suitable to form sulfur and disulfur bridges leading to the formation of volatile compounds affecting the organoleptic properties of the wine. It is known that this amino acid is involved in the aromatic qualities of Sauvignon Blanc wine. Some flavour compounds found in the must include the form of S-cysteine conjugate precursors [12].

A varietal is a wine made principally from one grape variety and carrying the name of that grape. For example, a wine made from 100% Merlot would be called a Merlot varietal or cultivar. Combining the juices from more than one variety of grapes makes a wine called a "blend".

Several studies on the determination of amino acids in wine have been made. A representative summary of some of the results is shown in Table 1.

Amino Acid	Abbreviation	Seven French Wines [5]	Pinot Noir [7]	One Grape Variety [6]
Arginine	ARG	23-64	6.9	2.20-88.5
Hydroxyproline	HYP	3.3-8.9	3.8	-
Serine	SER	7.1-14	7.8	1.28-51.2
Aspartic acid	ASP	8.6-19	10.3	0.65-26.2
Glutamic acid	GLU	26-54	24.5	0.93-37.4
Threonine	THR	5.4-10	4.4	0.87-35.0
Glycine	GLY	11-20	11.3	0.78-31.3
Alanine	ALA	26-64	24.1	1.30-52.8
Proline	PRO	380-790	552.3	-
Methionine	MET	-	-	1.19-44.7
Valine	VAL	3.6-9.1	-	0.90-36.0
Phenylalanine	PHE	7.3-22	5.9	0.89-35.6
Isoleucine	ILE	2.4-5.9	2.1	0.50-22.8
Leucine	LEU	6.0-13	5.9	1.07-42.8
Histidine	HIS	3.5-11	2.6	0.60-24.1
Lysine	LYS	7.5-17	12.1	2.37-94.8
Tyrosine	TYR	2.0-7.5	5	1.10-43.6

Table 1: Concentrations of amino acids in different wines according to previous studies. All concentrations are reported in mg/L.

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Chapter 2. Fundamentals of liquid chromatography

2.1 The chromatographic process

The "official" definition of the International Union of Pure and Applied Chemistry (IUPAC) of chromatography is as follows: "Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction. Elution chromatography is a procedure in which the mobile phase is continuously passed through or along the chromatographic bed and the sample is fed into the system as a finite plug [1,2].

The stationary phase is either a solid, porous, surface-active material in small particle form or a thin film of liquid coated on a solid support or column wall. The mobile phase can be either a gas or a liquid. If a gas is used, the process is known as gas chromatography; if a liquid is used, it is referred to as liquid chromatography. This also includes thin layer chromatography. In the following discussion, the focus is on liquid chromatography, since this was the method used in this study.

Whilst the sample is carried through the chromatographic bed, the analytes partition between the mobile and stationary phases according to their relative affinities for the respective phases. This leads to separation of the analytes. A schematic diagram in Figure 1 shows this process, called elution. Phase preference can be expressed by the distribution coefficient K:

$$K_X = \frac{c_{stat}}{c_{mob}} \qquad \textbf{(1)}$$

where c_{stat} is the concentration of compound x in the stationary phase and c_{mob} is the concentration of x in the mobile phase.

The retention factor k (formerly termed capacity factor k') is defined as:

$$k_X = \frac{n_{stat}}{n_{mob}} \qquad \textbf{(2)}$$

where n_{stat} is the number of moles of x in the stationary phase and n_{mob} is the number of moles of compound x in the mobile phase.

The stationary and mobile phases must be in direct contact with each other in order to ensure a distribution balance. It is obvious that the distribution coefficients, and therefore retention factors, of the different analytes in the sample mixture must not be the same otherwise separation is not possible. Different distribution coefficients give different retention times for the analytes [2]. The distribution coefficient is constant for a specific analyte under identical chromatographic conditions. This thermodynamic constant is temperature dependent.

The distance taken to establish a new balance between the two phases in a chromatographic bed is known as the theoretical plate. The longer the chromatographic bed, the more theoretical plates it contains. This generally corresponds to a better degree of separation. However, this is lessened by the effect of band broadening. The sample zones becomes broader the longer they stay in the column.

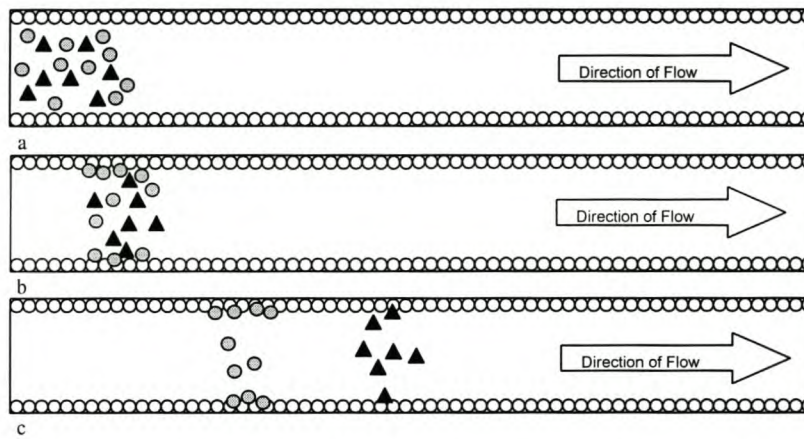

Figure 1: Graphic representation of the chromatographic process a) injection of sample into the system, b) the analytes distribute themselves amongst the stationary and mobile phases according to their distribution coefficients, c) the analytes are separated and elute from the column, where they are detected.

2.2 Band broadening in liquid chromatography

There are four main factors that contribute towards band broadening in liquid chromatography. They are Eddy diffusion, flow dispersion, longitudinal diffusion, and mass transfer effects.

1. Eddy diffusion:

Due to the different size and shape [3] of the stationary phase particles, as well as the fact that they are not packed entirely perfectly, different mobile phase molecules may follow different paths through the chromatographic bed. Some will travel in an approximately linear direction, whilst others will follow a curved, or windy, path. This results in solute molecules leaving the chromatographic bed sooner than others. This effect is minimised when the size distribution of the stationary phase is narrow, and the particle size is small.

2. Flow dispersion (Figure 2)

It is assumed that the mobile phase flow is laminar between the stationary phase particles. However, in practice, it is found that the flow is slower closer to the stationary phase particles. The flow is faster in the 'channel' between the particles.

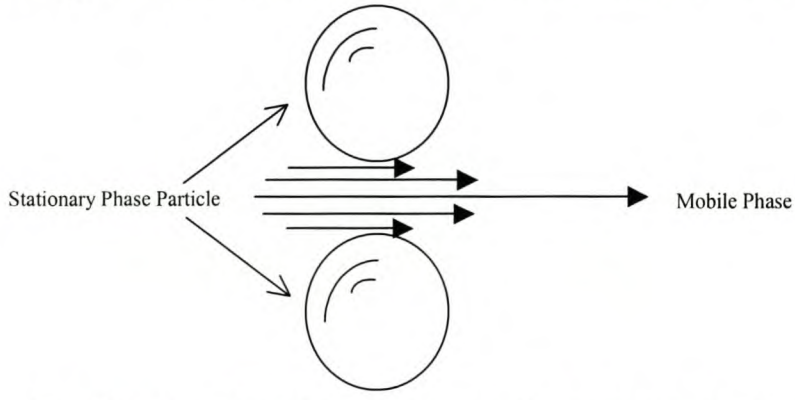

Figure 2: Mobile phase flow velocity decreases further away from the "channel" between two stationary phase particles.

The above two effects are minimised by lateral diffusion, which is the movement of solute from one flow path to another. It tends to equalise the speed of solute species in the column. The longer a solute species spends in the column, the more lateral diffusion will occur, so flow dispersion is slightly flow-dependent, being reduced by the use of low mobile phase flow rates [4].

3. Longitudinal diffusion (Figure 3)

Longitudinal (axial) dispersion occurs when the sample diffuses in an axial direction through the mobile phase. This fortunately does not play a large role in liquid chromatography, due to the relatively small analyte diffusion coefficients in a liquid phase. However, the longer the sample stays in the chromatographic bed,

the greater the effect of longitudinal diffusion. Therefore the effect is minimised by a faster mobile phase flow rate.

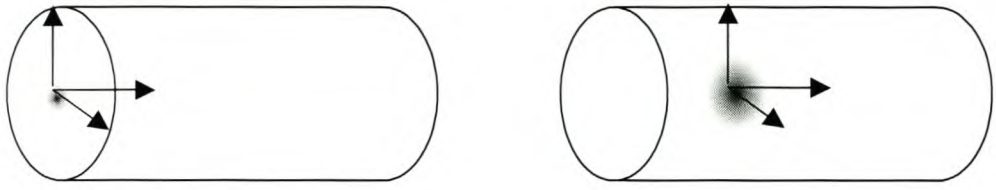

Figure 3: Illustration of the effect of longitudinal (axial) diffusion. On the left, the volume the analyte occupies is still relatively small, directly after injection. On the right, diffusion plays a role in the increasing volume.

4. Mass transfer effects:

Mass tranfer is the most important factor of band broadening in liquid chromatography. The stationary phase particle consists of 'pores', some of which go straight through the particle and others that are closed off. The pores are filled with mobile phase, which does not move (it stagnates). Here, the sample molecule moves by diffusion only. Two things can happen: firstly, the molecule may diffuse back into the mobile phase, and secondly, the molecule may adsorb/absorb to the stationary phase particle. Both processes take time, in which the other sample molecules in the mobile phase have moved further along in the chromatographic bed. This factor of band broadening increases with increasing mobile phase flow velocity. The effect of mass transfer is reduced when small particles, or those with a thin, porous surface layer are used as stationary phase. This ensures that the molecules do not have to travel far to return to the mobile phase.

There is an opposite effect with regards to the optimal flow rate. To reduce longitudinal diffusion, a higher flow rate is needed, however, to minimize mass tranfer effects, a low flow rate is necessary. There is an optimal flow rate where the combination of all band broadening effects produces minimum dispersion [4]. To determine the optimal flow rate, the Van Deemter equation is used:

$$H = A + \frac{B}{u} + Cu \qquad \textbf{(3)}$$

where $A = 2\lambda d_p$, $B = 2D_M$ and $C = \frac{\int(k)d_p^2}{D}$ **(4)**

H is the height of one theoretical plate. *A* combines the Eddy diffusion and flow dispersion term, and is independent of the linear mobile phase velocity *u*, *B* is the longitudinal diffusion term, *C* the mass transfer term, d_p is the particle diameter, λ the packing factor, *f(k)* a function of the retention factor, *D* the diffusion coefficient that represents diffusion in both the stationary and mobile phases and D_M the diffusion coefficient in the mobile phase only [4]. The plot H versus *u* is represented in Figure 4.

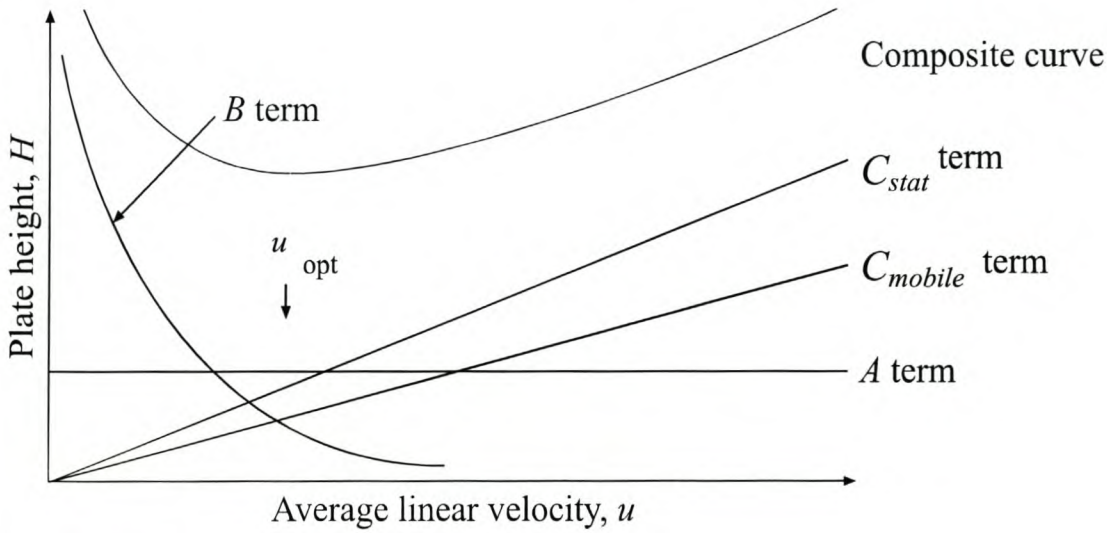

Figure 4: Van Deemter plot

The slope of the resulting curve after the minimum depends on the particle size of the packing material. In analytical HPLC, where we are using small particle size packing we can use relatively high flow rates (giving shorter analysis times) without sacrificing too much in loss of efficiency [4]. Flow rates are, however, limited by high-pressure drops over packed liquid chromatography columns.

2.3 Efficiency in HPLC

The most common method of measuring efficiency of a chromatographic column is through the number of theoretical plates *N*. Theoretical plates can be calculated from the chromatogram by the following equations:

$$N = \left(\frac{t_R}{\sigma}\right)^2 = 16\left(\frac{t_R}{w_b}\right)^2 = 5.54\left(\frac{t_R}{w_h}\right)^2 \quad \textbf{(5)}$$

where t_R is the retention time of the peak. For a Gaussian peak, σ is used to express the peak width similar to its use to express the width of a standard distribution. The width of the peak is 2σ at the inflection point, 4σ at the base (w_b) and 2.354σ at half height, w_h.

The theoretical plate number is a representation of the amount of band broadening that occurs in the chromatographic system. High values of N are indicative of an efficient system. Typical values for modern HPLC columns range between 10 000 and 20 000.

The height equivalent of a theoretical plate (HETP) or the plate height, H, of a column is given by:

$$H = \frac{L}{N} \quad \textbf{(6)}$$

where L is the length of the column and H is the distance over which chromatographic equilibrium is achieved during separation. From this equation it is possible to see that the more efficient the column, i.e. the greater the number of theoretical plates, the smaller the value of H.

H can also be described in terms of its reduced plate height, h, and the particle diameter, d_p:

$$H = hd_p \quad \textbf{(7)}$$

This means that it is possible to express the plate number by the following equation:

$$N = \frac{L}{hd_p} \quad \textbf{(8)}$$

For a packed column with small particles it can be shown that h ~ 2 [3].

2.4 The chromatogram

The analytes are separated in the column and pass through the detector by the mobile phase, where they are recorded as Gaussian curves. The signals are known as peaks

and the whole entity is known as the chromatogram (Figure 5). The peaks give both qualitative and quantitative information on the sample analytes [3]. Each peak will have a specific retention time under identical conditions. This means that an analyte can be characterised through its retention time. The area and/or height of the peak are proportional to its quantity. By setting up calibration curves, it is possible to quantitate the amount of an analyte in a given sample.

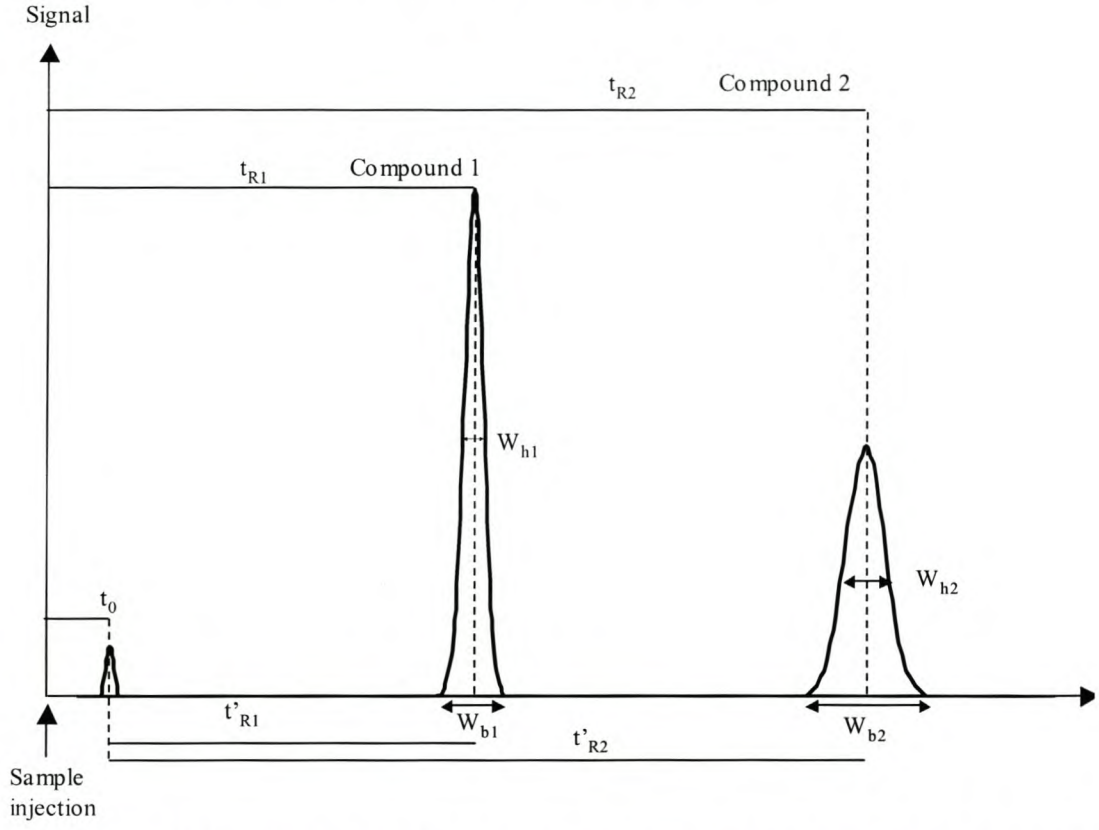

Figure 5: Graphical representation of a chromatogram, illustrating the calculation of peak width, and retention times.

t_o is the dead time or retention time of an unretained solute, i.e. the time required by the mobile phase to pass through the column (also called the breakthrough time). This means that the linear flow velocity, u, can be calculated by the following equation:

$$u = \frac{L}{t_0} \tag{9}$$

where L is the column length. t_R is the retention time of an analyte that interacts with the chromatographic system. It is the period between sample injection and recording of the peak maximum. Two compounds can be separated if they have different retention times. t_R' is the net retention time or adjusted retention time:

$$t_R = t_0 + t_R'. \quad \textbf{(10)}$$

All molecules spend the same amount of time in the mobile phase represented by t_0. t_R' is a measure of the amount of interaction that occurs between the stationary phase and the analyte [5]. The longer a compound interacts with the stationary phase, the later it elutes.

Retention time is a function of mobile phase flow velocity and column length. At low flow rates or for long columns, t_o is large and hence so is t_R. t_R is therefore only suitable for characterising a compound on a given column and flow rate. The retention factor or k value is preferred and is chromatographically related to the previously discussed factor (equation 2) as follows:

$$k_X = \frac{n_{stat}}{n_{mob}} = \frac{t_R'}{t_0} = \frac{t_R - t_0}{t_0} \quad \textbf{(11)}$$

k is independent of the column length and mobile phase flow-rate.

Retention factors between 3 and 10 are preferred. If the k values are too low, the compounds will pass through the chromatographic system too quickly and separation will be inadequate due to the lack of stationary phase interaction. High k values are accompanied by long analysis times. The k value is connected with the distribution coefficient described earlier in the following way:

$$k = \frac{KV_{stat}}{V_{mob}} \quad \textbf{(12)}$$

where V_{stat} is the volume of stationary phase and V_{mob} the volume of mobile phase in the column.

To assess whether two compounds are separated, their relative retention, α, is used:

$$\alpha = \frac{k_2}{k_1} \quad \textbf{(13)}$$

The separation factor α is a measure of the chromatographic system's selectivity. In HPLC, changing the properties of both the stationary and mobile phase vary the value of α. The type of forces, which play a significant role in the selectivity, include dispersion forces, dipole-dipole interactions, hydrogen bonding, π-π interactions, etc.

2.5 Resolution

Resolution (R) is a measure of the degree of separation between two peaks. R can graphically be calculated as follows:

$$R = 2\frac{(t_{R2} - t_{R1})}{(w_1 + w_2)}. \quad \textbf{(14)}$$

Factors that influence the resolution of two peaks include the separation factor, α, the number of theoretical plates, N, and the retention factor, k. By combining equations 5, 11 and 13 with equation 14, it can be shown how the resolution value can be calculated chromatographically :

$$R = \tfrac{1}{4}\frac{(\alpha - 1)}{\alpha}\sqrt{N}\frac{k_2}{(1 + k_2)} \quad \textbf{(15)}$$

From this equation, it can be seen that resolution can be improved by changing either α, N or k. By varying the composition and characteristics of either the mobile or stationary phases, α is varied. The k value is varied by changing the stationary or mobile phase composition or the temperature of analysis and N can be changed by increasing the column length or by decreasing the size of the stationary phase particles [3].

2.6 Separation modes in liquid chromatography

2.6.1 Normal phase chromatography

Normal phase chromatography (NP) is defined as having a polar stationary phase and a non-polar mobile phase. It was the first chromatographic technique used and therefore designated as "normal". NP is typically used for non-polar to semi-polar analytes. It is not used for the separation of polar analytes as long retention times arise due to the high affinity of the polar compounds with the stationary phase.

NP is often referred to as adsorption chromatography. The mechanism of separation is the reversible adsorption of analytes on the polar, weakly acidic surface of silica gel, which serves as the stationary phase.

Bonded phase NP chromatography is a more modern approach to NP. Here the silica is used as a support material, upon which polar functionalities are covalently bonded to the surface. These columns include cyanopropyl, aminopropyl and diol phases. The separation mechanism is rather partitioning than adsorption. This chemical modification of the stationary phase results in durable and stable columns [2].

2.6.2 Reversed phase chromatography

Due to the fact that the polar compounds give many problems in the normal phase separation technique, reversed phase chromatography was developed. Reversed phase chromatography (RP) gets its name from the fact that the polarity of the mobile phase and stationary phases in the process are the reversed as opposed to normal phase chromatography: the mobile phase is polar and the stationary phase is non-polar [2]. This technique was introduced by Howard and Martin. It is the most popular method of liquid chromatography, due to its versatility and ability to handle polar analytes. RP is a more reproducible method compared to NPLC.

Octadecylsilane (ODS), consisting of an n-alkane with 18 carbon atoms chemically bonded to spherical silica particles, is the most frequently used stationary phase. C_8 and shorter alkyl chains and also cyclohexyl and phenyl groups provide other alternatives [3].

2.6.2 Ion-exchange chromatography

The basic principles of ion-exchange chromatography are based on charges on the analytes. The analyte can be ionised into its corresponding cation or anion by adjusting the pH of the mobile phase. Amino acids are zwitterionic and can therefore be ionised into either a cation or anion, depending on the resin used as the stationary phase.

$$R(NH_2)COOH \Leftrightarrow RCOO^- + H^+$$

or

$$RNH_2(COOH) + H^+ \Leftrightarrow RNH_3^+$$

The stationary phase will have a positive charge when separating anions and a negative charge when dealing with cations. The mobile phase contains ions of the same charge as the analytes. These ions and analytes compete for sites on the stationary phase. This is the basis for ion-exchange chromatography. The technique therefore takes advantage of the coulombic interaction (attraction of opposite charges) between the analytes and the stationary phase. Factors of great importance include the pH, ionic strength, and type of counter ions within the mobile phase, as well as the type of ion exchanger used [2, 3].

2.6.4 Size exclusion chromatography

Size exclusion chromatography is different from other forms of chromatographic separation by the fact that separation is performed via molecule size classification rather than interaction between the analytes and stationary phase. A porous material with well-defined pore sizes is used as the column packing [3].

2.6.5 Affinity chromatography

Affinity chromatography is a specific chromatographic method. Its specificity is due to the fact that the two participating compounds are ideally suited to each other both spatially and electrostatically. A ligand is bonded to a support on the stationary phase and only a particular analyte, containing a suitable receptor site for the anchored ligand, is retained from the sample solution. This is a reversible process. Any other analytes within the sample solution do not match the ligand and are therefore not retained on the column [3].

2.6.6 Ion chromatography

Ion chromatography was developed for the separation of small inorganic ions and organic acids. The stationary phases used have a lower exchange capacity than those applied in ion-exchange chromatography as discussed in section 2.6.3. This means that the ionic strength of the eluent can be low, which in turn gives low conductivity facilitating conductive detection. However, this is often not enough to give low background conductivity and suppression techniques are used to eliminate background conductivity [3].

2.7 Detection in HPLC

A schematic drawing of a state-of-the-art LC instrument is shown in Figure 6.

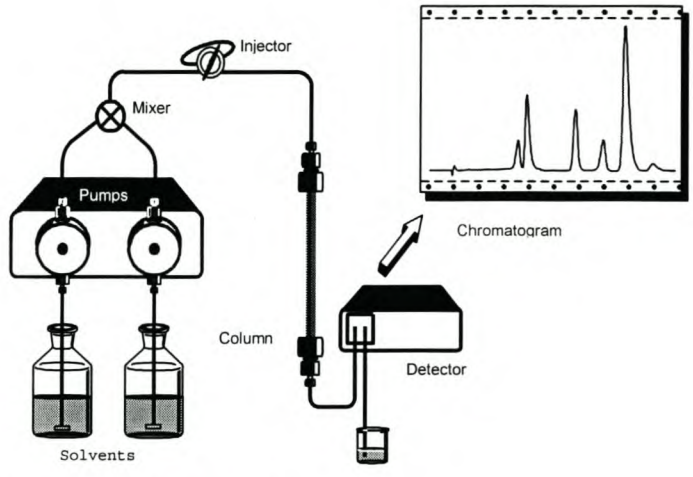

Figure 6: State-of-the-art LC instrument

The system is built up of pumps, an injector, a column, a detection device and a data handling system. Most of the parts are standard and well-known and will not be discussed in the framework of this thesis. Detector selection however is of utmost importance for amino acid analysis and this aspect of the instrumentation will be discussed in depth.

The function of a chromatographic detector is to monitor the content of the mobile phase emerging from the column. The output of the detector is an electrical signal that is proportional to some property of the mobile phase and/or the solutes. The most important characteristics required for an ideal detector are:

(a) Sensitivity: is the ratio of output to input. An ideal detector will supply a large detector signal for a small amount of solute. Although all detectors suffer from instrumental noise, it essential to keep the noise to a minimum as it determines the minimum amount of solute that is detectable. The sensitivity of detectors is often given as a noise equivalent concentration, C_N, which means the concentration of solute that produces a signal equal to the detector noise level. C_N will depend on the nature of the solute analysed.

(b) Linearity: a linear detector has a response that is directly proportional to the amount or concentration of solute. The linear range of the detector is that concentration range over which this proportionality is obeyed.

(c) Universal and predictable response: a universal detector will detect all the analytes in the sample, whereas a selective detector will detect only certain components. In analytical work we need both types. The best we can hope for is that we can predict how the response of the detector will change for different chemical compounds, and that the response does not change too much if there are small changes in the operating conditions (e.g. column temperature or flow rate).

(d) Low dead volume: Dead volume in the detector adds to extra-column dispersion, so it must be kept to a minimum. The dead volume includes the cell volume of the detector itself, and also the length and bore of any tubing associated with it. For spectrometric detectors a reduction in the cell volume is likely to lead to a loss of sensitivity.
Other requirements of an ideal detector are also that it is non-destructive, cheap and easy to use [4].

2.7.1 UV/Absorbance detectors

The principle behind the UV/VIS detectors is that the mobile phase, which contains the analyte, is passed through a small flow cell placed at the end of the column. This cell is held in front of the radiation beam of a UV/VIS photometer or spectrophotometer. The requirement is that the solute is able to absorb UV radiation. It is required that the mobile phase absorbs little or no radiation.

The Beer-Lambert law describes light absorption by solutes. It can be defined as the logarithm of the quotient between the intensities of the radiation entering the sample, I_o, and the radiation leaving the sample, I. It is a function of concentration, c, and is formulated as:

$$A = \log(I_o/I) = ecb \qquad \textbf{(16)}$$

where A = absorbance, b = path length of the cell and e = molar absorptivity, which is a constant for a given solute and wavelength. The Beer-Lambert law only applies to monochromatic radiation [4].

There are three different types of UV detectors. The fixed wavelength UV detector is the simplest version. It usually employs a low-pressure mercury vapour lamp as its UV light source. The mercury lamp provides several distinct lines of UV radiation, with the 254 nm wavelength being the most intense. The light source is then passed through a filter to remove any extraneous wavelengths before being passed through the flow cell [2]. The principal of the fixed wavelength detector is shown in Figure 7. A detail of the flow cell is presented in Figure 8.

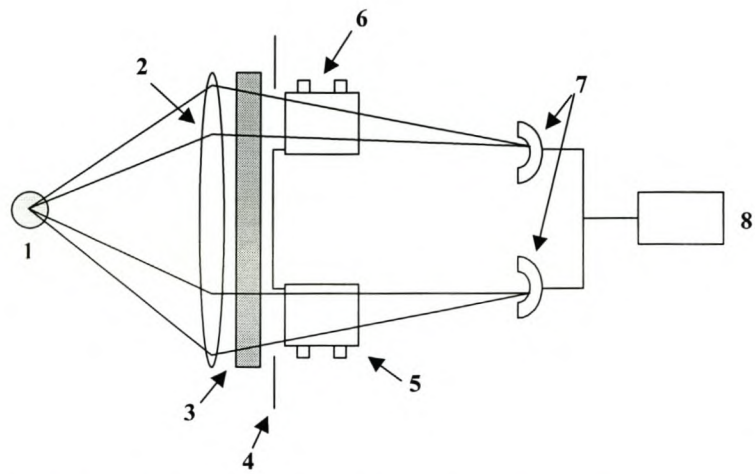

Figure 7: Principles of a fixed wavelength detector. 1: low pressure mercury lamp, 2: lens, 3: UV-filter, 4: diaphragm, 5: measuring cell, 6: reference cell, 7: photodetector, 8: signal amplifier.

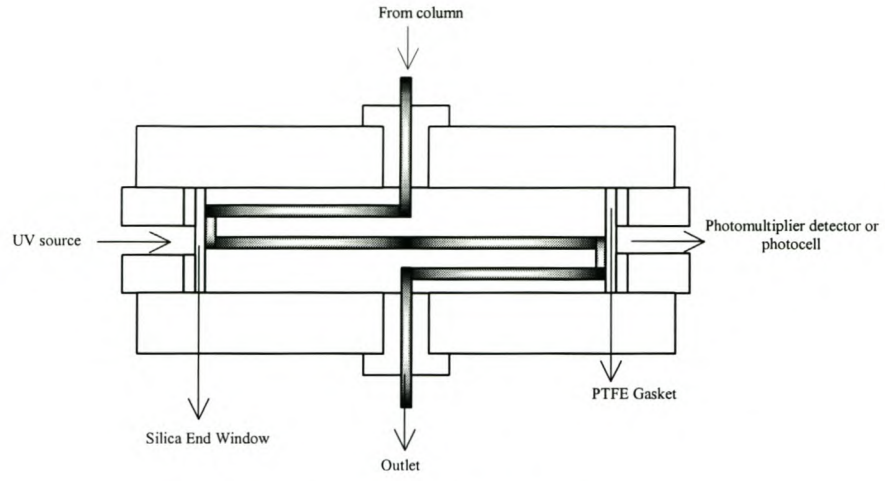

Figure 8: The UV detector flow cell.

The variable wavelength UV detector (Figure 9) has a monochromator added into it that enables the wavelength of the light source to be chosen. This has the advantage of detecting compounds that do not absorb UV light at 254 nm. A deuterium lamp is used as a light source, producing a wide spectrum of UV light, from about 190 to > 800 nm. The light is then bounced off a grating, which separates the various wavelengths. The grating is placed on a moveable platform, which allows the user to select any single wavelength from the spectrum [2].

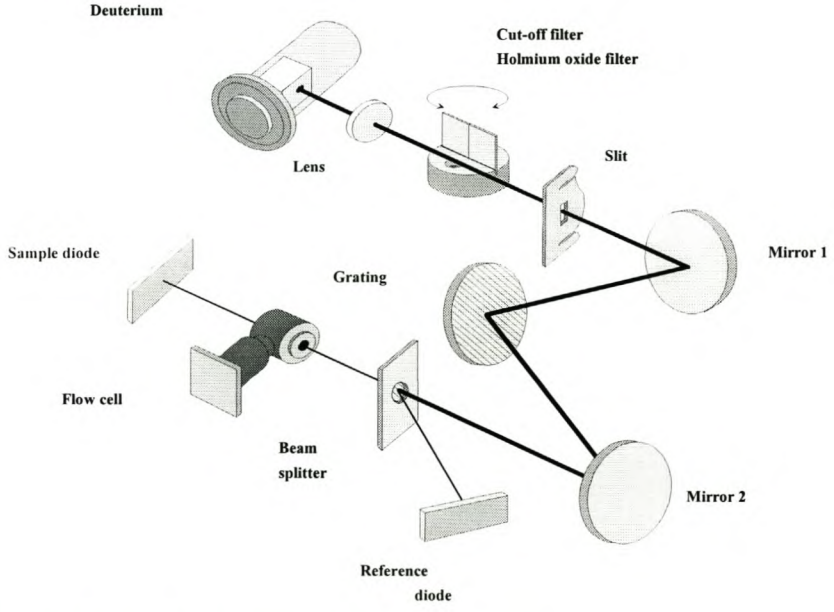

Figure 9: Principle of a variable wavelength UV detector

The photodiode array detector (PDA) has the extra advantage over the variable wavelength detector in the fact that it is possible to access all of the wavelengths in the spectrum simultaneously (Figure 10). The principle is the same as that of the variable wavelength detector, in that the light source is bounced off a grating, however, in this case, the grating is stationary and the single detector is replaced with an array of photodiodes. Because of the parallel data acquisition, processing and storage of a spectrum can be done typically in about 0.5 s, compared with several minutes for a conventional UV spectrophotometer.

As there are no moving parts to wear out, wavelength-resetting errors are reduced and the instrument is likely to require less maintenance than does a conventional spectrophotometer. Also, the ability to make use of multiple wavelengths and the speed of data acquisition mean that various signal-averaging techniques can be used to reduce noise and improve sensitivity. Added to these are the specific advantages of combining a PDA with HPLC. The spectrum of the peaks can be stored and compared against spectra of known standards. It is easier to find the optimum wavelength for single wavelength detection and it is simple to program wavelength changes at the required times in a chromatogram. Use of a PDA also allows to represent data in a contour plot, showing the relationship between absorbance, wavelength and time. This helps with identification of unknowns in the chromatogram/sample [3].

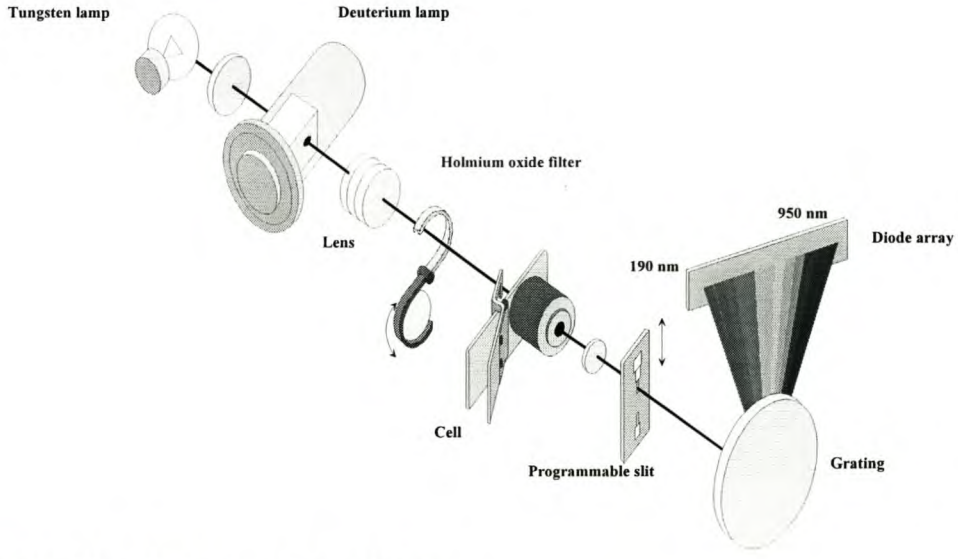

Figure 10: Principle of a diode array detector

2.7.2 Fluorescence detectors

Some compounds are capable of absorbing UV radiation and subsequently emitting radiation of a longer wavelength, either instantly (fluorescence) or after a time delay (phosphorescence) [4]. The fluorescence detector is one of the most selective and sensitive detectors in liquid chromatography, allowing for sub-ppb (µg/L) detection limits. A schematic representation of an LC florescence detector is shown in Figure 11. However, only a small percentage of molecules have the ability to fluoresce so its application is limited [2]. Compounds that fluoresce naturally have a conjugated cyclic structure like polycyclic aromatic hydrocarbons (PAHs). Non-fluorescent compounds can be converted to fluorescent derivatives by treatment with suitable reagents [4]. This procedure called derivitisation is applied in this study.

The fluorescent power is proportional to the number of molecules in excited states, which in turn is proportional to the radiant power absorbed by the sample:

$$P_F = \Phi_F (P_0 - P) \tag{17}$$

where P_F is the radiant power of fluorescence, Φ_F is the quantum efficiency, P_0 is the radiant power incident on the sample and P is the radiant power emerging from the sample.

The radiant power absorbed by the sample is $P_0 - P$. The quantum efficiency, Φ_F, is the ratio of the number of photons emitted as fluorescence to the number of photons absorbed by the sample.

Applying Beer's law to equation 17 yields:

$$P_F = \Phi_F P_0 (1 - e^{-\varepsilon bc}) \tag{18}$$

By expanding this equation into a power series, we obtain:

$$P_F = \Phi_F P_0 \varepsilon bc[1 - \frac{\varepsilon bc}{2!} + \frac{(\varepsilon bc)^2}{3!} - .... + \frac{(\varepsilon bc)^n}{(n+1)!}] \tag{19}$$

If εbc is small, only the first term in the series is significant and equation 19 can be written as

$$P_F = \Phi_F P_0 \varepsilon bc \tag{20}$$

This equation indicates a linear relationship between fluorescent power and the concentration, provided that εbc is small, i.e. the concentration is very dilute.

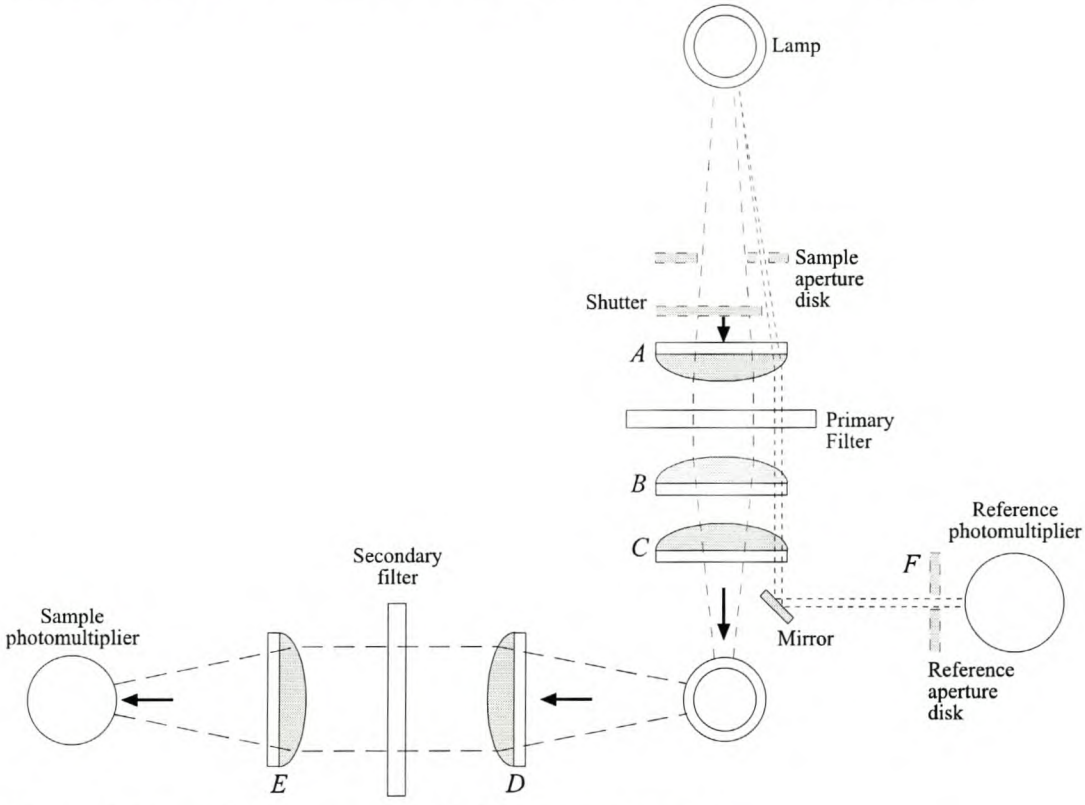

Figure 11: Schematic representation of a fluorescence detector

Because fluorescence emission is occurring in all directions, detection is performed at an 90 degree angle from the excitation direction, where the stray light (background signal) is reduced to a minimum.

2.7.3 Mass spectrometry

Mass spectrometry (MS) is the most universal detection device. Mass spectrometric detection consists of three basic steps: ion production, mass separation and detection. Today, one of the most popular ionisation modes for LC-MS is atmospheric pressure electrospray ionisation (ESI). In ESI the ions are produced by accelerating the solutes eluting from the column in an electric field of 2-3 kV towards a heated capillary leading inside the MS (Figure 12). The droplets eluting from the column become charged and by the help of a nebulizing gas, flowing around the column exit and in the ionisation chamber, the solvent is evaporated. This leads to shrinkage of the charged droplets while they preserve their charges. The repulsion of the charges present at the surface of the droplet becomes larger than the cohesive forces of the

droplet and the droplet explodes into a multitude of smaller droplets, which in turn undergo the same process. In conclusion, all the solvent is evaporated and only charged analytes remain. These charged analytes are attracted into the MS while neutral solutes are removed. ESI is known as a "soft" ionisation mode because the ions are stable and do not tend to fragment. This allows easy observation of the molecular ion of the solutes. Once the ions are produced they can be separated based on their m/z ratio in several ways. Quadrupole MS are the most commonly used. The resulting mass spectrum gives the relative abundance of individual ions having different m/z ratios.

Detection can occur in two modes: either simultaneously detecting a range of masses via the scan mode or to detect only those masses characteristic of a specific compound using the selected ion monitoring (SIM) mode. The spectra resulting from the scan mode have signals covering a certain mass range. This has the greatest application in qualitative analysis or structure elucidation. In the SIM mode, only a few m/z ratios are monitored. SIM is mostly used for detection of target compounds and the m/z values are set in advance. This provides higher sensitivity.

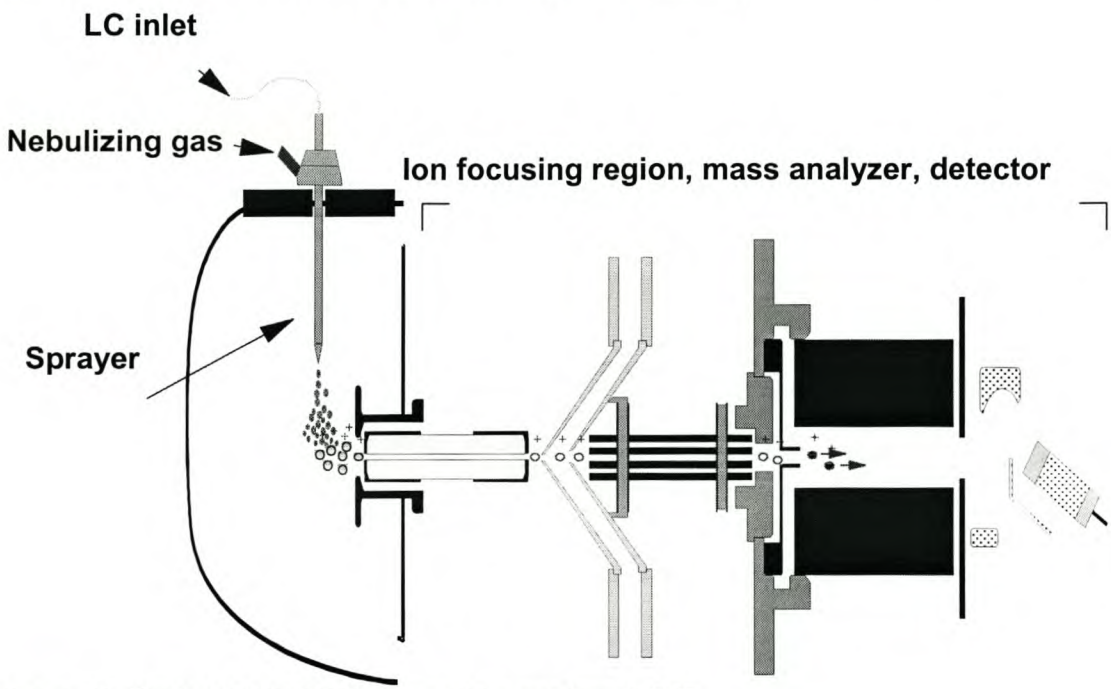

Figure 12: Typical LC-ESI-MS set-up with a quadrupole MS.

2.8 Derivatisation

Because UV and fluorescence detectors are not universal, derivatisation strategies are often required in order to provide the solutes with a suitable chromophore group [3].

This is often aimed at increasing the sensitivity for specific solutes in a complex matrix, such as biological fluids or environmental samples [4]. In this study the matrix is wine and the aim was to achieve selective detection of amino acids with a minimum of interferences from other wine constituents.

Derivatisation can be performed before the separation (pre-column derivatisation) or after the separation (post-column derivatisation) [3,4]. The procedure can also take place off-line or on-line with regards to the chromatographic system. Two of the more commonly used techniques are pre-column off-line and post-column on-line, as discussed below [4].

2.8.1 Pre-column derivatisation

One of the advantages of pre-column derivatisation, is that the chromatographic process is not at all affected by the reaction time of the derivatisation step, allowing chromatographic conditions to be freely chosen [3, 4]. It also means that any excess of the reaction reagent can be removed prior to analysis to reduce the effects of interference from these and other compounds within the matrix. This is especially useful when solutes of the reagent solution itself interfere with sample peaks. In general, this derivatisation process may also be regarded as a purifying or cleaning step and it often improves the chromatographic properties of the samples [3,4].
This derivatisation procedure is quantitative as long as the derivatives formed are reasonably stable and well defined, which is not always possible [4, 3].

2.8.2 Post-column derivatisation

In this case a reagent is added on-line after separation, i.e. after the eluent has left the column. Generally, a high concentration of reagent solution is preferred as this reduces dilution effects to a minimum. A quick and complete reaction is required in order to limit band broadening and to allow quantitative analysis. Therefore it is preferable to heat the reactor, as chemical reactions generally occur faster at higher temperatures.

However, there are a number of shortcomings when using this technique. The mobile phase must be carefully selected, since it may not react with the derivatisation

reagents. Also, the derivatisation reagent itself may not give a strong response [3], moreover the instrumental set-up has to be modified to perform post-column derivatisation, leading to higher costs. A typical example of post-column derivatisation is the reaction of amino acids with ninhydrin as will be explained later.

2.9 Quantitative analysis in HPLC

Because in this work amino acids are quantified, a brief overview of the different quantification procedures is presented.

2.9.1 External calibration

When the injection volume can be reproduced very well, as is the case in HPLC, this is generally the method of choice. By comparison of injection of a known amount of analyte X (Q_{xs}) giving peak area A_{xs} obtained upon injection of an unknown amount (A_x), the quantity (Q_x) can be calculated as follows:

$$Q_x = Q_{xs}\frac{A_x}{A_{xs}} \qquad \textbf{(21)}$$

More reliable results are obtained by using a series of quantities of the standard (Q_{xs1}, $Q_{xs2}, \ldots . Q_{xsn}$), such that a range of concentrations can be covered. This leads to the construction of a calibration curve as shown in Figure 13:

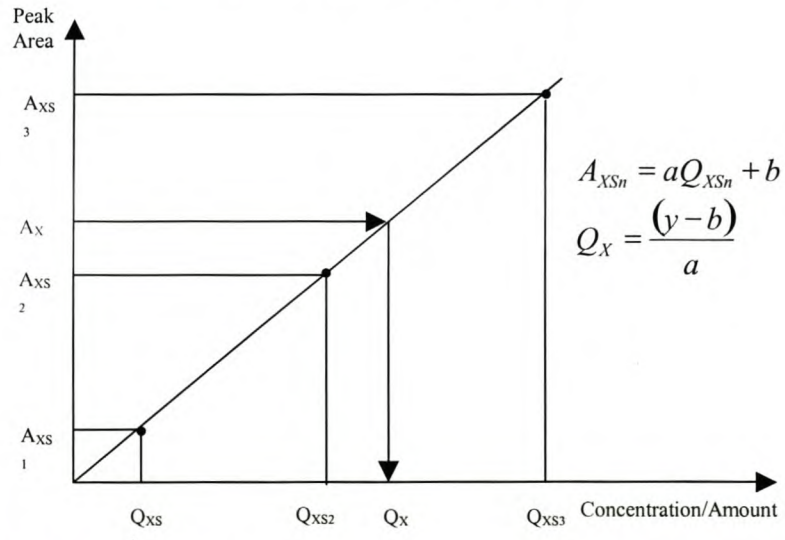

Figure 13: External calibration method.

From the obtained function for the calibration curve:

$$A_{xsn} = aQ_{xsn} + b \quad \textbf{(22)}$$

the concentration of the unknown can be calculated by :

$$Q_x = \frac{(A_x - b)}{a} \quad \textbf{(23)}$$

The external standard method has been used for the separation and quantification of amino acids, however, it was noted that in the case of a number of samples, analytical precision could be increased by utilising the internal standard method [7].

2.9.2 Internal calibration

An internal standard is a substance that is added to the sample to compensate for solute losses during sample preparation and to correct for irregularities during chromatographic analysis. This method is particularly useful for techniques that are not too reproducible, and for situations where one does not (or cannot) recalibrate often.

The properties of an ideal internal standard (IS) include the following:

1. The IS should resemble the analytes of interest as closely as possible, and not react with any of the analytes or components within the sample
2. The IS should be resolved from the analytes of interest, yet not elute too far away form the analytes
3. The IS should not be found naturally within the sample, it should be incorporated within the sample matrix in the same way as the analytes.
4. The IS should be available in high purity.

There are two ways of using the internal standard method. Firstly, a known amount of an internal standard (Q_{st}) is added to the mixture with an unknown amount of the compound(s) to be analysed (Q_x). The peak areas of the internal standard (A_{st}) and the unknown (A_x) are related to the concentrations by:

$$Q_{st} = f_{st} A_{st} \quad \textbf{(24)}$$

$$Q_x = f_x A_x \quad \textbf{(25)}$$

or

$$Q_x = Q_{st} \frac{f_x A_x}{f_{st} A_{st}} \approx Q_{st} \frac{A_x}{A_{st}} \quad \textbf{(26)}$$

This equation is only valid if it can be assumed that the response factor of the unknown (f_x) is the same as the response factor of the internal standard (f_{st}).

In the second approach, a calibration curve is constructed from the ratio of the detector response to the analyte divided by the response to the internal standard plotted against the concentration (amount) of analyte. The ratio of the detector response to the sample unknowns and internal standard is then used for all quantitation measurements (Figure 14).

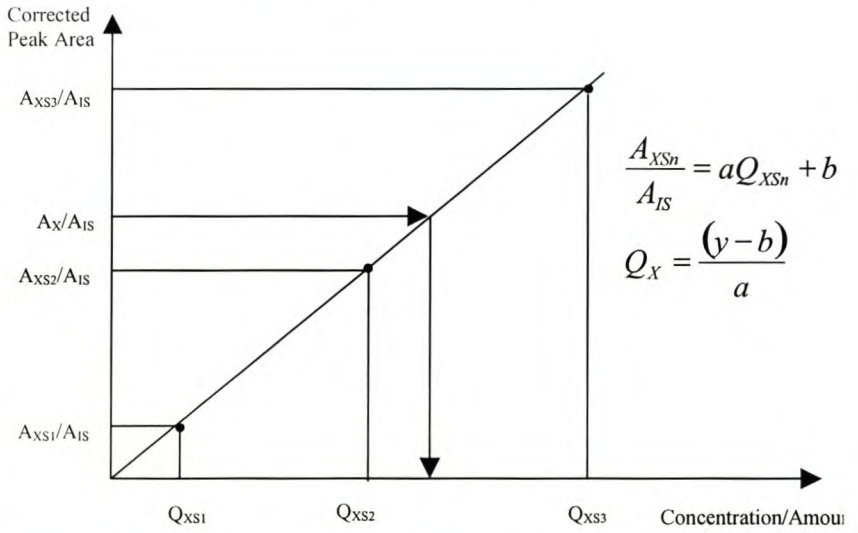

Figure 14: Construction of a calibration curve with the internal standard method

For calibration of the analytes, at least three different solutions of the analyte should be prepared. In each of the solutions, the same amount of internal standard is added and the results are calculated by using the calibration curves.

2.9.3 Standard addition calibration

In this method, the standard is the same as the analyte of interest. The principle of this method is that the additional, incremental signal produced by adding the standard is proportional to the amount of standard added. This proportionality can be used to determine the concentration or amount of the analyte in the original sample.

The calibration curve shown in Figure 15 illustrates that this principle is more easily seen graphically then by using calculations. A signal is present when no standard has been added to the sample solution. The concentration of the analyte within the original sample solution can be found by extrapolating the calibration curve towards the cross section with the x-axis [2].

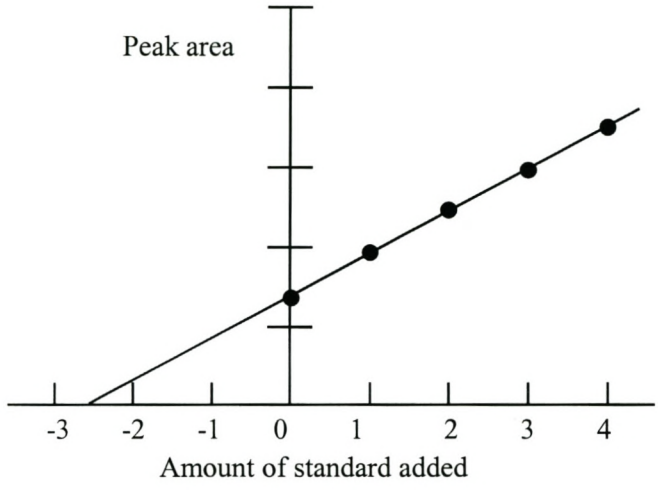

Figure 15: Calibration curve using the standard addition method

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Chapter 3. The Analysis of amino acids by HPLC

In this chapter the different derivatisation strategies used for amino acid analysis are described followed by an overview of the different methods used for their analysis. An introduction to the chemometrical evaluation of amino acid data in wine is included.

3.1 Derivatisation

The problem of amino acid derivatisation is concentrated on the selective and sensitive detection of amino acids [1]. Direct detection by UV absorption is only possible at very low wavelengths, which leads to poor sensitivity and interference from background absorption. The common amino acids need to undergo a chemical reaction in order to be detected effectively by either UV or fluorescence detection. In the past, many different reagents have been used for the derivatisation of amino acids. The best known reagents are listed in Table 2 and are discussed in detail.

Nr	Derivatisation Reagent	Reference
1	Ninhydrin	3
2	Fluorescamine	2, 3
3	Phenylisothiocyanate (PITC)	38, 39
4	Dansyl chloride (DHS-Cl)	28, 40
5	o-Phthaldialdehyde (OPA)	21, 41, 44
6	9-Fluorenylmethylchloroformate (FMOC)	42
7	Iodoacetic acid (IDA)	43
8	7-Fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD)	1, 8

Table 2: Derivatisation reagents used in previous amino acid analyses [11].

3.1.1 Ninhydrin

This complex reaction (Figure 16) results in the formation of a very intense purple coloured derivative. Because the distinctive amino acid side chain group is not included in the derivative and, hence, all amino acid derivatives are the same, this reaction can only be used in post-column derivatization applications. The chromatographic process is therefore based on ion exchange.

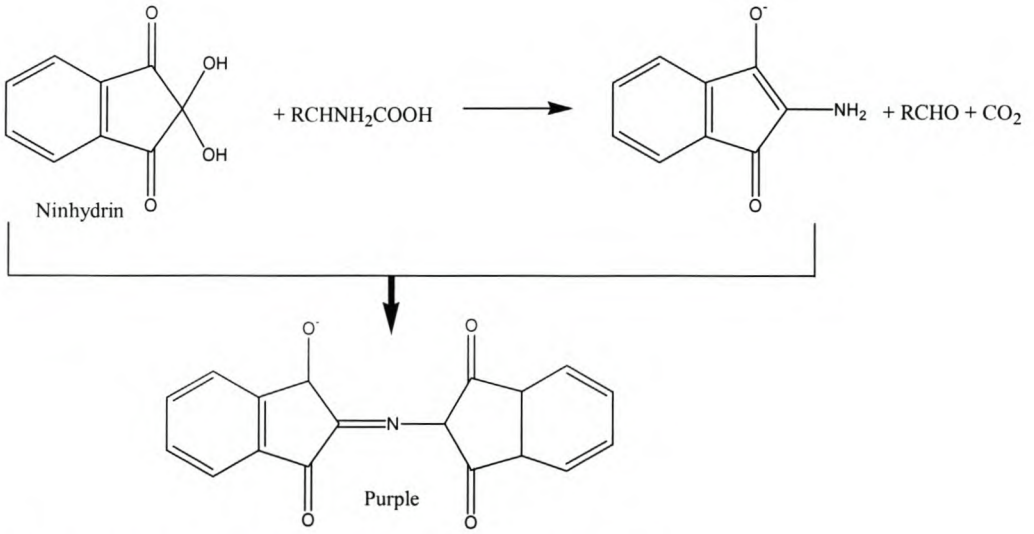


Figure 16: Reaction mechanism of ninhydrin and amino acids

3.1.2 Fluorescamine

Although this reagent readily reacts (Figure 17) and gives good fluorescence with all primary amino acids, including cysteine, it was found that interference of a fluoram[1]-HCl impurity was highly restrictive when using this reagent [2]. Fluorescamine rapidly hydrolyses into unwanted nonfluorescent products when dissolved in water. Therefore it is normally dissolved in acetone [3]. Although the reagent does not react normally with proline or hydroxyproline [4], Felix and Terkelsen adapted the amino acid analyser based on fluorescamine to allow for the additionally detection of hydroxyproline [5]. Georgiadis and Coffey used post-column derivatisation with fluorescamine to analyse amino acids in protein hydolysates [6].

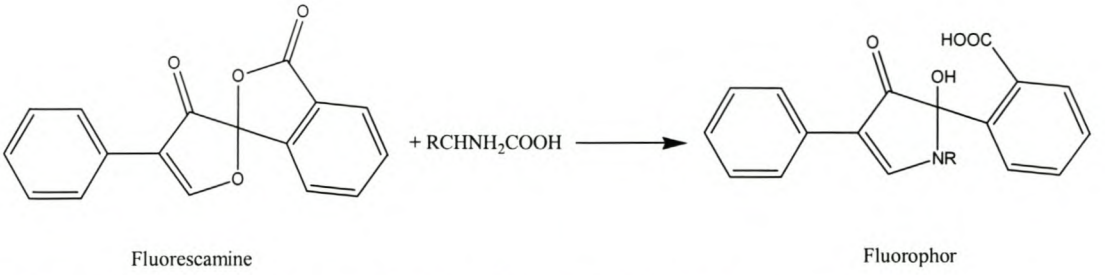


Figure 17: Reaction mechanism of amino acids with fluorescamine. (4)

3.1.3 Phenylisothiocyanate

When amino acids are reacted with the Edman reagent phenylisothiocyanate, the phenylthiocarbamyl derivatives of the amino acids are formed, which can be separated on RPLC and detected with UV detectors (Figure 18).

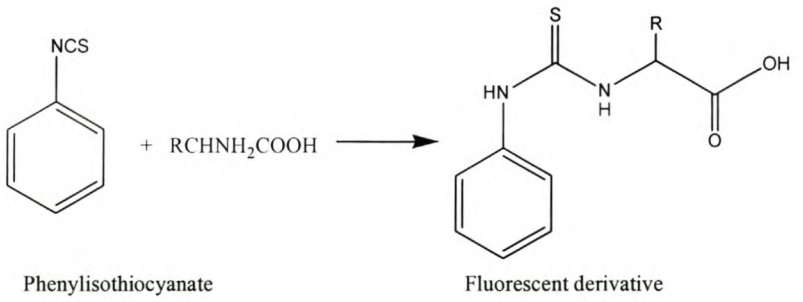
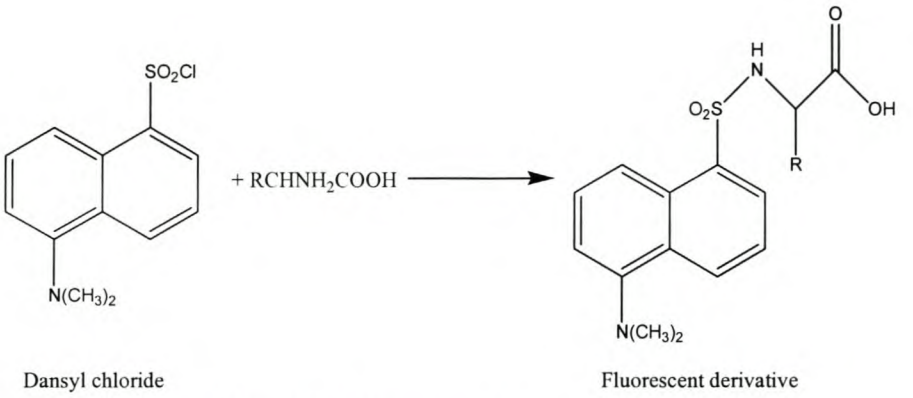


Figure 18: Reaction mechanism of phenylisothiocyanate with amino acids

3.1.4 1-Dimethylaminonaphthalene-5-sulfonyl chloride (dansyl chloride) derivatives

The reaction with dansyl chloride (Figure 19) is widely used, as it can be performed quickly and enables the amino acids to be detected at the nanomolar level [7]. Dansyl derivatives can be detected by both UV and fluorescence detectors [1,8] However, this method does not allow good resolution of the cysteine peak with those of leucine and isoleucine [9].

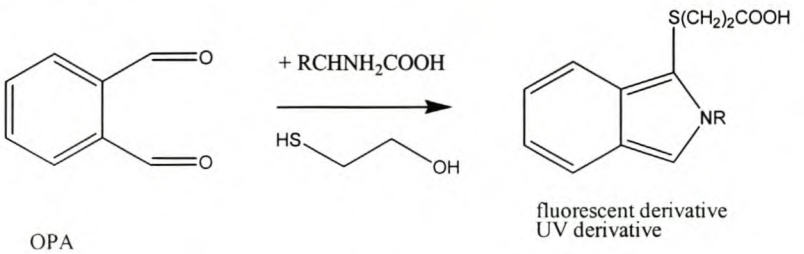


Dansyl chloride Fluorescent derivative

Figure 19: Reaction of dansyl chloride with amino acids

3.1.5 o-Phthaldialdehyde (OPA)

Marc Roth [10] did pioneering work in the derivatisation of amino acids with o-phthaldialdehyde (OPA). He found that amino acids react with OPA in an alkaline medium, in the presence of the reducing agent 2-mercaptoethanol (MCE), to give strongly fluorescing compounds. These compounds were later identified as 1-alkylthio-2-alkyl-substituted isoindole structures (Figure 20) [11]. Heating was not necessary. The mercapto-compound is incorporated into the isoindole structure [1]. Originally o-diacetylbenzene was used. However, he soon found that adding a strongly reducing reagent and substituting o-diacetylbenzene with o-phthaldialdehyde fluorescence was obtained for all amino acids except cysteine, proline and hydroxyproline [10].



OPA fluorescent derivative
UV derivative

Figure 20: Reaction mechanism of OPA and amino acids in the presence of 3-mercaptopropionic acid

Benson and Hare [3] showed that the initial conditions of Roth's [10] work could be optimised to improve detection of the amino acids, making OPA a more suitable derivatisation reagent than the previously favoured fluorescamine and ninhydrin. The modifications included increasing the concentration of 2-mercaptoethanol (MCE) and

the addition of Brij into the reagent solution. They also found that the derivatisation of amino acids with OPA yeilded 5 to 10 times more sensitivity than with fluorescamine and ninhydrin [3].

The use of this reagent has since grown rapidly. Its fast reaction and reasonably stable derivatives have made the procedure very successful. The separation on a reversed phase column, followed by fluorescence detection constitutes a rapid, sensitive and selective detection method for all primary amino acids [1].
Over the years the thiol-containing reagent has been changed and optimised in order to find the most stable derivative. It has been found that the isoindole structure obtained with MCE undergoes a sulfur-to-oxygen rearrangement to give a 2,3-dihydro-1H-isoindol-1-one structure (see Figure 20) [11,12]. It was therefore assumed that substitution of the MCE with a thiol lacking the β-hydroxyl group would give derivatives with greater stability [12]. This instability could also be attributed to the amino acid selected in the derivatisation procedure. For example, glycine and histidine were shown to have a faster degradation rate than the rest of the amino acids [13,14]. Simons and Johnson first substituted MCE with n-propylamine and ethanethiol, in order to determine the derivative structure. These isoindoles appear to be the smallest compounds yet described for the fluorescent detection of amino acids [11]. Those tried and tested include: ethyl mercaptane [1], ethanethiol [12], ethanedithiol [1], propyl mercaptane [1], propanethiol [1], N-acetyl-L-cysteine [15] and 3-mercaptopropionic acid (MPA) [16]. It was found that the OPA/MPA combination gives the most stable and fluorescent derivative products [15,17,18]. It was also found that by increasing the concentration of the thiol-containing reagent and by adding Brij, the derivatives had even greater stability, as well as making lysine fluorescent , which had not been achieved in previous studies [2,3].

However, the OPA/MPA reagents are not able to derivatise the secondary amino acids (or imines) proline and hydroxyproline. This, along with the fact that a few amino acids, including methionine and glycine, give more than one derivative with the OPA/MPA reagent, are the primary disadvantages when using this reagent. It has been found, through investigation of the above compounds by on-line HPLC-electrospray ionisation MS, that the initial structure of the derivatives proved to be

isoindoles (I in Figure 21) and that the transformed species (VII in Figure 21) all contain an additional OPA molecule within their structure. A reaction mechanism for this process has been suggested as shown below [19]. The double OPA derivatives of the amino acids (VII) were thereby observed by ESI-MS as the molecular ion minus water (VIII).

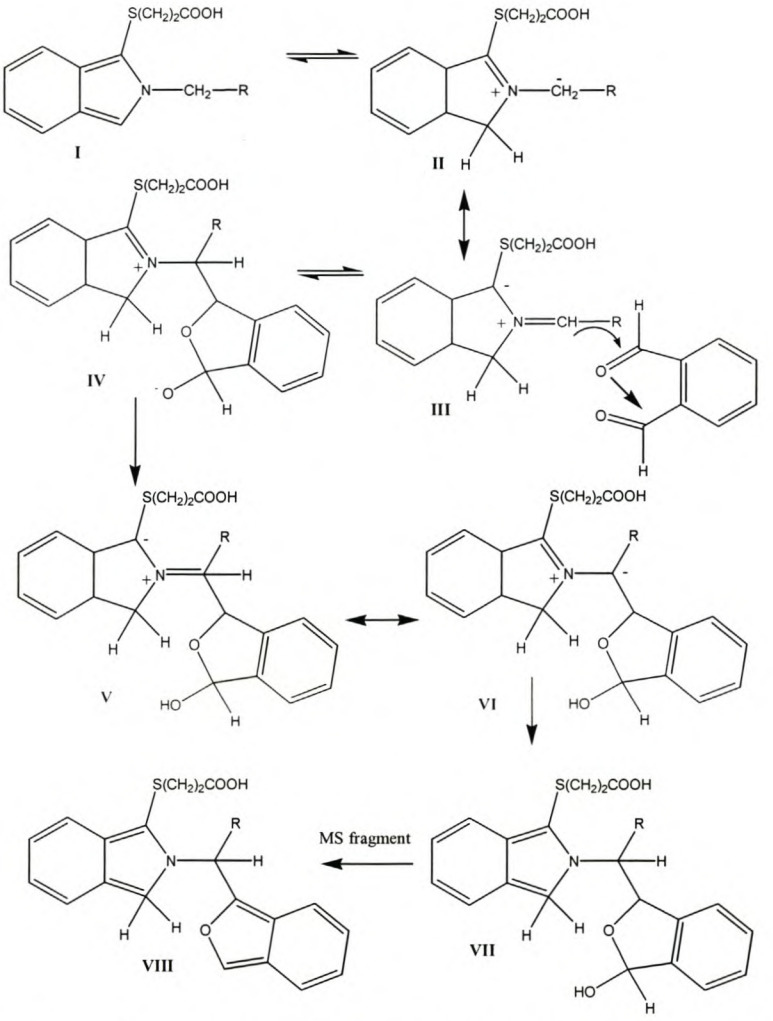


Figure 21: Reaction pathway of the formation/transformation of the multiple amino acid OPA/MPA derivatives. R is the amino acid side chain group.

Many attempts have been made to combine the derivatisation of the primary amino acids with one enabling the secondary amino acids to be transformed.

3.1.6 9-Fluorenylmethylchloroformate (FMOC)

A successful reagent is 9-fluorenylmethylchloroformate (FMOC) (Figure 22) [20]. By combining with OPA derivatisation, stable, fluorescent derivatives of both the secondary and primary amino acids are formed.

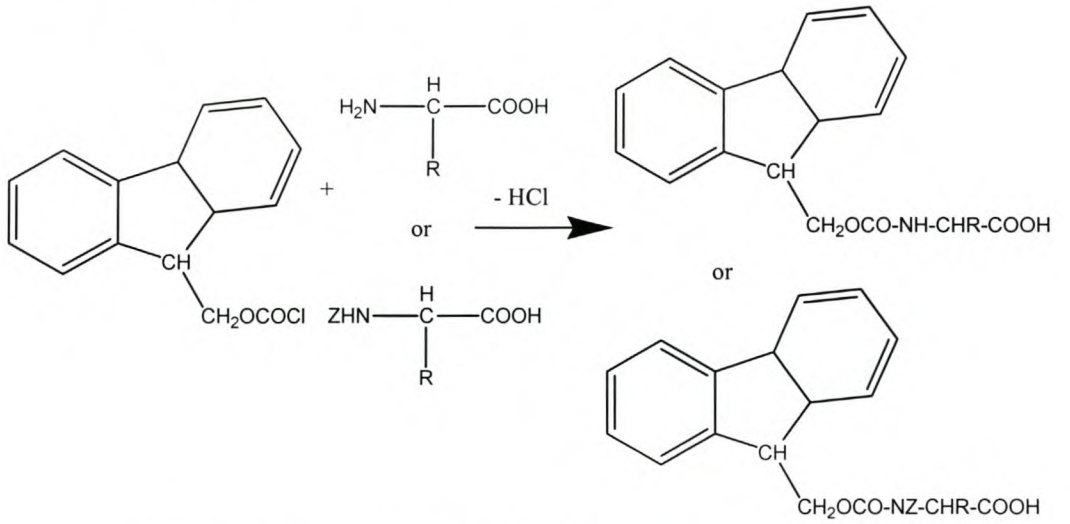


Fig 22: Reaction mechanism of FMOC with primary or secondary amino acids, forming fluorescent derivatives.

The main disadvantage of the FMOC reagent is the fact that it forms many unwanted side products that cause interference during separation. There have also been problems encountered with its insolubility and the reagent is therefore dissolved in acetonitrile [16]. Attempts have been made to remove excess impurities from the reaction solution [2] involving removal of a fluoram[1]-positive impurity.

3.1.7 Iodoacetic acid (IDA)

The reaction products of cysteine and cystine exhibit weak fluorescence when reacted with either OPA or FMOC [1]. In order to detect cysteine the introduction of iodoacetic acid into the derivatisation procedure was found to yield excellent results [9,21,22,23]. Alkylation of cysteine with iodoacetic acid is performed before the OPA reaction (Figure 23). The iodoacetic acid is normally included in the borate buffer solution. Another procedure that has been tried is oxidation of cysteine to its cystine sulphonic acid counterpart with a reagent such as chloramine T [1].

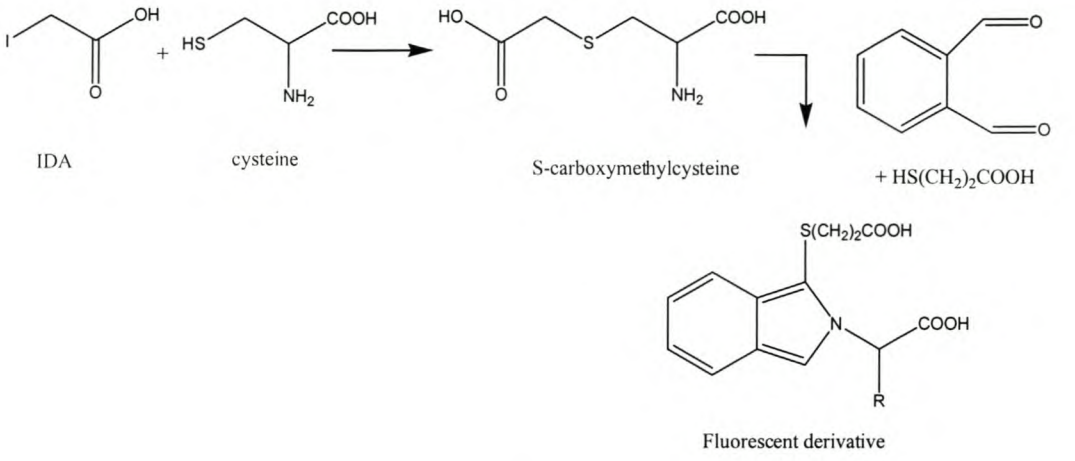


Fig 23: Reaction mechanism of IDA and cysteine before reaction with OPA in the presence of 3-mercaptopropionic acid.

3.1.8 7-Fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD) and other derivatising reagents

7-Chloro and 7-Fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD) are reagents that can compete in sensitivity of detection with OPA and whose amino acid derivatives are easy to separate by RPLC. Weigele *et al.* described a method for detecting secondary amino acids by using N-chlorosuccinimide to form primary amines, which could then react with fluorescamine [3]. Unfortunately, this is not possible in conjunction with OPA, since the N-chlorosuccinimide oxidises the OPA, rendering it useless.

3.2 Chromatographic analysis of amino acids

Many techniques are used to analyse amino acids. These include GC [24], HPLC, etc. Ion exchange chromatography has been used to separate amino acids since 1956 [25,26]. This is based on the work of Moore and Stein, where the amino acids are separated by cation exchange chromatography, followed by post-column derivatisation with ninhydrin, fluorescamine or OPA [16]. For example, Georgiadis and Coffey used an ion-exchange column (DC-4A Resin) with fluorescamine post-column derivatisation [6]. Generally, the amino acids are separated in the protonated form on a strong acid, polymeric, cation exchanger with stepwise changes in pH, from about 3 to 9.6 [26].

Roth [10] used ion exchange chromatography to separate amino acids in his pioneer work involving OPA as an alternative to fluorescamine and ninhydrin. Benson and Hare compared the sensitivities of OPA, fluorescamine and ninhydrin, using a DC-4A cation exchange resin [3]. Cronin and Hare evaluated the separation of OPA-derivatised amino acids by cation-exchange chromatography using the same resin [27]. They also used sodium citrate buffers, at various pH's, ranging from pH 2.9 to 10.7. It was found that the results were in excellent agreement with those obtained by ion exchange chromatography, followed by ninhydrin derivatisation and that the method was less expensive with greater baseline stability [28].

However, the disadvantages of a long analysis time, as well as resulting peak broadening and high costs, have led to the development of new techniques. The incorporation of pre-column derivatisation followed by RP separation has gained momentum in recent years [9,21,29].

Several RPLC procedures using pre-column derivatisation for the determination of amino acids in biological samples have been described, most of them favouring the combined derivatisation with OPA/MCE and FMOC. Carducci *et al* used a Nova-Pak C_{18} (4μm) (300 x 3,9 mm I.D.) for the determination of amino acids in urine [21]. Cooper *et al* demonstrated the stability of OPA/MCE derivatives of amino acids using reversed phase HPLC with a 5 μm Ultrasphere ODS column [14]. The later study was conducted in two ways: initially, by holding the derivatives within the injection loop at varying time intervals before injection, and secondly, 2 minutes after injection the flow rate of the mobile phases was stopped at varying time intervals to determine the stability of the derivatives on the column itself. It was found that the derivatives demonstrate varying fluorescent responses at different time intervals after reaction and also different stabilities. It was also found that the optimum pH to yield maximum fluorescence was from pH 9.5 to 10.0 for all the amino acids examined. Casoli and Colagrande used a C_8 reversed phase column to separate 22-dansylated amino acids, with a multistep gradient elution program. Cheytor utilised a 3μm Ultrasphere ODS column in order to separate and quantify amino acids in fruit juices with OPA in pre-column derivatisation. Marchand *et al.* used a Lichrospher RP 18 (125 x 4 mm I. D., 5 μm) for the separation and quantification of amino acids in wine and musts [9].

Today the prevalent method in amino acid analysis is reversed phase LC, with pre-column derivatisation with OPA/MPA and/or FMOC. In this study, the combination of OP/MPA, FMOC and IDA was evaluated.

3.3 Chemometrical evaluation of the amino acid composition of wines

Characterisation and differentiation of wine has recently received increasing attention [30-35]. Classification by sensory analysis is often not possible due to practical limitations and subjectivity. The use of instrumental analytical parameters has been investigated as an alternative method. Different classes of wine constituents have been proposed as suitable for differentiation purposes. These include the organic acids, minerals, phenolic compounds, amino acids, several classes of volatiles as well as classical enological parameters, and combinations of some of these classes.

Multivariate statistical analysis of the often complex analytical data gathered for wines has been used in a number of studies where differentiation was the final aim [30-35]. Mostly, the chemometric methods of principal component analysis (PCA) and hierarchical cluster analysis (HCA) have been used in data exploration to determine inherent patterns in the data. Discriminant analysis (DA) has also been used to find functions able to assign unknown samples to pre-defined classes.

PCA is an unsupervised multivariate method whose aim is to find principal components (PCs) which are linear combinations of the original variables (in this case quantities of different amino acids) describing each case or object (wine). Each PC is calculated to account for the maximum variation, which usually leads to a much smaller number of useful PCs than the original number of variables. These PCs can then be plotted to visually determine similarities between cases (i.e. those wines lying in close proximity on a scores plot). HCA is another unsupervised method where objects are aggregated into clusters on the basis of inter-object distance in a high dimensional space. The resulting dendrogram can be used to detect groups of similar objects [36,37]. The data collected in this thesis have been subjected to HCA and PCA however as preliminary evaluation only.

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Chapter 4. Development of a robust method for amino acid analysis

4.1 Introduction

One of the main tasks of this thesis was to filter out of the many analytical procedures described for amino acid analysis, one specific method that was producing reliable data for the wine matrix. Because "routine use" was a key word, simplicity of the method had to be considered as well. A review is presented on the different steps towards the developed and optimised method, which is then described in detail.

4.2 Initial experiments

4.2.1 Evaluation of a standard operating procedure

A standard operating procedure was presented in the application literature [1] for the analysis of amino acids. The method is based on automated in-line derivitisation using OPA for primary amino acids and FMOC for secondary amino acids. The analytical conditions were strictly followed. These are briefly summarised below.

The mobile phases were prepared as follows:

A: An aqueous solution of 40 mM Na_2HPO_4 pH 7.8 [5.5 g NaH_2PO_4 monohydrate + 1 litre water, adjusted to pH 7.8 with NaOH (10N)]

B: ACN:MeOH:water (45:45:10, v/v/v)

The column used was a Zorbax Eclipse-AAA (150 x 4.6mm, 5µm) and the mobile phase gradient is shown in Table 3:

Time	Flow Rate (ml/min)	%A	%B
	2	100	0
1.9	2	100	0
18.1	2	43	57
18.6	2	0	100
22.3	2	0	100
23.2	2	100	0
26	2	100	0
27	0	100	0

Table 3: Initial mobile phase gradient conditions

The amino acids analysed are presented in Table 4.

Number	Amino Acid	Number	Amino Acid
1	Aspartate	13	Alanine
2	Glutamate	14	Tyrosine
3	Methionine	15	Valine
4	Cysteine	16	Norvaline (I.S.)
5	Asparagine	17	Tryptophan
6	Serine	18	Phenylalanine
7	Glutamine	19	Isoleucine
8	Histidine	20	Leucine
9	Glycine	21	Proline
10	Cystine	22	Lysine
11	Threonine	23	Hydroxyproline
12	Arginine		

Table 4: The 23 amino acids analysed in this study

The derivatisation procedure used is outlined in Figure 24, while the content of the derivitisation solutions is specified in Table 5.

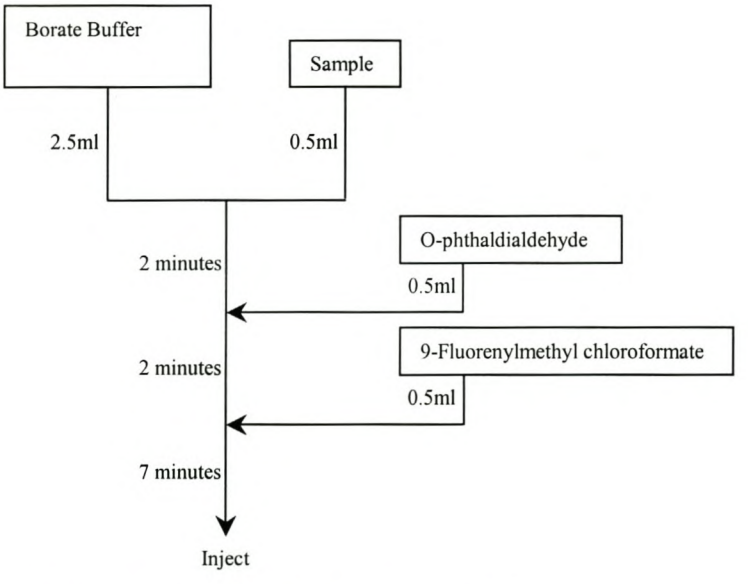


Figure 24: Schematic diagram of the derivatisation procedure.

OPA [2]	FMOC [3]	Borate buffer
Brij-35: 1000 ppm OPA: 7.6 mM MeOH: 2% (v/v) 3-MPA: 1% (v/v) Boric acid: 40 mM KSCN: 36 mM KOH: 53 mM Dilute with water to 10 ml	FMOC: 2.7 mM Dissolved in 25 ml acetonitrile	Boric acid: 133 mM Adjust pH to 10.2 with 10 M NaOH Dilute with water to 10 ml

Table 5: Derivatisation reagent solutions

10 µl of the resulting mixture was injected in the HPLC. UV detection was performed at 338 nm and 262 nm for the primary and secondary amino acids, respectively.

An example of the resulting chromatograms is shown in Figure 25. It can be seen that the primary amino acids, derivatised with OPA, elute first from the column. These are followed by the more apolar FMOC-derivatised amino acids (visible at 262 nm) and finally by the excess derivatising agents and side products. Note that a "hump" is also visible in Figure 24B. However, this does not interfere with the analysis of the later eluting FMOC derivatised amino acids. It was found that these conditions were not ideal. Resolution between Gly and Thr, as well as between Phe and Ile was insufficient. In addition, retention times of the amino acids were not sufficiently reproducible, whilst continuous problems resulting from precipitation of phosphate salts were encountered, even with extensive daily flushing to remove excess salts and with the use of a pre-column.

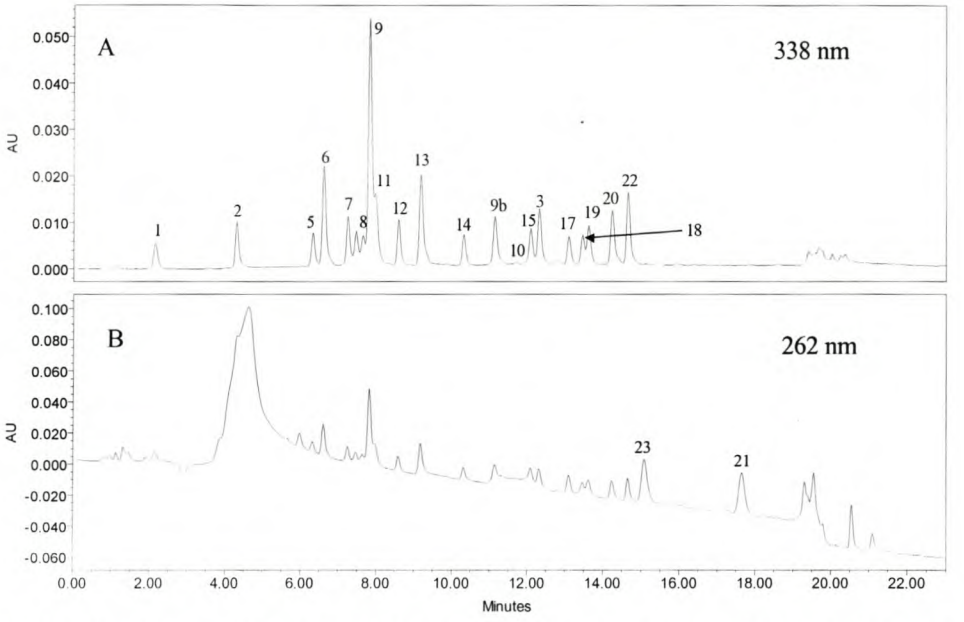


Figure 25: Chromatogram of OPA/FMOC-amino acids using conditions found in the application literature [1]. The amino acids are assigned according to Table 4.

4.2.2 Modification of the standard operating procedure

Ammonium acetate was used as alternative to phosphate in phase A, in an attempt to overcome some of these problems. The volatile ammonium acetate has the added advantage of making the method compatible with mass spectrometric detection. A mobile phase containing 50 mM ammonium acetate at pH 7.8 in A and $CH_3CN/MeOH/H_2O$ (45/45/10) in B was used under the same conditions as in paragraph 4.2.1. Although problems associated with precipitation were avoided with this new mobile phase, the reproducibility of the method was not yet sufficient. Changing the pH to 7.2 as reported in literature [5-8] did not improve the separation.

In an attempt to overcome reproducibility problems, the Zorbax-AAA column was replaced by a LUNA C18 (2) (150 x 4.6 mm I.D., 5 μm) column from Phenomenex. This resulted not only in an increase in efficiency, but also improved retention time reproducibility.

4.2.3 Conclusion

The standard operating procedure was found to be not suitable for the analysis of the amino acid standards. Problems relating to poor robustness and reproducibility were overcome by using ammonium acetate as buffering reagent instead of phosphate, while resolution was improved when using a LUNA column instead of the Zorbax AAA column. Based on these more promising results, it was decided to optimise this modified standard operating procedure for the analysis of amino acids in wine.

4.3 Improved method

4.3.1 Introduction

The method chosen for optimisation is based on a standard operating procedure for the simultaneous determination of primary and secondary amino acids after derivitisation with OPA and FMOC. In this study, however, derivatisation was performed off-line as opposed to on-line, since the HPLC hardware used did not allow on-line derivatisation. IDA was added to the derivatisation scheme in order to allow derivatisation of cysteine. Furthermore, the SOP was adapted specifically in terms of the mobile phase and the column used, as this improved the robustness and resolving power of the method. In this section further optimisation of the method towards the final conditions is described.

4.3.2 Experimental

4.3.2.1 Materials

All chemical were of analytical grade. The amino acids, MeOH, KOH and Brij 35 were purchased from Sigma-Aldrich (Steinheim, Germany). Boric acid was obtained from Saarchem (Muldersdrift, South Africa). Acetonitrile and THF were provided by Riedel-de Haen (Seelze, Germany). OPA, 3-mercapto propionic acid (3-MPA), 9-fluorenylmethocycarbonlychloride (FMOC-chloride), KSCN and iodoacetic acid (IDA) were purchased from Fluka (Buchs, Switzerland).

4.3.2.2 Methods

Standard solutions of amino acids were prepared in two separate solutions to avoid solubility problems. The first solution contained 2mg/ml of the following amino acids in 0.01 M HCl:

Serine	Histidine	Lysine
Alanine	Valine	Glutamic Acid
Threonine	Proline	Glutamine
Arginine	Aspartic Acid	Glycine
Hydroxyproline	Cysteine	Methionine
Norvaline (I.S.)		

The solution was made by weighing out 100 mg of each of the above amino acids and dissolving them with of 0.01M HCl in a 25 ml volumetric flask.

The second solution of amino acids contained 2 mg/ml of the following amino acids in 0.1 M NaOH:

Tyrosine	Tryptophan	Cystine
Asparagine	Leucine	Isoleucine
Phenylalanine		

These solutions were made by dissolving 100 mg of each amino acid in 0.1 M NaOH in a 50 ml volumetric flask.

The solutions were stored at 4°C and were stable for approximately three weeks. Prior to derivatisation, sample solutions one and two were mixed in the ratio 1:2, ensuring equal concentrations for all amino acids (1333 ppm). The solutions were freshly mixed prior to derivatisation, as the mixed solution proved to be unstable.

The final derivatisation reagent solutions were prepared according to Table 6.

OPA	FMOC	IDA
Brij-35: 1000 ppm OPA: 7.6 mM MeOH: 2% (v/v) 3-MPA: 1% (v/v) Boric acid: 40 mM KSCN: 36 mM KOH: 53 mM Dilute with water to 10 ml	FMOC: 2.7 mM Dissolved in 25 ml acetonitrile	Boric acid: 133 mM IDA: 100 mM Adjust pH to 10.2 with 10 M NaOH Dilute with water to 10 ml

Table 6: Derivatisation reagent solutions

All three solutions were stored in the refrigerator at 4°C. FMOC and OPA solutions were kept for approximately nine days before being replaced. The IDA/buffer solution was stable for months.

The mobile phase composition was optimised as discussed in section 4.3.2.2. The final mobile phases were prepared according to the following procedure:

Phase A:

100 mM Ammonium acetate

pH 7.00 (± 0.02)

0.5% THF

Ammonium acetate (7.708 g) was dissolved in 700 ml of de-ionised water. Tetrahydrofurane (5 ml) was added to the solution and the pH was adjusted to 7.00 (± 0.02) with NaOH. The solution was then filled with de-ionised water in a 1 l volumetric flask.

Phase B:

Acetonitrile : methanol : water

(50:40:10 ; v/v/v)

It was important to measure the volumes of the solvents separately before adding them together, since a volume retraction occurs when acetonitrile and methanol are mixed. Both mobile phases were degassed and filtered through a 0.45 µm filter (Millipore Corp., Bedford) prior to use.

For optimisation of the separation conditions, a Zorbax Eclipse AAA column (150 x 4.6 mm, 5 µm) was used. Once the separation, detection and derivitisation parameters were optimised, a Luna C18 (2) column (5 µm 150 x 4.6 mm, Phenomenex), with a guard column of the same phase was used. The columns were heated to 40°C and 10 µl was injected. Details of the optimised gradient (see section 4.3.2.2) are presented Table 7.

Time	Flow	%A	%B
	2	99.5	0.5
6	2	99	1
8	2	97	3
9	2	93	7
11	2	85	15
18	2	81	19
29	2	65	35
32	2	0	100
35	2	0	100
38	2	99.5	0.5
43	2	99.5	0.5
44	0	99.5	0.5

Table 7: Optimized mobile phase gradient program.

The experiments were carried out on an Alliance Waters 2690 Separations module, which consisted of a quaternary pump, a degassing unit, an injection unit, and a column heater. The system was equipped with a Waters 996 Photodiode Array detector and a Waters 474 Scanning Fluorescence detector, all from Waters Corp. (Milford, MA, USA). The data analysis was done using Millenium[32] Chromatography Manager software, also from Waters.

4.3.3 Results and discussion

4.3.3.1 Column and mobile phase selection

Mobile phase optimisation was performed on the Zorbax Eclipse AAA column. Phosphate was substituted by ammonium acetate in mobile phase A, as this improved the robustness of the method and made it compatible with mass spectrometric detection (section 4.2.2). 25 mM, 50 mM and 100 mM ammonium acetate were compared at pH 7.2. Better efficiency was obtained at the latter ionic strength, and 100 mM ammonium acetate was subsequently used. However, the separation of the amino acids was not sufficient (Figure 26), and additional parameters were investigated.

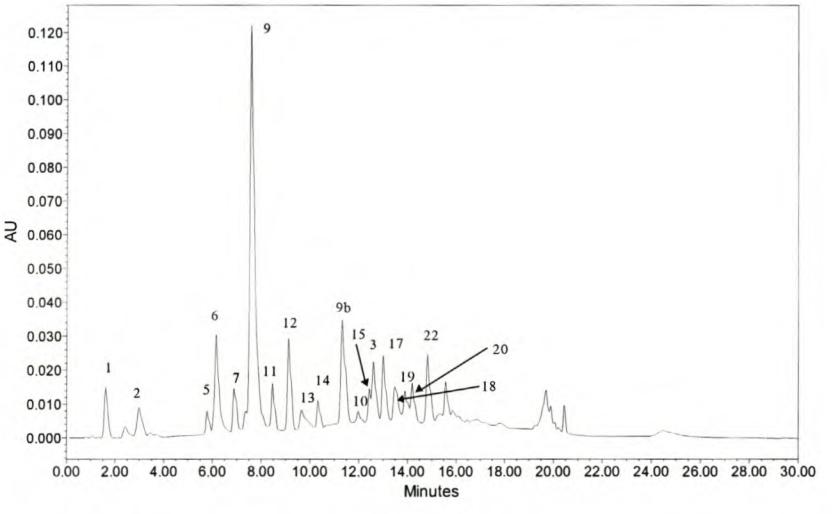


Figure 26: UV chromatogram (338 nm) of OPA-amino acids with ammonium acetate mobile phase A.

The effect of addition of an organic modifier to mobile phase A was studied. Methanol proved unsuccessful, but with the addition of tetrahydrofurane (THF) an improvement in separation and peak shapes could be obtained [7]. The amount of THF was varied from 0.5% to 5%, but the best results being obtained at the lowest percentage (Figure 27). Addition of THF to both phases did not improve the separation.

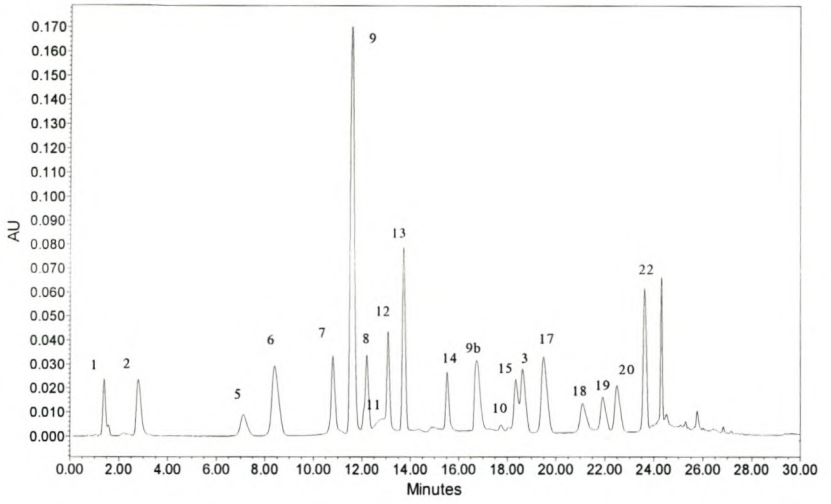


Figure 27: UV chromatogram of OPA-amino acids after addition of 0.5% THF into mobile phase A.

The pH of mobile phase A was also varied. Because acetic acid has a buffering capacity at pH 4.2, the pH was adjusted to 5, however, this resulted in co-elution of several amino acids. The range of pH 7 to 7.4 was investigated. At pH 7.0 the best separation was achieved and this pH was used in further experiments (Figure 28).

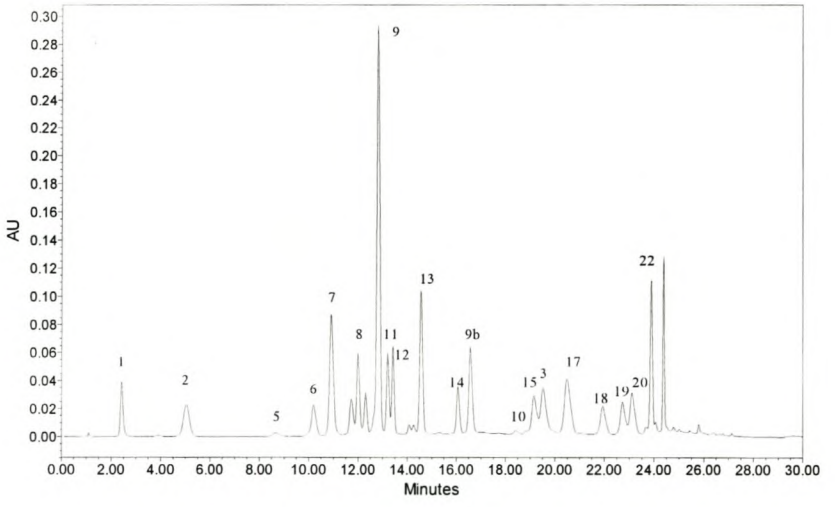


Figure 28: UV chromatogram of OPA-amino acids after pH optimisation.

The ratio between acetonitrile and methanol in phase B was also altered in order to further improve the method. Solutions of 90% ACN and 90% Methanol were both

attempted. In the former case, it was found that although the last eluting peaks were well-resolved, earlier peaks co-eluted. In the latter case, it was found that the later eluting peaks could not be separated. After testing various ratios of acetonitrile, methanol and water, the optimum was established at 50:40:10. An example of a chromatogram obtained under these conditions is shown in Figure 29.

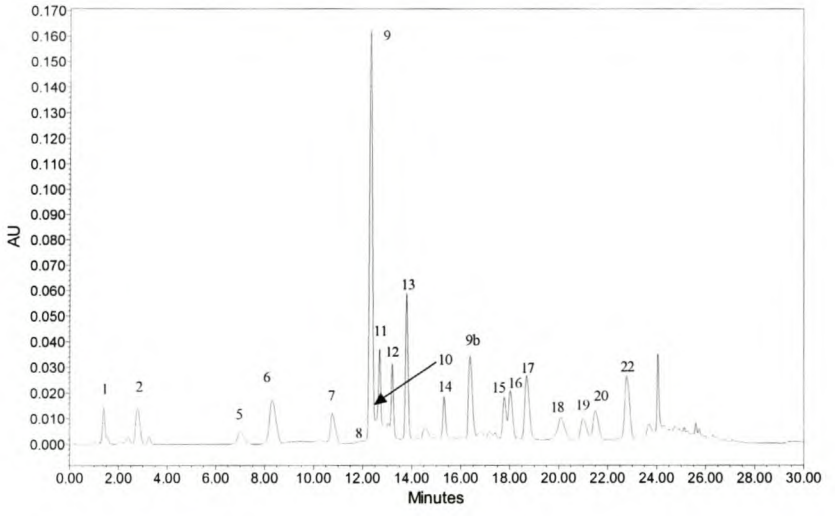


Figure 29: UV chromatogram of OPA-amino acids with optimised phase B.

4.3.3.2 *Derivatisation optimisation*

Initially, the derivatisation method included only OPA and FMOC. These solutions were made in the same way as the commercial reagents from Fluka and were not optimised further. According to reproducibility studies, the derivatives formed from the reagent solutions were consistent in both retention times and peak areas and it could be assumed that 100% reaction occurred between the amino acids and the derivatisation reagents.

However, with this reagent mixture it was not possible to quantify cysteine and its derivative cystine, as these amino acids do not react with either OPA or FMOC. Thus, an IDA derivitisation step was incorporated into the derivatisation procedure, before reaction with OPA [9]. The reagent was added to the borate buffer at different concentrations in order to optimise the response for cysteine. The concentrations evaluated include: 0.1 M, 0.5 M, 1.0 M and 2.0 M. 100 mM IDA provided the best results and was used in subsequent analyses.

To the best of our knowledge, the derivatisation strategy with incorporation of OPA, FMOC and IDA has not before been performed so far for the analysis of amino acids in wine. The derivatisation procedure itself is identical to that shown in Figure 24, with the exception that the borate buffer now contains 0.1 M IDA.

The timing of the derivatisation procedure was kept constant throughout the study in order to improve reproducibility. The time period between the mixing of the derivatisation reagents and injection was also kept constant at 7 minutes. An example of a chromatogram obtained with the three step derivitisation procedure is shown in Figure 30. Note the appearance of cysteine as an early eluting peak (peak number 4).

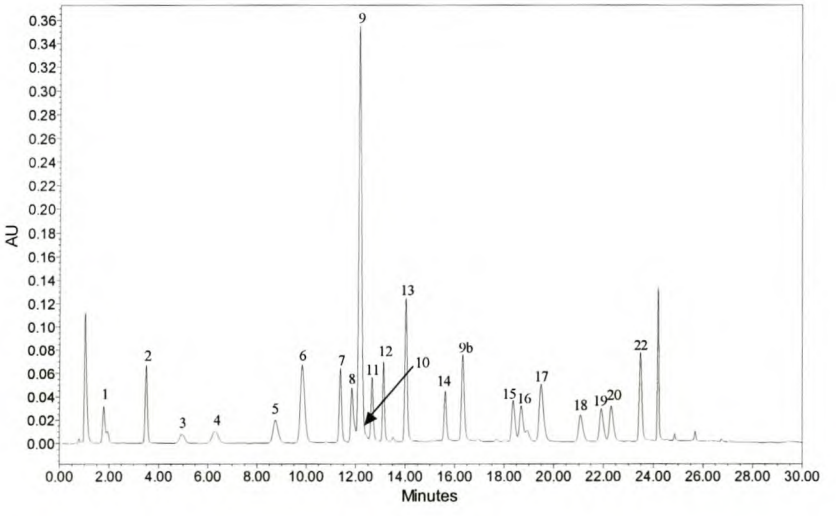


Figure 30: UV chromatogram of amino acids derivatised with proposed reaction scheme (peak 4 = cysteine).

Another aspect of the OPA reagent, one that is noted in the literature [8] (see also Figure 21), is that of the occurrence of multiple reaction products for various amino acids. In this study, it was observed that mainly two amino acids gave double peaks, namely glycine and methionine. It has been proposed that addition of a second OPA molecule to certain amino acids takes place. Figures 31 and 32 depict the double peaks for both glycine and methionine. For quantitation, the sum of the two peak areas was used, and the response was found to be linear.

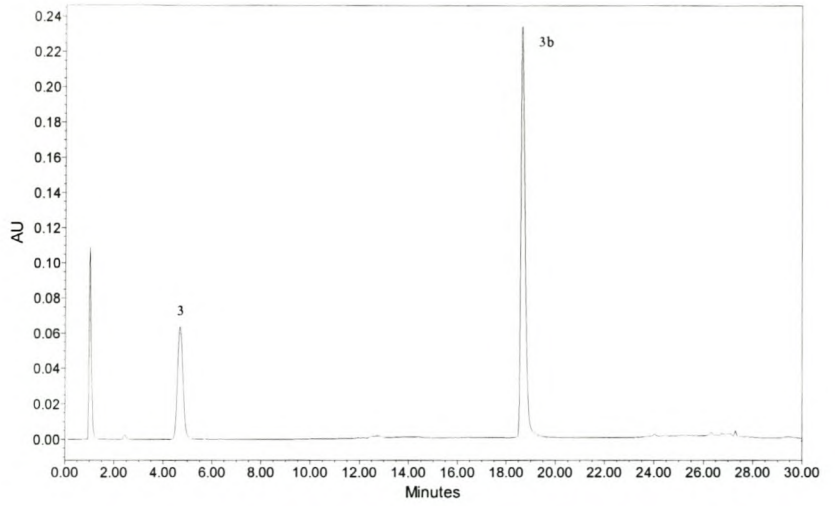


Figure 31: Chromatogram showing the double derivatives of methionine

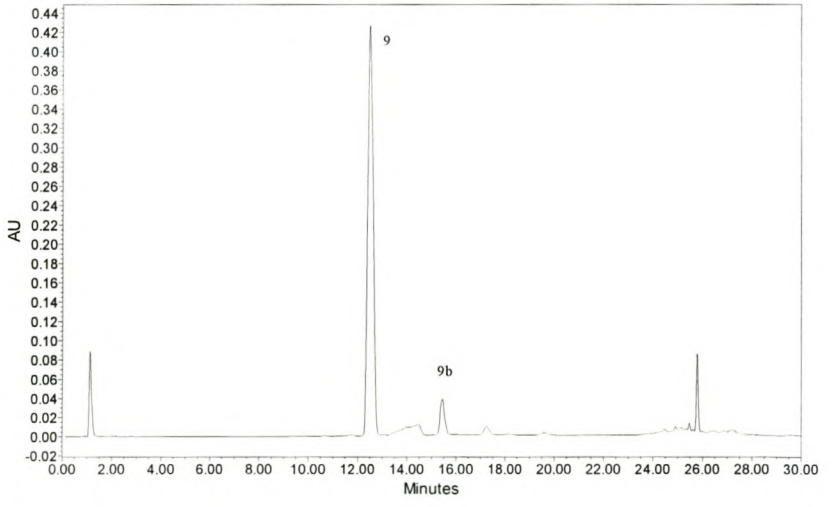


Figure 32: Chromatogram showing the double derivatives of glycine

4.3.3.3 *Detection*

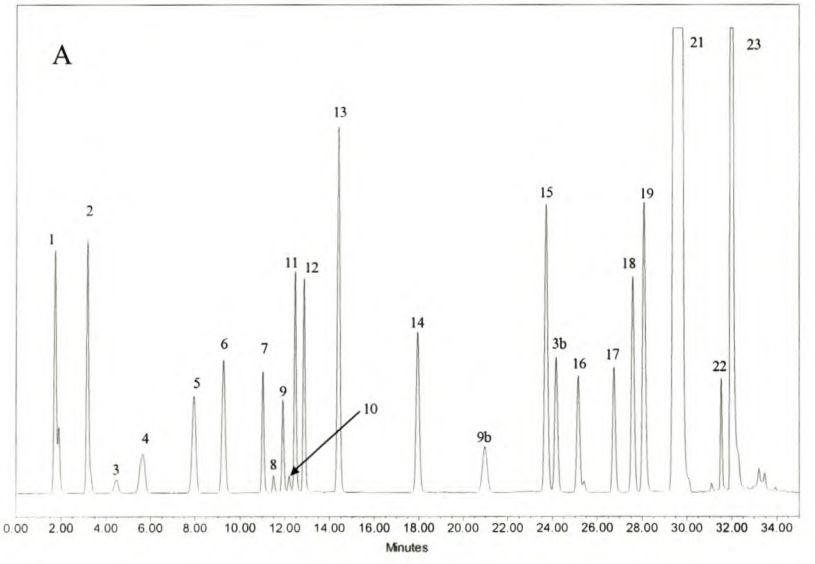
UV detection was performed at 338 nm for primary amino acids and 262 nm for secondary amino acids. Fluorescence detection can be up to 1000 times more sensitive than UV detection. This is essential in this study, since the concentration levels of the amino acids in wine are generally quite low. The primary amino acids (except leucine) can be detected by fluorescence detection at an excitation wavelength of 337 nm and an emission wavelength of 450 nm and the secondary amino acids at an excitation wavelength of 265 nm and emission wavelength of 310 nm. In order to

detect both the primary and secondary amino acids in the same chromatogram, the fluorescence detector was programmed to change the excitation and emission wavelengths at specified times. The program sequence is shown in Table 8. There was insufficient resolution between the peaks of proline and leucine. Therefore the leucine concentrations were calculated by UV detection. Table 9 summarises the detection methods used for quantitation of all 23 amino acids.

Time	λ_{ex}	λ_{em}
0.0	337	450
29.0	265	310
30.0	337	450
31.8	265	310
32.2	337	450

Table 8: Fluorescence detector timed sequence

With the finalised detection parameters, optimisation of the method was complete. The Zorbax Eclipse column was now exchanged for a LUNA C18, and the finalised method was applied to the analysis of amino acid standards (Figure 33). This figure also illustrates the difference in sensitivity between fluorescence and UV detectors. The comparison illustrates the need for fluorescence detection in this study.



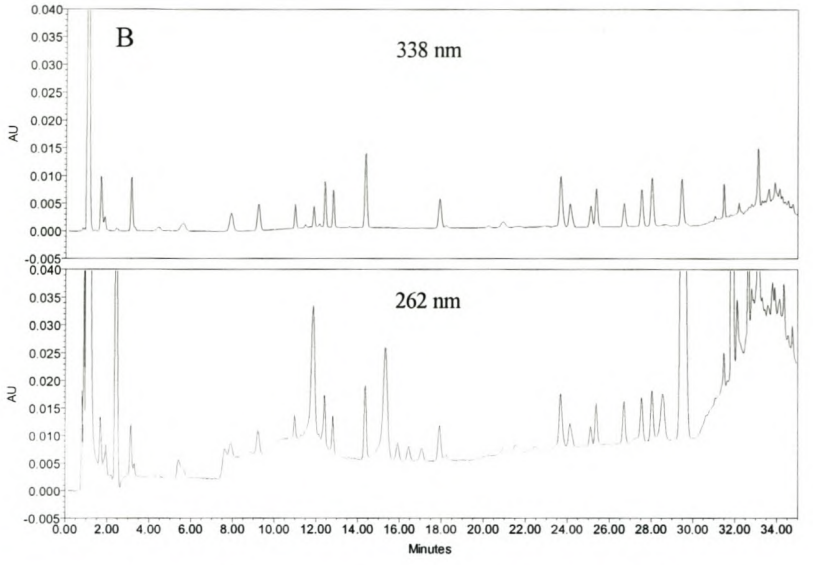


Fig 33: Chromatogram of standard solution of amino acids, excluding leucine. Figure A: fluorescence detection, Figure B: UV detection.

Amino acid	UV 338 nm	FL $\lambda_{ex}/\lambda_{em}$ (337/450 nm)	FL $\lambda_{ex}/\lambda_{em}$ (265/310 nm)
Asp		×	
Glu		×	
Met		×	
Cys		×	
Asn		×	
Ser		×	
Gln		×	
His		×	
Gly		×	
Cy2		×	
Thr		×	
Arg		×	
Ala		×	
Tyr		×	
Val		×	
Nor (I.S.)		×	
Trp		×	
Phe		×	
Ile		×	
Leu	×		
Pro			×
Lys		×	
Hyp			×

Table 9: Summary of the detection modes used to quantify each of the amino acids.

4.3.3.4 Application of developed method for LC-MS analyses

The new method proposed in this work for the analysis of amino acids was also briefly evaluated with electrospray mass spectrometric (ESI-MS) detection. It was expected that this would be unproblematic considering that the volatile ammonium acetate was used in the mobile phase. Due to its volatility the ammonium acetate is completely evaporated in the ESI interface without creating any salt deposit affecting the ionization process and, hence, the sensitivity, reproducibility and robustness of the method. Ideally methanol is the most suitable solvent to obtain maximal sensitivity with electrospray ionization but as has been shown in this work, acetonitrile and THF are required to obtain a better separation. Although these solvents are known to reduce the sensitivity they were not expected to cause serious problems. In order to test the suitability of the method for LC-MS some tentative experiments have been performed here with a set of amino acid standards and one red wine sample (wine 10 = red wine blend of good quality).

The reversed phase liquid chromatography separations were done on a narrow bore Luna $C_{18}(2)$ 250 mm x 2 mm x 5 µm column from Phenomenex. The mobile phase composition and the gradient have been described before. The flow rate was 0.2 ml/min and the flow was fully introduced into the ESI-MS. UV detection was performed at 338 nm. MS-detection was done with a benchtop LCQ MS^n from Thermo Finnigan equipped with an electrospray ionization source. In all the experiments the sheat gas flow (N_2) was operated at 95% and the nebulizing gas was set at 5%, the spray voltage was 4 kV and the transfer capillary was heated at 270°C. Collision induced dissociation (CID) was operated at 2 V to avoid the formation of clusters. The MS scan range covered 50-1200 amu.

Upon analysis of the derivatized amino acid standards (except norvaline) with LC-MS in the positive ionisation mode the following UV chromatogram and base peak chromatogram was obtained (Figure 34A and 34B).

It can be seen in the UV chromatogram that the separation efficiency was not optimal on the column used for this experiment. The relatively broad peaks and the poor separation complicated sensitive detection of all the amino acids. The signals were therefore not very intense in the base peak chromatogram. However, the amino acids could be observed by monitoring the individual extracted ion traces. In Figure 33C

and 33D the reconstructed ion chromatograms of the $[M+H]^+$ ions of the derivatives of the serine, glycine, alanine and tyrosine and valine can be seen, respectively. Isobaric derivatives of the different amino acids could easily be distinguished by comparing the MS data with the expected retention times.

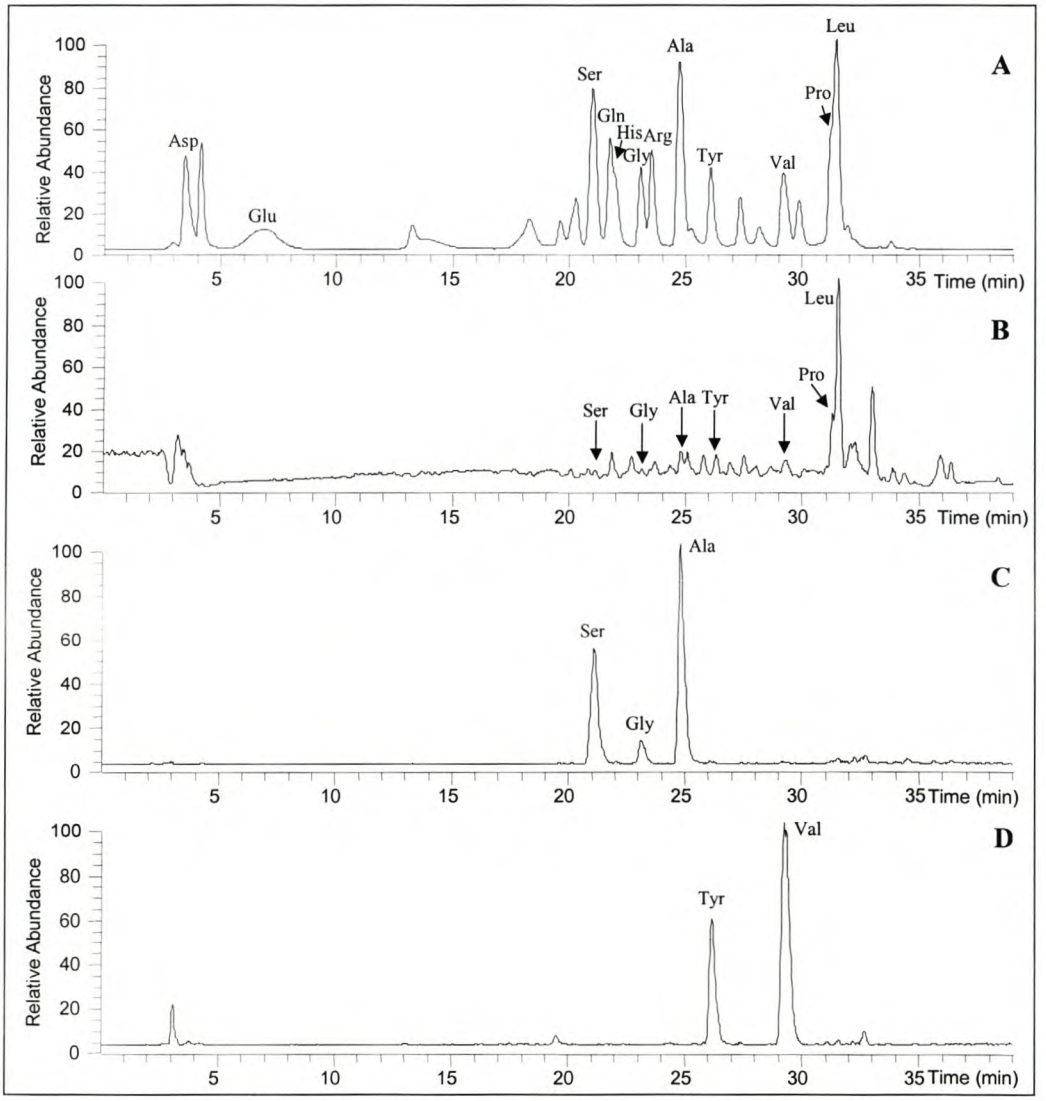


Figure 34: Analysis of the mixture of 23 derivatised amino acids by LC-MS in the positive ionisation mode. A: UV Chromatogram, B: Base peak chromatogram, C: reconstructed ion chromatogram for serine, glycine and alanine, D: reconstructed ion chromatogram for tyrosine and valine (conditions see text).

One of the aims of these LC-MS analyses was to determine the composition of the secondary derivatives of glycine, known to elute between tyrosine and valine, but the

sensitivity proved not sufficient for this signal and, hence, this structure could not be determined. This was also the case for methionine.

It appeared detection was possible both in the positive and in the negative ionisation mode. In the negative ionisation the highest sensitivity was obtained for the [M+Na-$2H^+$] ions. Substitution of one of the protons from the carboxylic functions by sodium is a common phenomenon in this mode.

The wine sample was analysed in the negative ionisation mode. The results are shown in Figure 35.

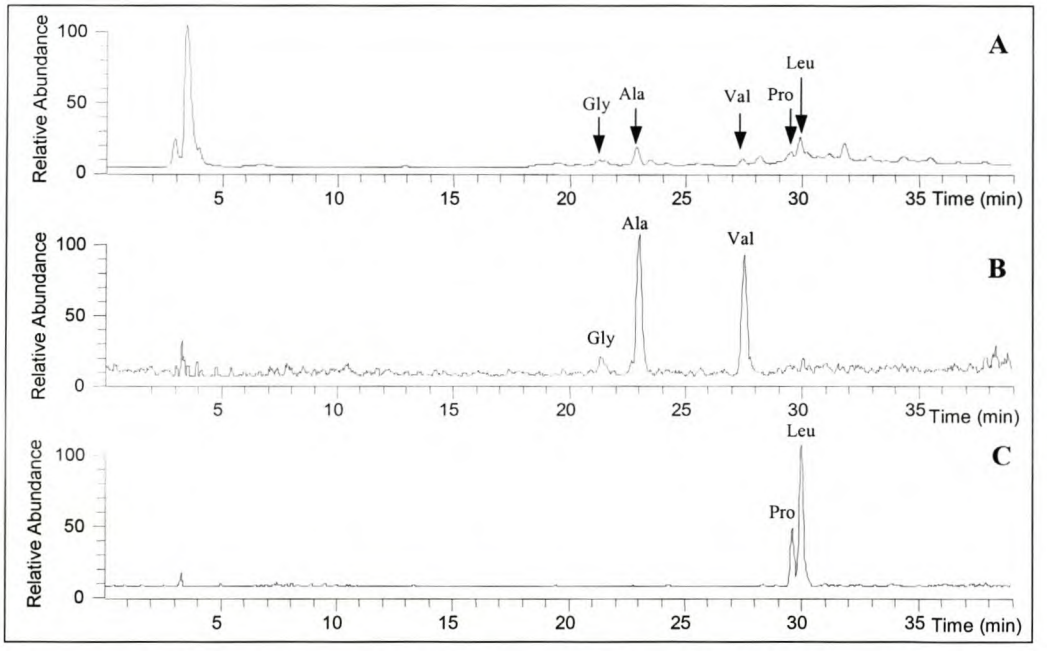


Figure 35: Analysis of wine sample 10 (= 1999 red wine blend of good quality) by LC-MS in the negative ionisation mode. A: UV chromatogram, B: reconstructed ion chromatogram for glycine, alanine and valine C: reconstructed ion chromatogram for proline and leucine (conditions see text).

It is clear that the amino acids can be identified as such. However, under these conditions a far better sensitivity was obtained by UV and FLD detection and the MS serves as a mere confirmation of the identity of the peaks. It can hence be concluded that the analytical procedure developed in this thesis can in principle be combined with ESI-MS. However, more time would be necessary in order to optimise all the parameters, such as to achieve a higher sensitivity and no peak broadening.

4.3.3.5 Figures of merit

Repeatability

Once the gradient, mobile phases, separation and resolution were optimised, the reproducibility of the method was tested by running 4 consecutive analyses, each with the same standard solution, whilst a fresh derivatisation was performed for each analysis. The relative standard deviations for peak areas were calculated. The obtained results are shown in Table 10. As can be seen, the RSD% values are satisfactory, excepting that of cysteine. This might be due to the fact that the derivatisation procedure was performed manually off-line. These conditions are not ideal, as better reproducibility can be achieved when automatically adding reagents to the sample solution using automated HPLC equipment.

In order to monitor the stability of the derivatives formed, the same standard solution of amino acids was injected 4 times over in succession. Most of the peak areas decreased with the exception of a few, namely glycine, tyrosine, valine, phenylalanine, proline, lysine and cysteine. The peak area of cysteine seemed to increase with increasing time after derivatisation and it was thought that the peak might be co-eluting with a reagent peak, as the reagent peaks tend to increase in time.

Name	RSD%
Aspartate	1.94
Glutamate	1.86
Methionine	1.87
Cysteine	12.83
Asparagine	5.39
Serine	4.92
Glutamine	6.73
Histidine	8.87
Glycine	2.00
Cystine	2.37
Threonine	6.12
Arginine	1.82
Alanine	1.44
Tyrosine	2.86
Valine	1.76
Norvaline	1.64
Tryptophan	2.35
Phenylalanine	1.63
Isoleucine	1.47
Proline	2.59
Lysine	7.95
Hydroxyproline	4.09

Table 10: Relative standard deviation values of the peak areas for 4 freshly derivatised and analysed amino acid mixtures.

Linearity

Calibration graphs were set up using the internal standard method. All the amino acids (excepting proline and hydroxyproline) were calibrated over the range 1 to 50 ppm, as this is the concentration range in which the amino acids are expected in wines. Proline and hydroxyproline were expected to appear in far higher concentrations than the other amino acids, since the secondary amino acids are generally not used during the fermentation process. They were calibrated with concentrations of 1000, 500, 200, 100 and 20 ppm. The concentration of the internal standard was kept constant at 20 ppm.

For the amino acids dissolved in HCl solution, 100 mg of each amino acid was weighed out and dissolved in 0.01 M of HCl in a 50 ml volumetric flask (2000 ppm). The solution was then diluted 10× with de-ionised water. For the NaOH solution, 50 mg of each amino acid was dissolved in a 50 ml volumetric flask with 0.1 M NaOH (1000 ppm). This was also diluted 10×. The solution containing proline and hydroxyproline had 200 mg of each in a 50 ml volumetric flask that was then filled with 0.01M HCl. The two HCl solutions were then mixed in the correct ratio to ensure that they had the above-mentioned concentrations. The internal standard solution had a concentration of 80 ppm and was dissolved in 0.01 M HCl. For calibration, 2 ml of the NaOH solution was added to 1 ml of the HCl solution. To this was then added 1 ml of internal standard, giving a concentration of 20 ppm for norvaline. Examples of the calibration curves obtained are shown in Figures 36 and 37. The corresponding calibration details are summarised in Table 11.

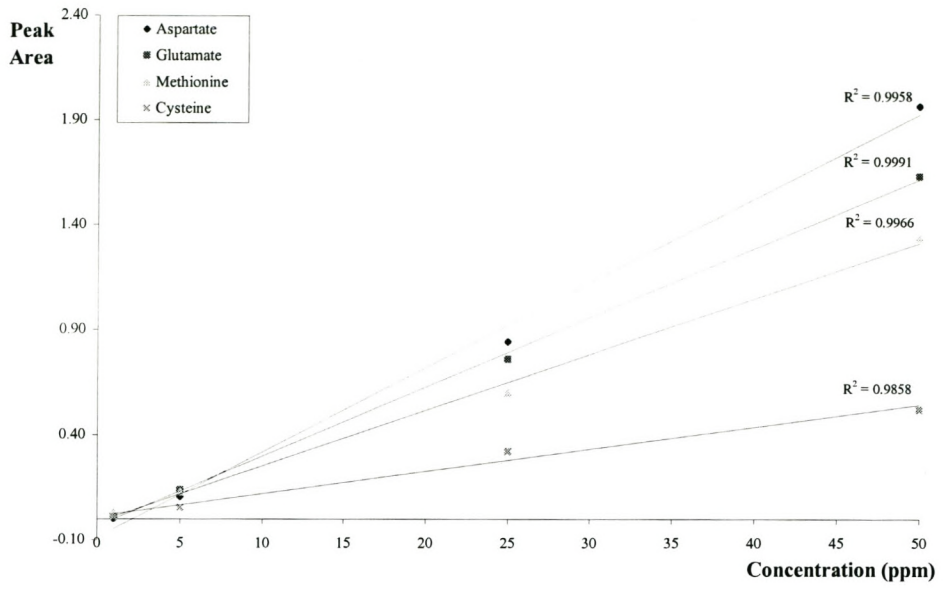


Figure 36: Calibration curves for Asp, Glu, Met and Cys.

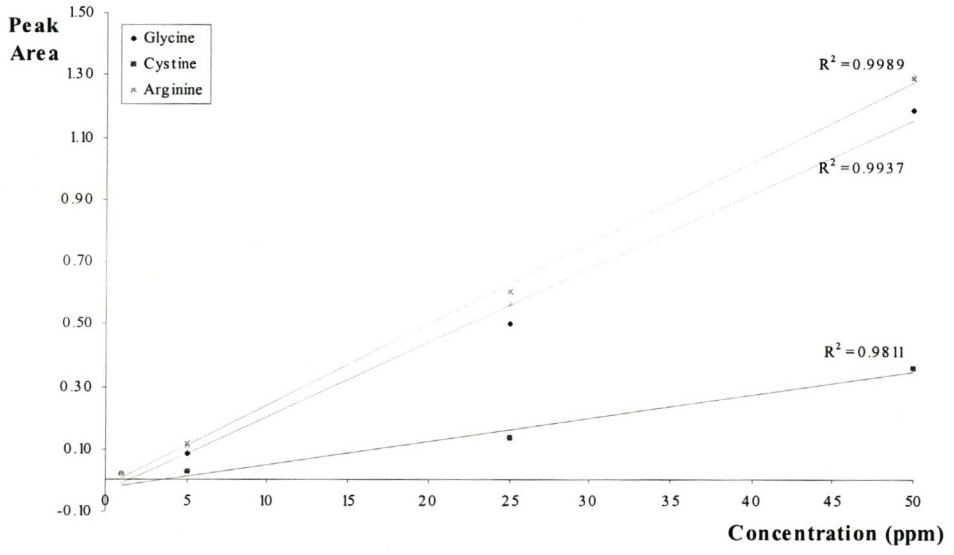


Figure 37: Calibration curves for Gly, Cy2 and Arg.

Amino acid	Range	R^2	Amino acid	Range	R^2
Asp	1-50 ppm	0.9958	Arg	1-50 ppm	0.9989
Glu	1-50 ppm	0.9991	Ala	1-50 ppm	0.9989
Met	1-50 ppm	0.9966	Tyr	1-50 ppm	0.9325
Cys	1-50 ppm	0.9858	Val	1-50 ppm	0.9995
Asn	1-50 ppm	0.9571	Trp	1-50 ppm	0.9791
Ser	1-50 ppm	0.9616	Phe	1-50 ppm	0.9836
Gln	1-50 ppm	0.9650	Ile	1-50 ppm	0.9829
His	1-50 ppm	0.9484	Leu	1-50 ppm	0.9595
Gly	1-50 ppm	0.9937	Pro	20-1000 ppm	0.8469
Cy2	1-50 ppm	0.9811	Lys	1-50 ppm	0.9990
Thr	1-50 ppm	0.9958	Hyp	20-1000 ppm	0.8579

Table 11: Calibration details for the amino acids.

Note the poor correlation coefficients for proline and hydroxyproline. These are due to saturation of the fluorescence detector at the high concentration values used for their calibration and can be improved by limiting the range.

4.3.4 Conclusion

A HPLC method for the determination of primary and secondary amino acids as well as cysteine after derivitisation with OPA, FMOC and IDA has been optimised. Optimum results were obtained on a LUNA reversed phase column. The mobile phase was optimised in terms of buffering reagent, ionic strength, pH, and organic modifier content. UV and flourescence detection were compared, with the latter proving more effective. Initial experiments demonstrating the compatibility of the method with electrospray-ionisation and MS detection have been performed.

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2. Product Declaration 092304/1, Fluka Chemie AG, Switzerland
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Chapter 5. Analysis of wine samples

5.1 Introduction

The optimised HPLC method for the determination of amino acids, discussed in Chapter 4, was now evaluated for the determination of the amino acid content of South African wines.

5.2 Experimental

The experimental part has been outlined in paragraph 4.3.2. This discussion is limited to the treatment of wine samples only. Wine samples were kindly supplied by KWV or bought from local stores. The selection included 18 wines of varying quality (seven Cabernet Sauvignon, four Chardonnay, one Sauvignon Blanc and six red blends).

A table summarising the wine properties, including origin and vintage is displayed in Table 12:

	Wine	Year	Blend	Origin
1	Cabernet '88	1998	no	Stellenbosch
2	Cabernet '92	1992	no	Stellenbosch
3	Cabernet '94	1994	no	Stellenbosch
4	Cabernet '95	1995	no	Stellenbosch
5	Cabernet '96	1996	no	Stellenbosch
6	Cabernet '97	1997	no	Stellenbosch
7	Cabernet '98	1998	no	Stellenbosch
8	Red Blend	1997	yes	Stellenbosch
9	Red Blend	1998	yes	Stellenbosch
10	Red Blend	1999	yes	Stellenbosch
11	Red Blend	2000	yes	Stellenbosch
12	Red Blend	2002	yes	Tulbagh
13	Red Blend	2002	yes	Boland
14	Chardonnay	1999	no	Stellenbosch
15	Chardonnay	2000	no	Stellenbosch
16	Chardonnay	2001	no	Stellenbosch
17	Chardonnay	2002	no	Robertson
18	Sauvignon	2002	no	Robertson

Table 12: Samples used in the wine analysis

Although the amino acids themselves are relatively stable in wine after fermentation, it is necessary to store the wines in an inert, cool environment with limited oxygen contact, in order to ensure an unchanged chemical profile of the wine. This also means that for each new analysis, the wine samples should be taken from a freshly opened wine bottle or from well-stored samples. The wine samples were transferred to smaller bottles under nitrogen in the following way: smaller 50 ml and 100 ml amber bottles were filled with liquid nitrogen. The wine bottle was then uncorked and the wine displaced by nitrogen pressure (see Figure 38). The amber storage bottles were filled to overflow before sealing with screw caps, in order to minimise contact with atmospheric oxygen. These samples were then kept in the refrigerator at 4°C until analysis.

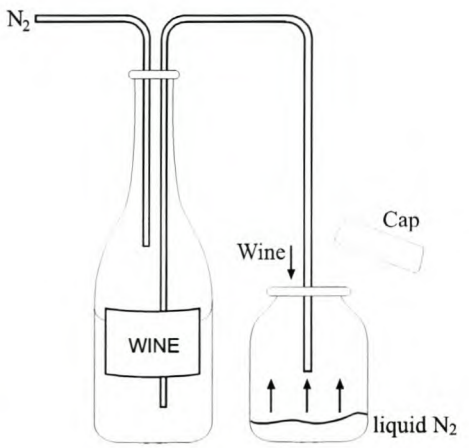

Figure 38: Schematic diagram of the transfer of wines

5.3 Results and discussion

The determined concentrations of 21 amino acids in 18 South African wines are summarised in Table A1 in the appendix. The chromatograms of these wine samples can also be seen in the appendix (Figures A1-A18).

5.3.1 Qualitative profiles

By comparing the red and white wine chromatograms and resulting concentrations, it can be seen that there is a definite difference in the amino acid profiles. As an

example a comparison between amino acid content of a red (sample 7) and a white wine (sample 14) of similar vintage and region is shown in Figure 39.

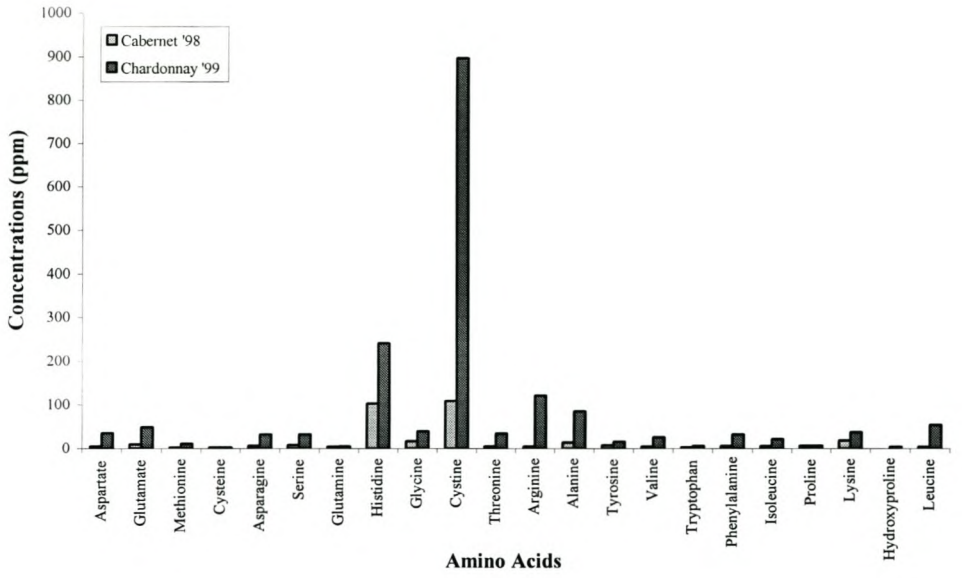

Figure 39: Comparison between amino acid content of red and white wines.

It is noted that white wines generally have higher concentrations of amino acids than the red wines. This may be due to the fermentation techniques that are used for red and white wines. Temperatures are higher during fermentation with red wine than white wine. During white wine fermentation, temperatures are kept low so as preserve CO_2, freshness and primary fruit aromas. This increase in temperature may boost the fermentation rate in red wines, thus utilising more amino acids than in white wines, which would account for the lower levels of amino acids in red wines.

5.3.2 *Quantitative data*

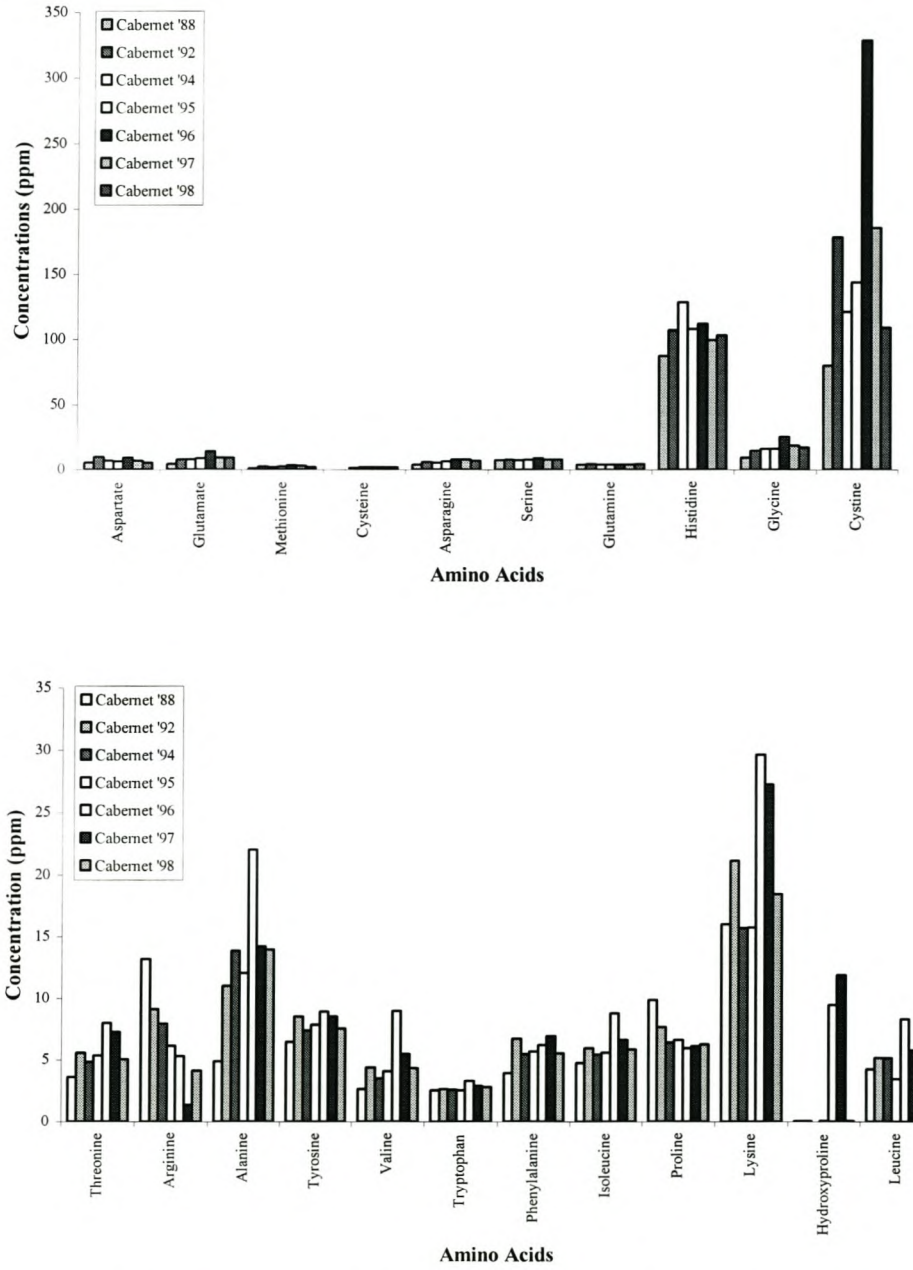

Figure 40: General trend of Cabernet Amino acid concentrations over a time span of ten years.

As can be seen from Figures 40, the general trend of the amino acid concentrations is a slight increase from 1988 to 1995/1996. After that the concentrations tend to diminish again to 1998. There are, however, exceptions, such as the absence of hydroxyproline before 1996 and after 1997. Also the levels of arginine and proline are

greatest in 1988. Also, histidine has its highest levels in 1994 and cysteine and glycine are highest in 1996.

The difference between the Cabernet Sauvignon series and the blended red wines are shown the Figure 41. It was decided to compare wines of the same age, thus the cabernet and blended wines of the years '97 - '99 were chosen.

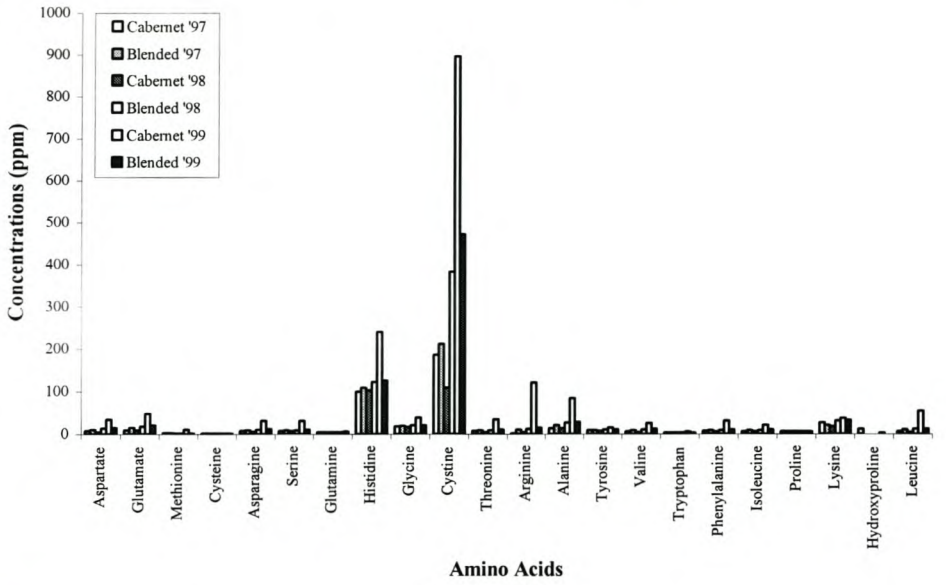

Figure 41: Comparison between Cabernet wines and blended red wines

If the peaks of cystine and histidine are removed, it is possible to see a more accurate trend of the amino acid concentrations as shown in Figure 42.

It can be seen from the figures that the blended wines tend to have higher concentrations of amino acids than the cabernet wines, with the exception of the 1999 cabernet, which has greater amino acid levels than the corresponding blended wine. The concentration of amino acids of the Cabernet '99 is consistently higher than any of the other wines in the series, which are more or less of a same amount.

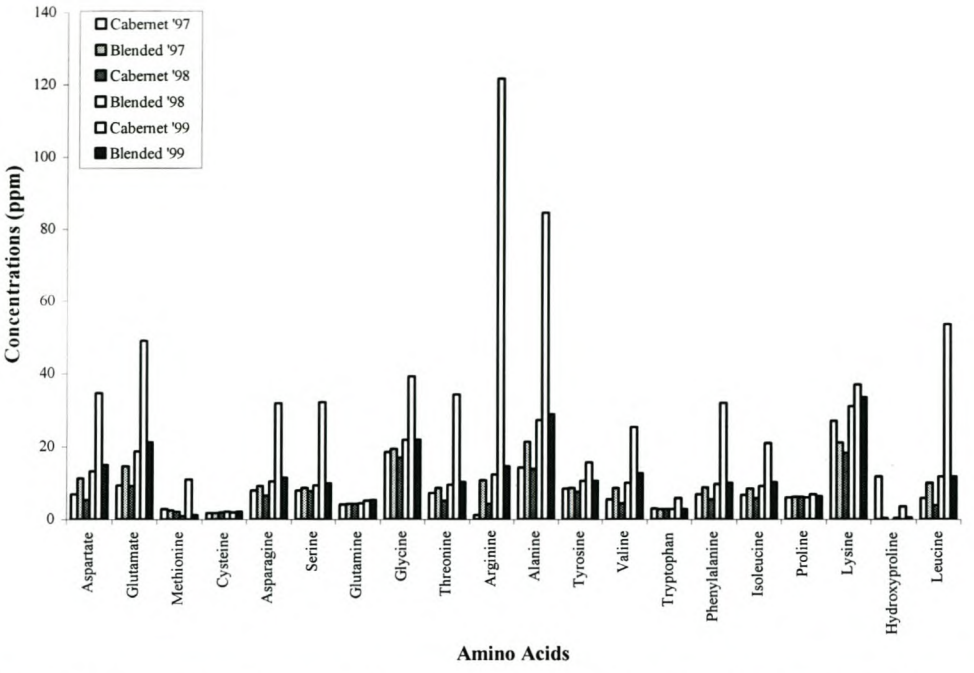

Figure 42: Comparison between Cabernet wines and blended red wines; excluding cystine and histidine

Comparison was made between the "good" and "poor" quality wines. The poor quality red wines are all blended and will therefore be compared with the blended red wines of good quality. The ages will be kept as close as possible. The Chardonnay wines will be compared, keeping the wines as close in age as possible as well. The results are shown in Figures 43 and 44.

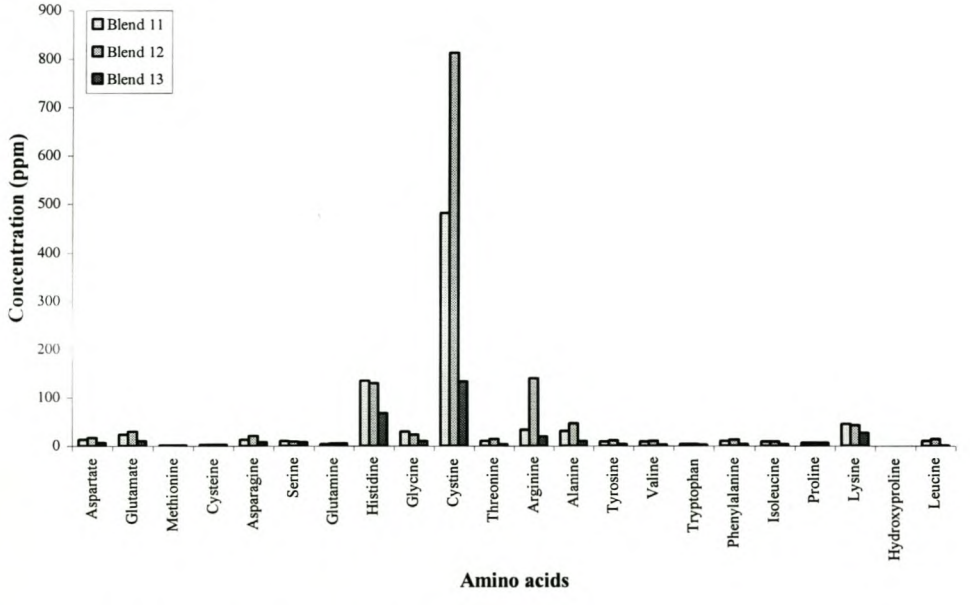

Figure 43: Comparison between the red wine blends.

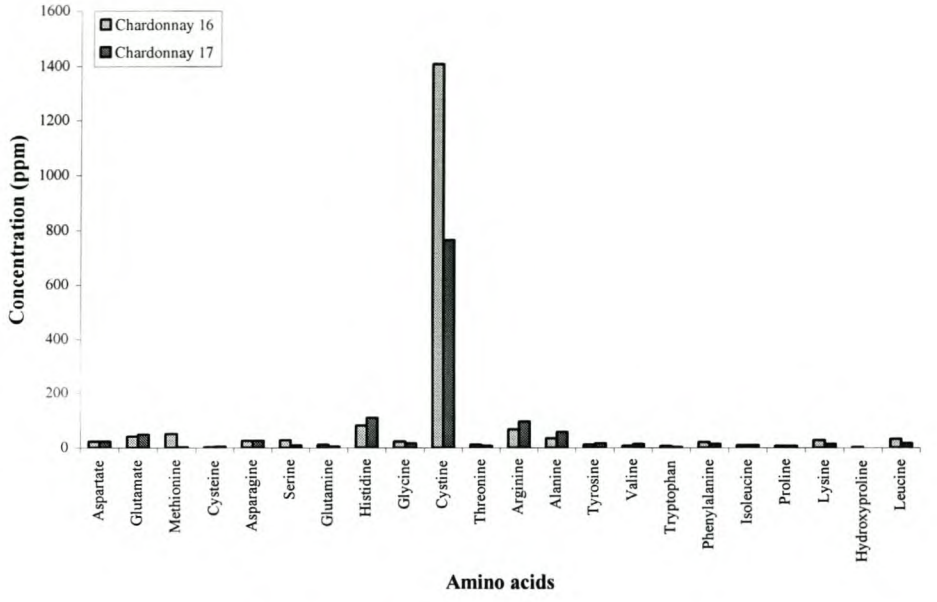

Figure 44: Comparison of a good quality chardonnay and a poor quality chardonnay.

There does not seem to be any trend visible with regard to the quality of the wines. This may be due to the different geographical origin of the wines. This would include different climates and fermentation techniques.

Generally, it has been found that the concentrations of proline are lower than expected from the literature. It is also observed that the cystine levels of all the wines are particularly high. This could be due to the fact that all wines noted in the literature have been of European origin as opposed to the South African wines analysed in this study. The different climate of South Africa, and particularly the Western Cape, may influence the original amino acid concentrations in the grapes prior to fermentation. Also, wine-making practices may lead to different concentrations of the amino acids.

5.3.3 Introduction to chemometric evaluation

The analysis of amino acids in wines presented in this thesis is part of a project where the aim is to differentiate between the wines samples based on their chemical content. With this in mind, multivariate techniques were applied to the results discussed above, in order to assess the possibility of differentiation between these wines based on their amino acid content.

PCA and HCA were performed using the STATISTICA software package for Windows, version 6 (Statsoft, Inc.). HCA was performed on the standardised data matrix using the single linkage method based on Euclidean distances. PCA was performed on the correlation matrix.

Figure 45 depicts the joining tree diagram for the amino acid data. Small linkage distances between wines can be interpreted as indicating a relatively high similarity. It is clear that the red wines cluster together and are easily distinguishable from the whites, demonstrating the (expected) inherent grouping in the amino acid data. Within these two main clusters, however, differentiation between the wines is not clear. It can be noted that the white wines, although separated from the reds, do not display any great degree of clustering. Especially the 3 Chardonnay varietal vintages (wine 14,15 and 16) appear to be very dissimilar in amino acid content.

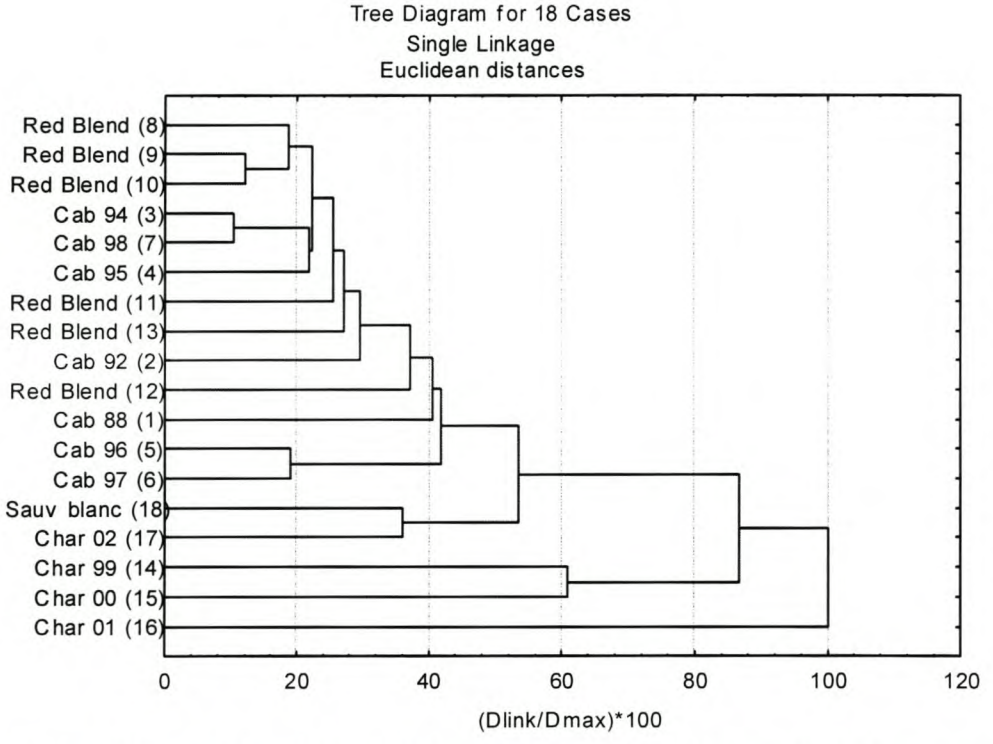

Figure 45: Single linkage hierarchical clustering dendrogram for 18 wines obtained from amino acid data.

In Figure 46 the loadings plot for the variables is depicted. From this it is evident that most of the amino acids have high negative loadings on PC 1. This means that these

groups of variables are highly correlated with this axis, and can be considered to maximally contribute to the variance explained by this component.

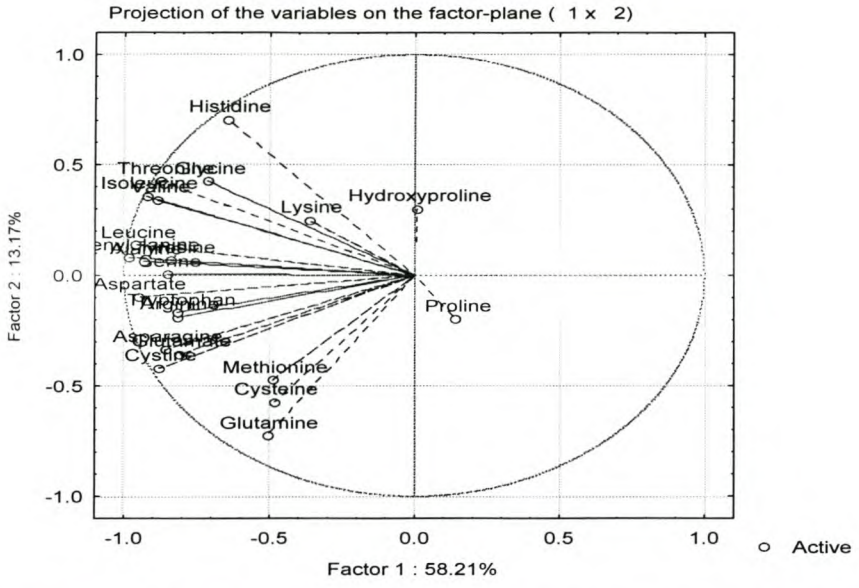

Figure 46: Loadings plot for the first 2 PCs, obtained from the amino acid data on 18 wines.

The scores plot of the first 2 PCs, accounting for 71.4% of the total variance, is presented in Figure 47. From this it is clear that red and white wines are easily separated by PC 1, with the latter having high negative factor coordinates. Thus, the fact that the white wines generally have higher amounts of amino acids can be seen to be responsible for their differentiation from red wines on PC 1. Once again, significant grouping of the different white wines is not evident. In the case of red wines, however, at least 2 groups can be distinguished – the 4 red blend vintages of good quality wines (samples 8,9,10,11) are grouped and separated from the 7 Cabernet sauvignon wines (samples 1,2,3,4,5,6,7). The wines of low quality are scattered all over the plot. Hence different vintages of the good quality wines appear fairly constant in their amino acid content. The close grouping of the Cabernet varietals suggests that a distinction between the different types of grapes can be made from this type of analysis.

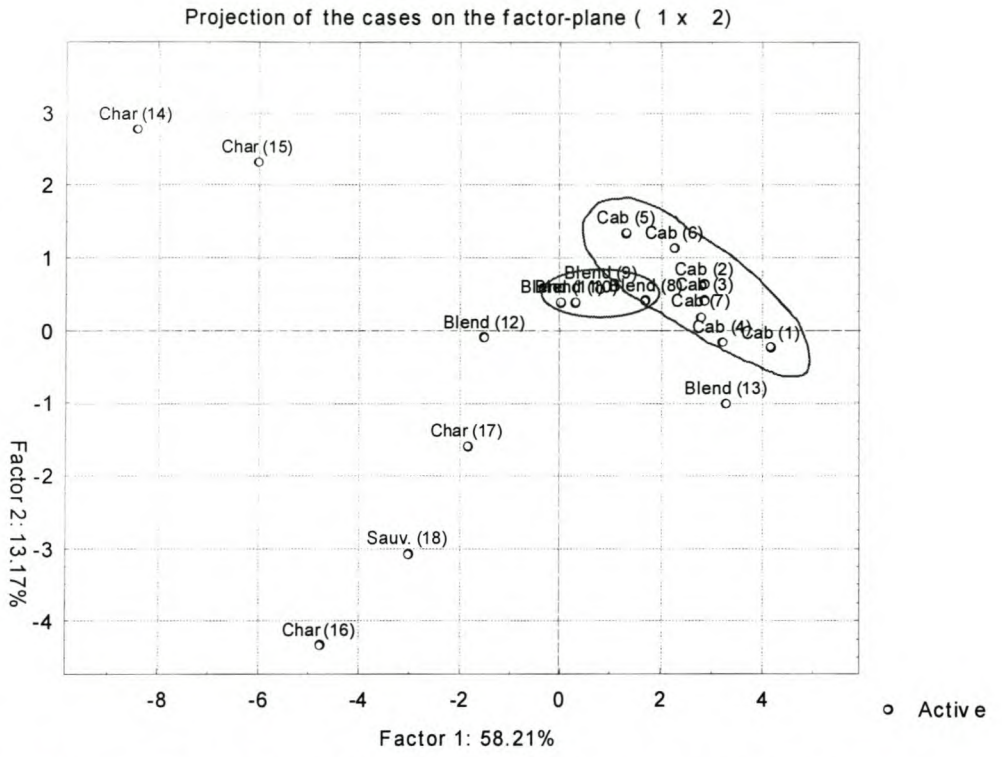

Figure 47: Scores plot for PC 1 vs. PC 2, obtained from the amino acid data on 18 wines.

It has to be noted that in the chemometric study discussed above a limited number of wine samples were used. However, the exploratory results appear promising, and the method established for the analysis of amino acids in wine can now be used to extend the data-base on these compounds. Further multivariate analysis of these data, as well as other chemical data obtained on the same wines, can then be used to improve the classification of the wines.

5.4 Conclusion

The developed method has been shown to be suitable for the analysis of amino acids in wine. 18 South African wines were analysed, and certain conclusions could be made based on their amino acid content. White wines consistently contain higher amounts of the amino acids compared to red wines. Mostly, blended red wines contain more amino acids than Cabernet Sauvignon wines. No distinction between high and low quality wines of the same cultivar could be made based on the amino acid content. Exploratory chemometrical analysis of the data shown promising results in terms of differentiating between single-cultivar wines.

General conclusions

The aim of this study was to provide a robust qualitative and quantitative method for the analysis of amino acids in wine. The robustness of the system has been found to be acceptable. While the sensitivity of the method (using flourescence detection) is more than adequate, the limited linear range for some of the amino acids does limit the overall usefulness of the method.

The effectiveness of the developed derivatisation technique can be seen in its simplicity and short reaction time. It is now possible to analyse all of the natural amino acids, including cysteine, proline and hydroxyproline.

The use of this technique for the analysis of wines has been demonstrated. With the proposed procedure many analyses can be performed without problems concerning reproducibility and sensitivity. Although no definite trend can be noted with regards to different wines from the quantitative data, as such, it could be shown that by chemometrical evaluation of the results a fairly good distinction between the different types of wines and grapes could be made.

The necessity of using both a UV and FL detector in this analysis has been described. Without the use of both detectors, it would not be possible to detect and quantify all of the amino acids. The fluorescence detector greatly enhances sensitivity, which is vital when dealing with low amino acid concentrations as are expected in wine samples. It is also essential to use the UV detector in order to quantitate leucine, which would otherwise co-elute with proline.

It is also observed that due to the nature of this method, further development is possible with regards to the incorporation of mass spectrometry. The mobile phase is volatile and therefore will not interfere with the analysis. It is hoped that this will be developed further in the near future.

APPENDIX

Wine	Cabernet Sauvignon							Blend					
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Aspartate	5.3	9.8	6.7	6.6	9.1	6.9	5.3	11.3	13.3	15	13.5	17.6	6.1
Glutamate	4.5	7.8	7.6	9	14.4	9.3	9.2	14.6	18.7	21.2	23.6	30.0	10.9
Methionine	1	2.6	2.1	2.3	3.2	2.8	2.1	2.5	0.9	1.2	1.1	1.4	0.7
Cysteine			1.7	1.8	1.9	1.8	2	1.8	2.1	2.1	2.7	2.6	2.8
Asparagine	4	6	5.5	6.4	7.7	8	6.6	9.2	10.4	11.5	12.8	20.7	8.3
Serine	7.5	7.7	7.6	7.7	8.6	8	7.8	8.7	9.4	9.9	10	9.1	7.9
Glutamine	4	4.2	4.1	4.1	4.1	4	4.2	4.2	4.5	5.4	4.5	4.7	4.9
Histidine	87.7	107.1	128.6	108.3	111.9	99.6	103.4	108.7	122.3	125.6	134.8	130.0	68.4
Glycine	9.5	14.5	15.9	16	25.3	18.5	17	19.4	21.9	21.9	30.2	23.1	10.0
Cystine	80.2	178.4	121.5	144.1	329.3	185.9	109	212.5	383.3	472.8	482.5	814.0	133.6
Threonine	3.6	5.6	4.8	5.3	8	7.3	5	8.6	9.6	10.2	10.6	14.4	4.1
Arginine	13.2	9.2	8	6.2	5.3	1.3	4.2	10.9	12.4	14.6	33.7	141.2	19.1
Alanine	4.9	11.1	13.9	12.1	22.1	14.3	14	21.4	27.4	28.9	31.9	46.7	10.5
Tyrosine	6.4	8.5	7.4	7.9	9	8.5	7.5	8.7	10.5	10.5	9.4	12.4	4.5
Valine	2.6	4.4	3.5	4.1	9	5.5	4.3	8.6	10.1	12.6	9.9	11.0	2.7
Tryptophan	2.5	2.6	2.6	2.5	3.3	2.9	2.8	2.8	2.9	2.9	4	3.3	2.6
Phenylalanine	3.9	6.7	5.5	5.7	6.2	6.9	5.5	8.8	9.7	10.1	10.3	12.7	4.2
Isoleucine	4.7	5.9	5.4	5.6	8.8	6.6	5.9	8.5	9.2	10.2	8.8	9.7	4.5
Proline	9.9	7.6	6.4	6.6	5.9	6.1	6.2	6.2	6	6.4	7	6.6	6.9
Lysine	16	21.1	15.7	15.7	29.6	27.2	18.4	21.1	31.3	33.7	46.2	43.9	27.4
Hydroxyproline	0	0.1	0	0	9.4	11.9	0	0.3	0.3	0.5	0.1	0.4	0.00
Leucine	4.2	5.1	5.2	3.4	8.3	5.8	3.8	10	11.8	11.8	10.6	14.5	1.0

Table A1: Summary of concentrations of amino acids in the wine samples (in mg/L)

Wine	Chardonnay				Sauvignon Blanc
	14	**15**	**16**	**18**	**17**
Aspartate	34.8	22.7	22.6	25.9	24.0
Glutamate	49.1	44.1	41	81.1	49.7
Methionine	11	8.7	51.7	1.0	1.3
Cysteine	2	2	3.4	4.3	3.7
Asparagine	32	14.3	24.7	30.9	25.0
Serine	32.3	18.5	27.9	9.6	9.4
Glutamine	5.2	4.9	12.5	7.2	4.8
Histidine	240.9	241	82.8	68.0	109.4
Glycine	39.3	31.3	23.3	19.7	16.9
Cystine	897.5	785.1	1408.7	918.5	764.3
Threonine	34.4	21.8	11.5	8.5	8.0
Arginine	121.8	109.5	68.7	140.3	96.0
Alanine	84.6	71.5	34.9	65.6	59.2
Tyrosine	15.7	24.5	12.1	16.5	16.8
Valine	25.3	26.3	7.5	14.2	13.9
Tryptophan	5.7	6.3	8.1	3.3	3.2
Phenylalanine	32.1	28.5	21.8	16.5	14.7
Isoleucine	20.9	17.7	10.4	9.2	9.8
Proline	6.8	6.5	6.8	6.7	7.7
Lysine	37.3	25.1	28.8	18.2	14.5
Hydroxyproline	3.6	0.2	1.5	0.1	0.1
Leucine	53.9	38.8	33.4	16.5	17.0

Table A1 cont: Summary of concentrations of amino acids in the wine samples (in mg/L)

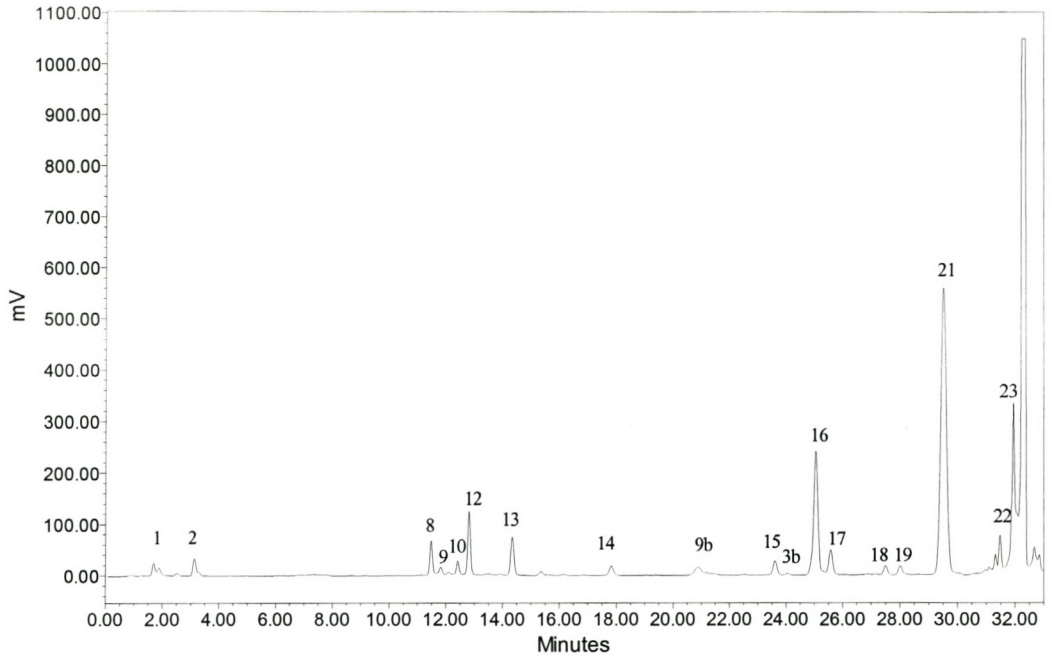

Figure A1: Wine sample 1 = 1988 Cabernet Sauvignon.

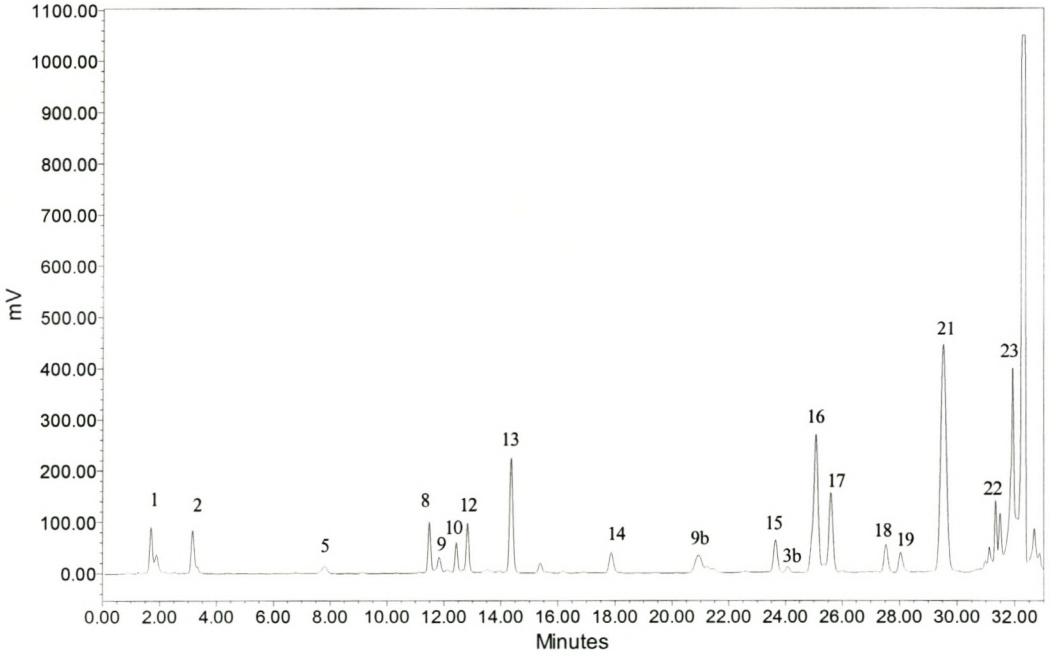

Figure A2: Wine sample 2 = 1992 Cabernet Sauvignon.

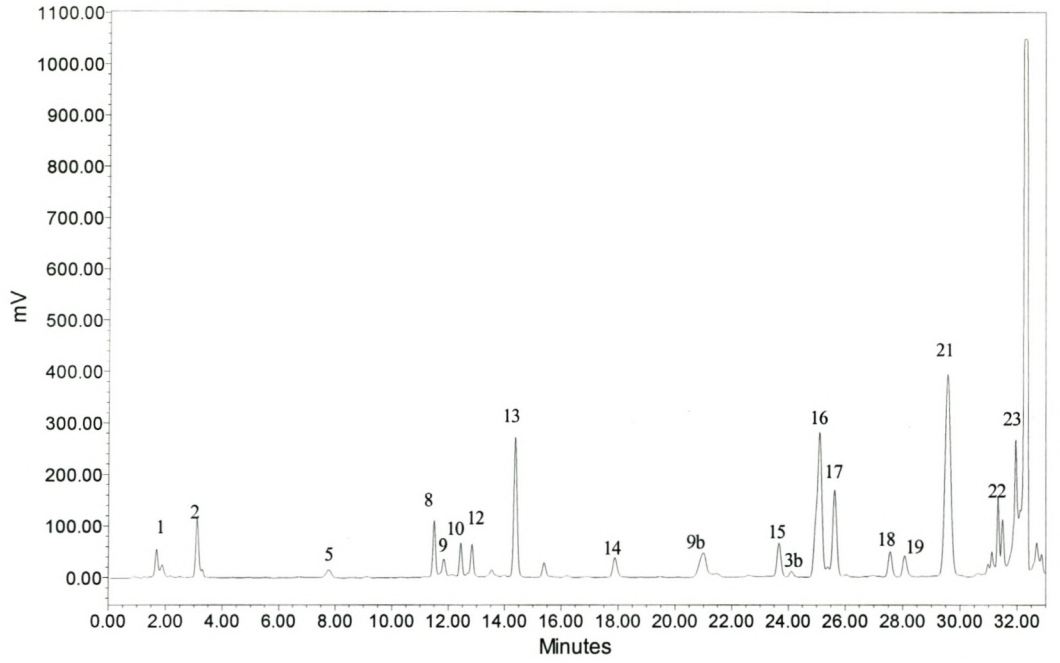

Figure A3: Wine Sample 3 = 1994 Cabernet Sauvignon.

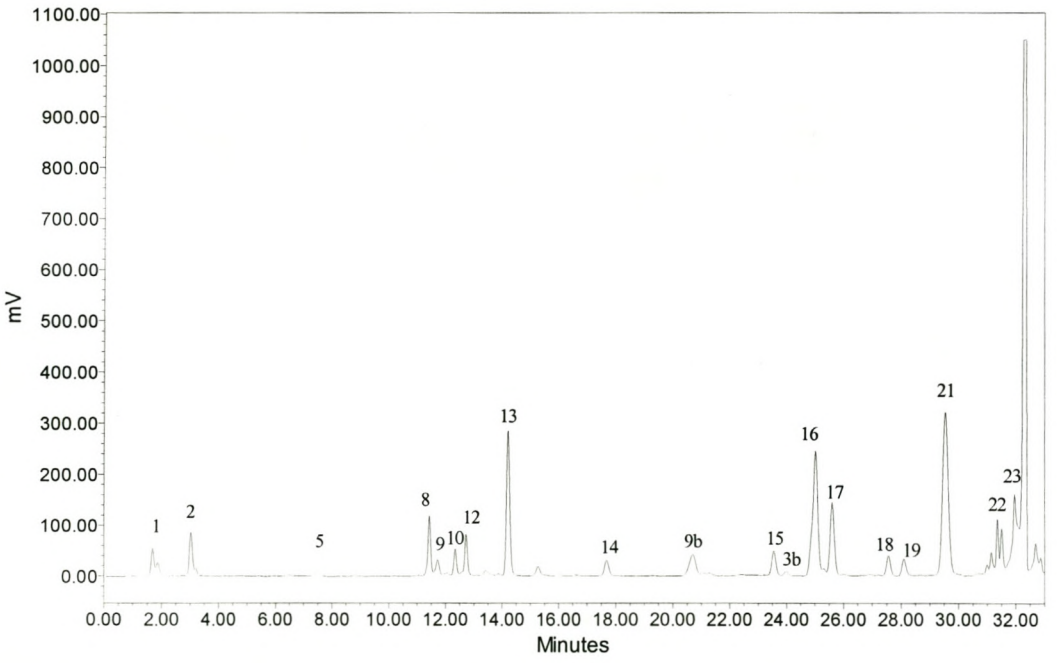

Figure A4: Wine Sample 4 = 1995 Cabernet Sauvignon.

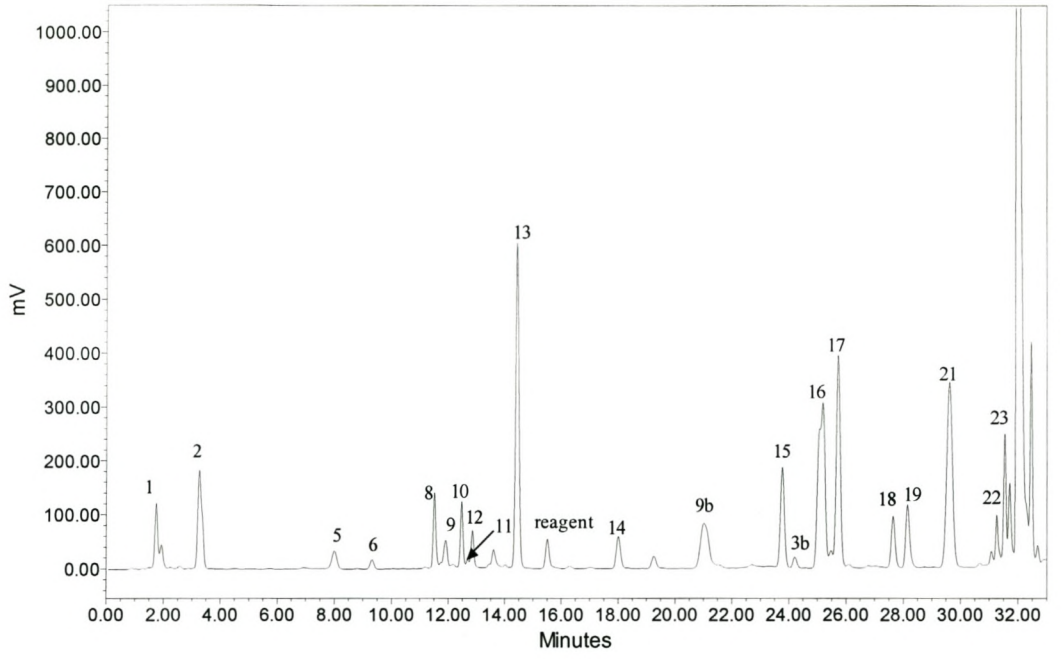

Figure A5: Wine sample 5 = 1996 Cabernet Sauvignon.

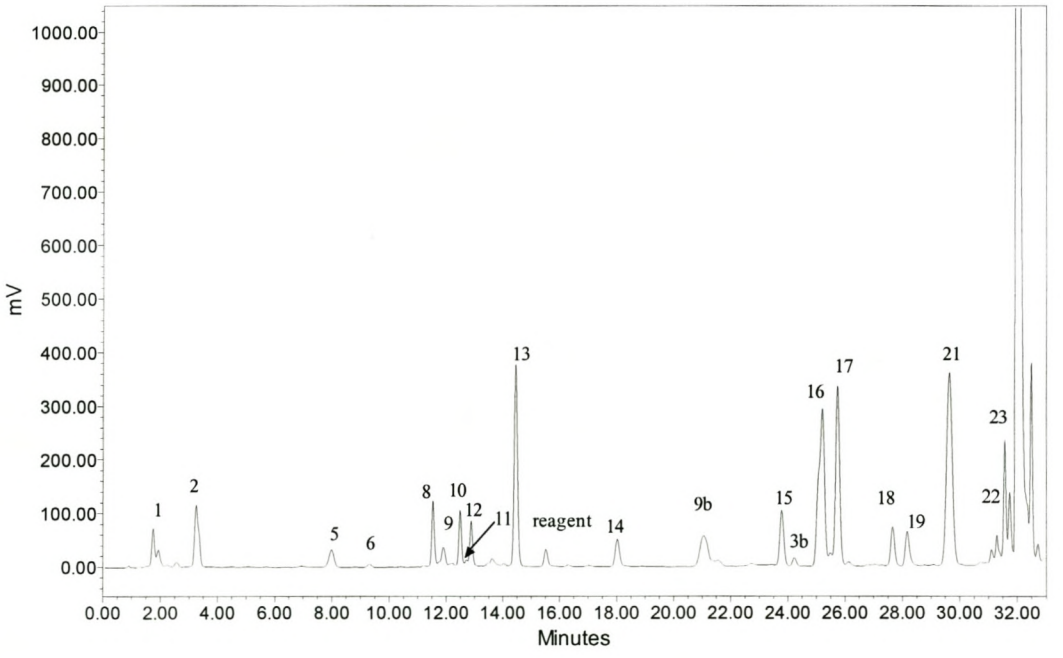

Figure A6: Wine sample 6 = 1997 Cabernet Sauvignon.

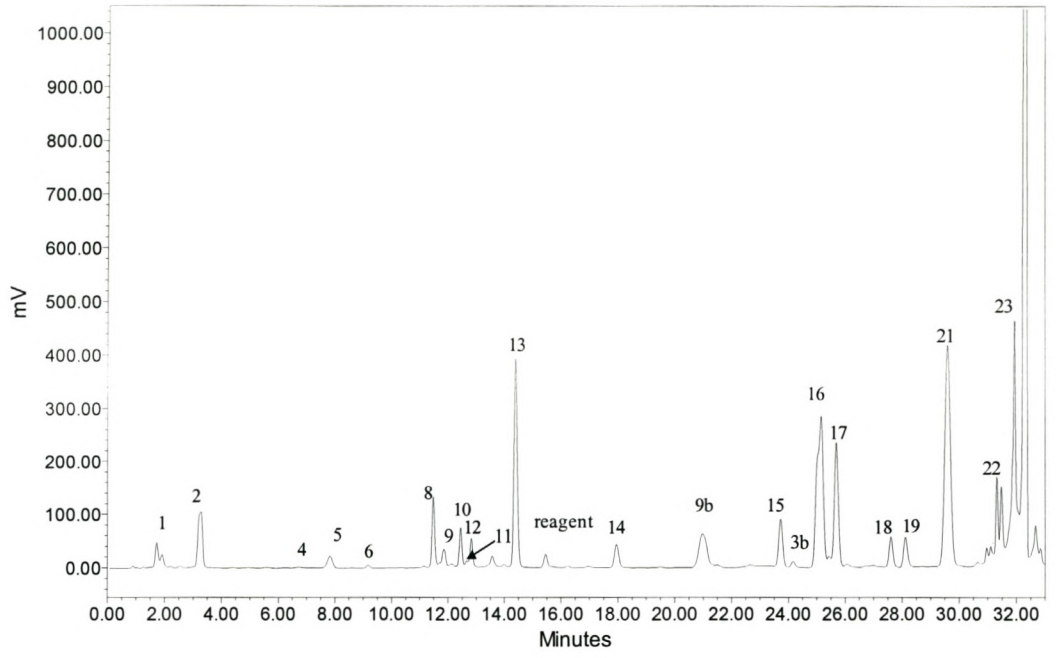

Figure A7: Wine Sample 7 = 1998 Cabernet Sauvignon.

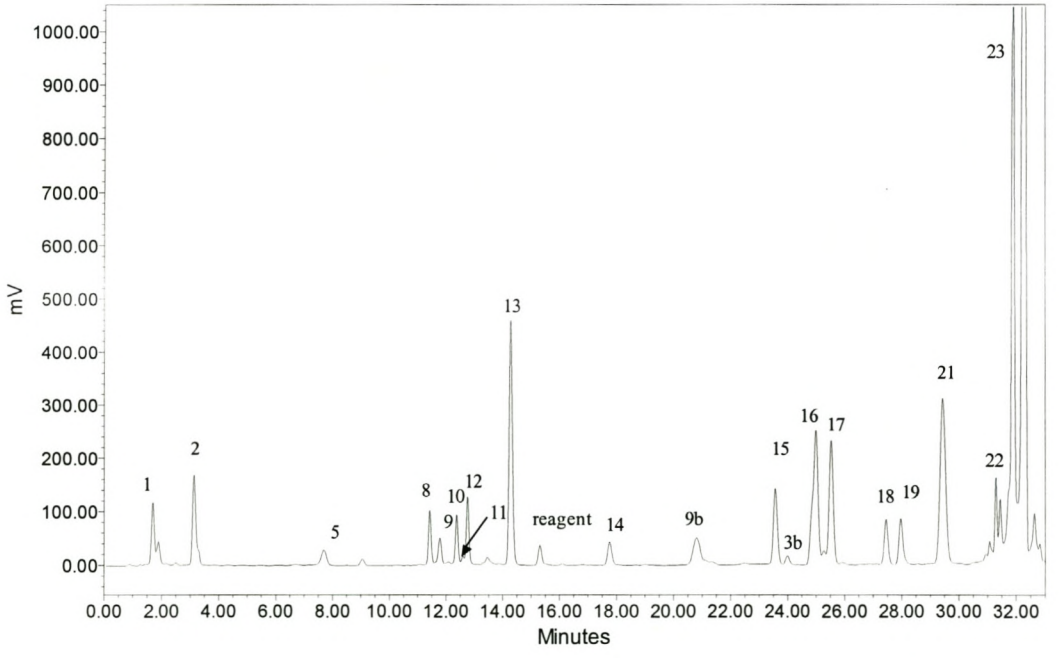

Figure A8: Wine Sample 8 = 1998 Red Wine Blend.

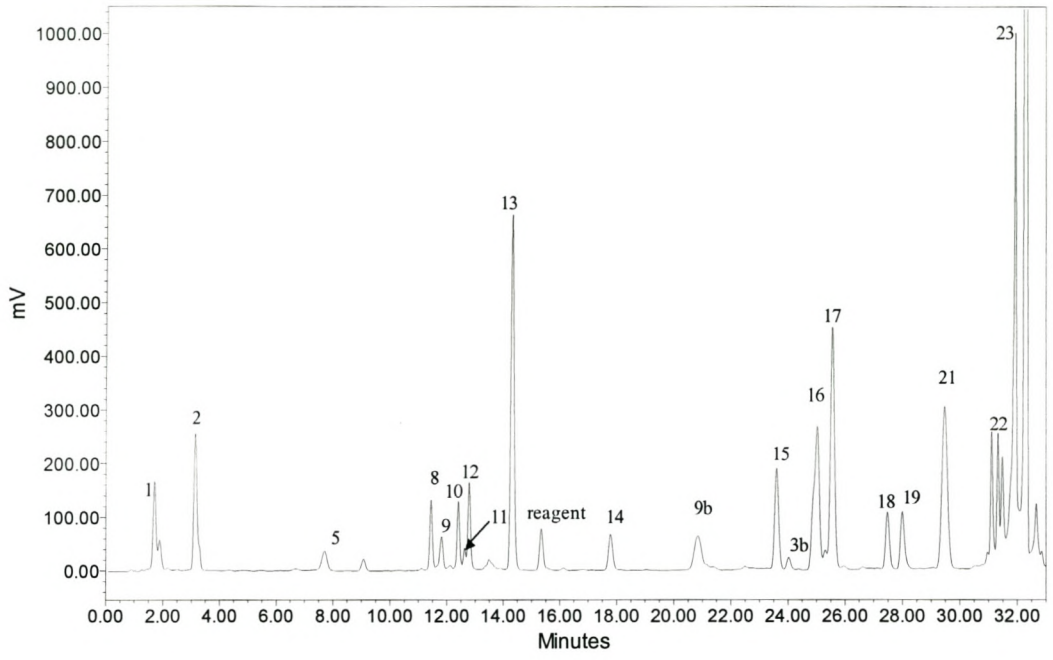

Figure A9: Wine sample 9 = 1998 Red Wine Blend.

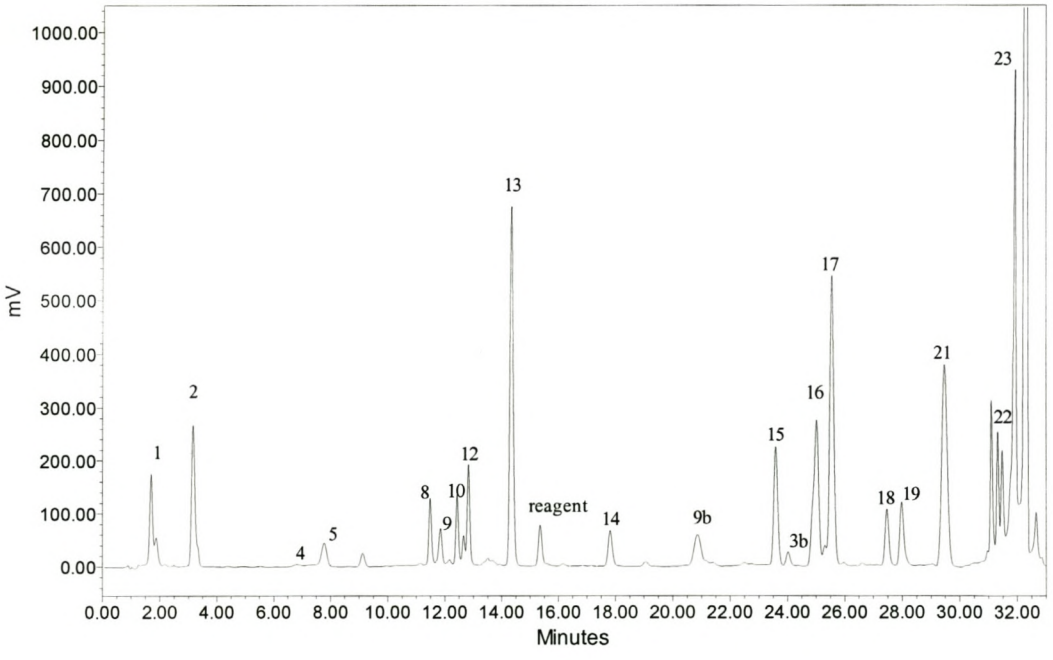

Figure A10: Wine sample 10 = 1999 Red Wine Blend.

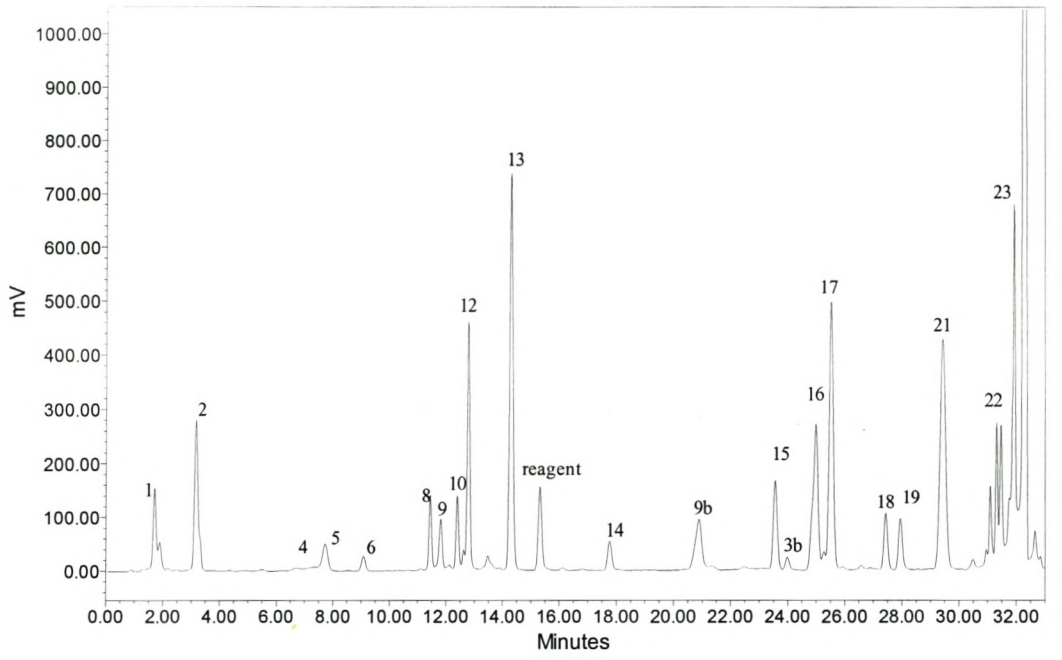

Figure A11: Wine Sample 11 = 2000 Red Wine Blend.

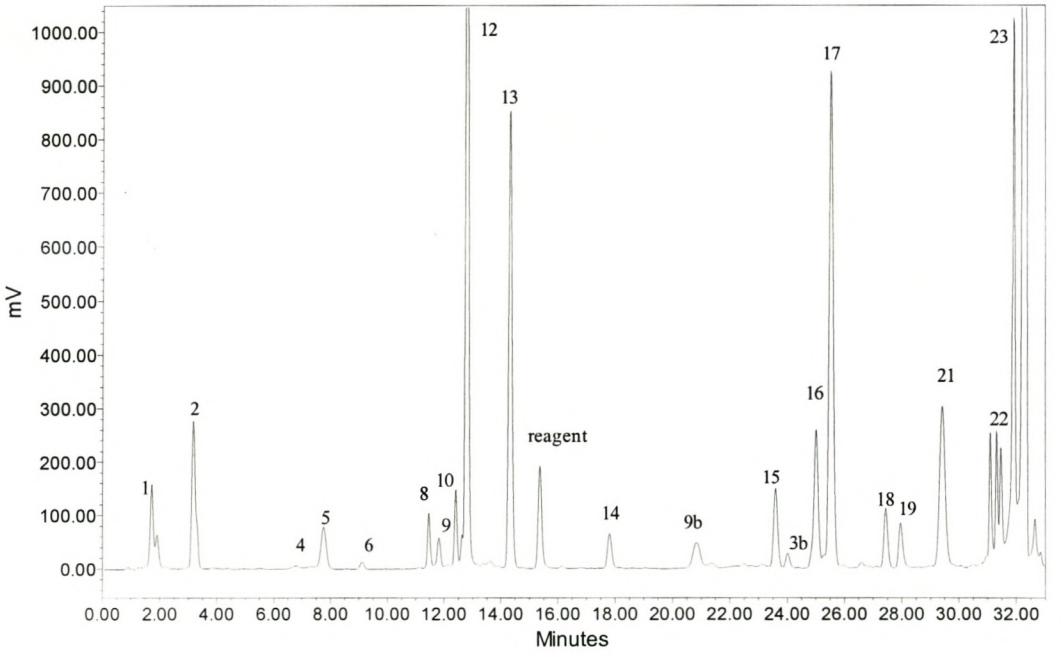

Figure A12: Wine Sample 12 = 2002 Red Wine Blend.

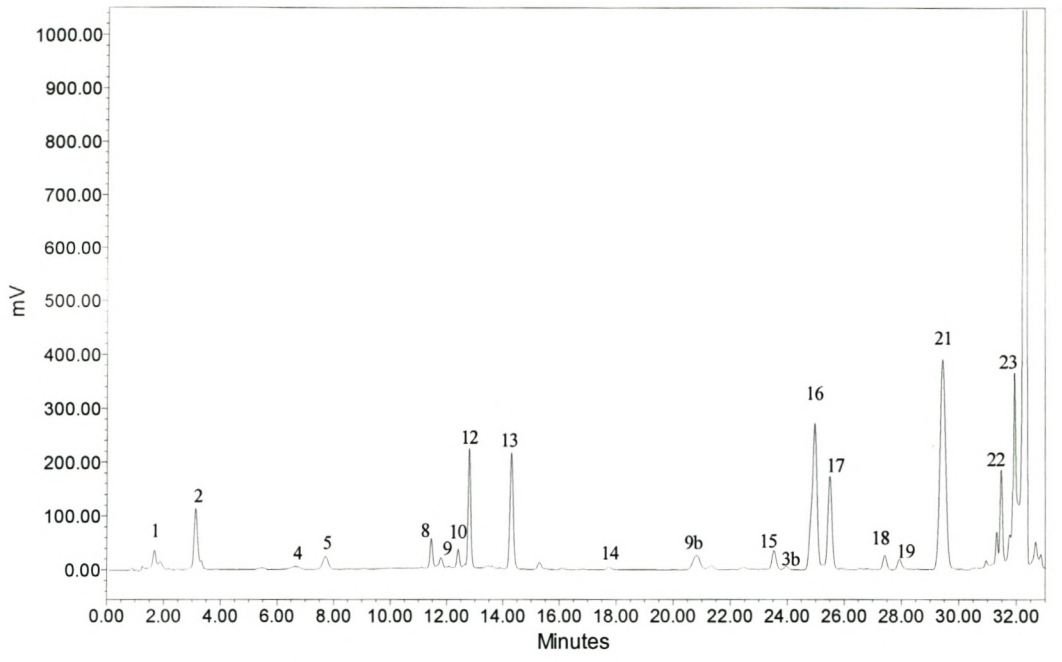

Figure A13: Wine sample 13 = 2002 Red Wine Blend.

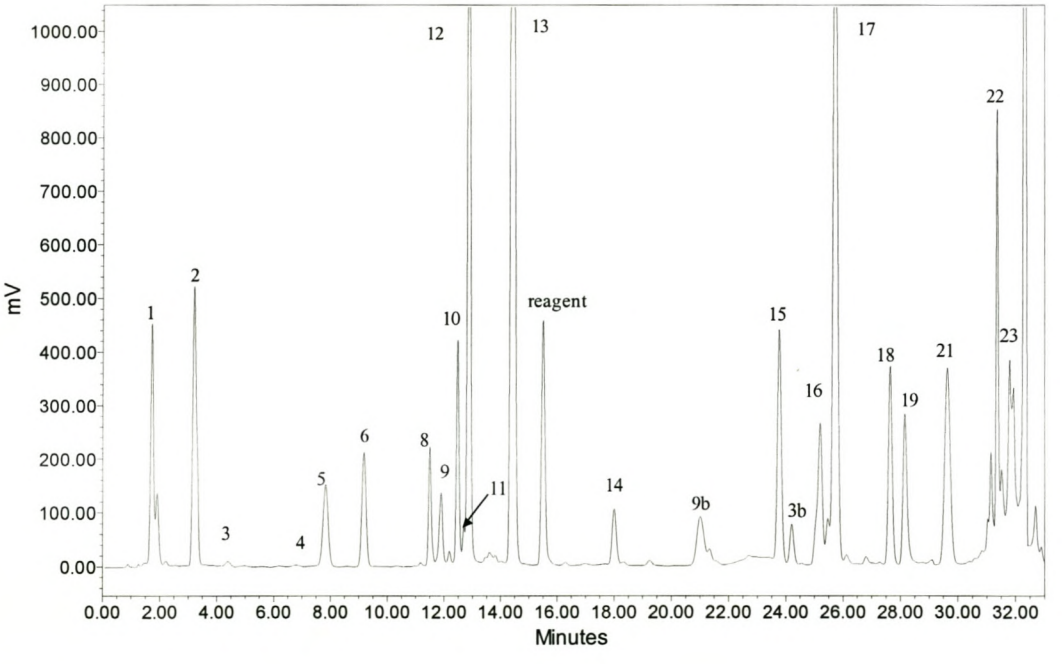

Figure A14: Wine sample 14 = 1999 Chardonnay.

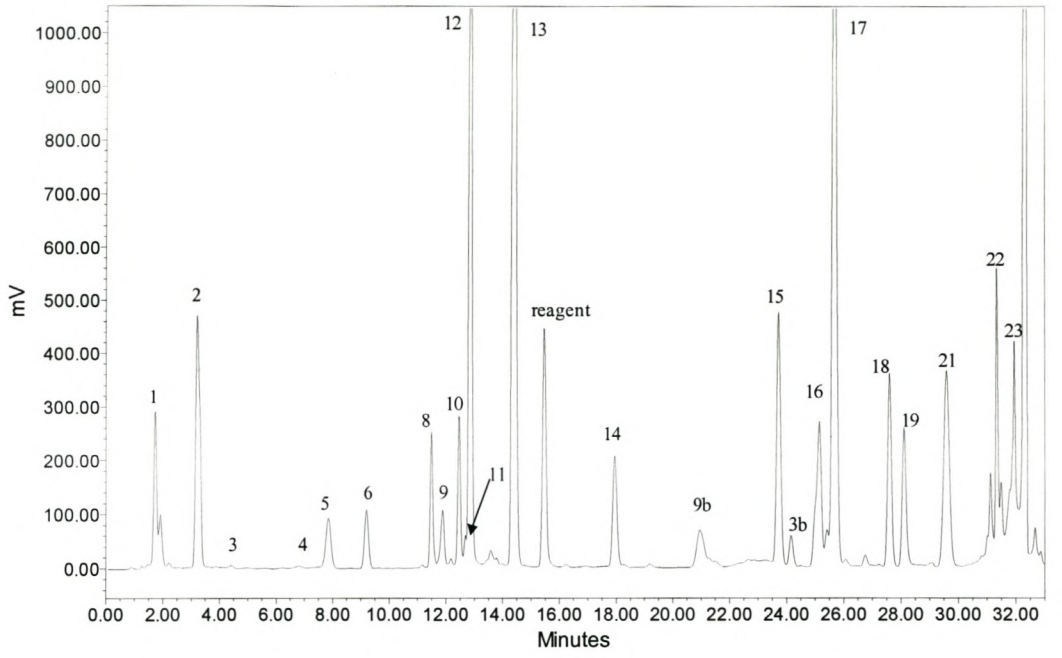

Figure A15: Wine Sample 15 = 2000 Chardonnay.

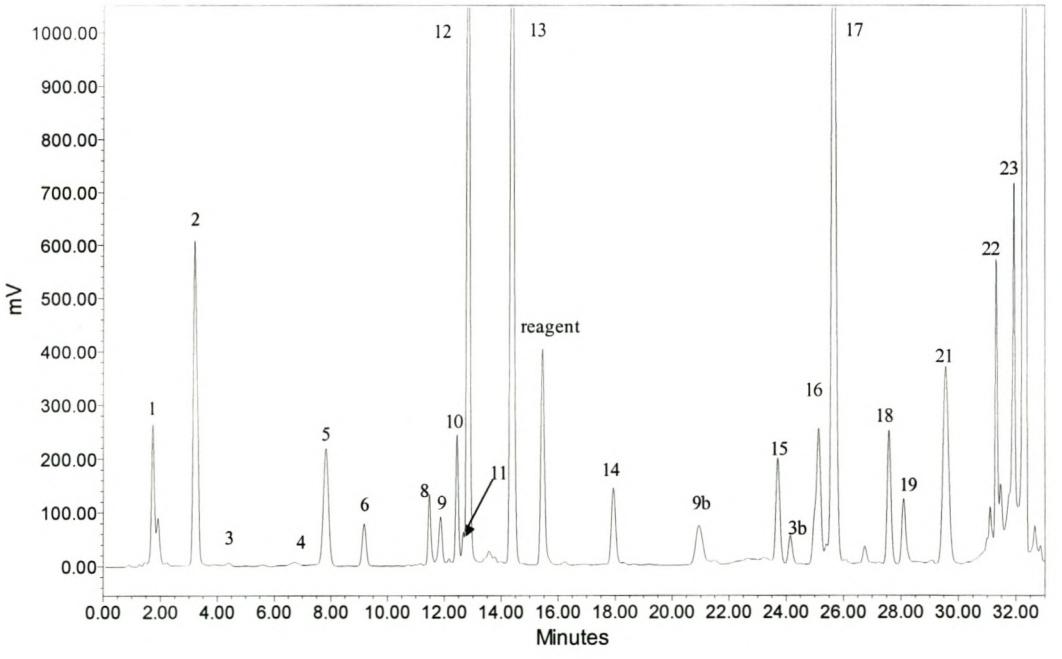

Figure A16: Wine Sample 16 = 2001 Chardonnay.

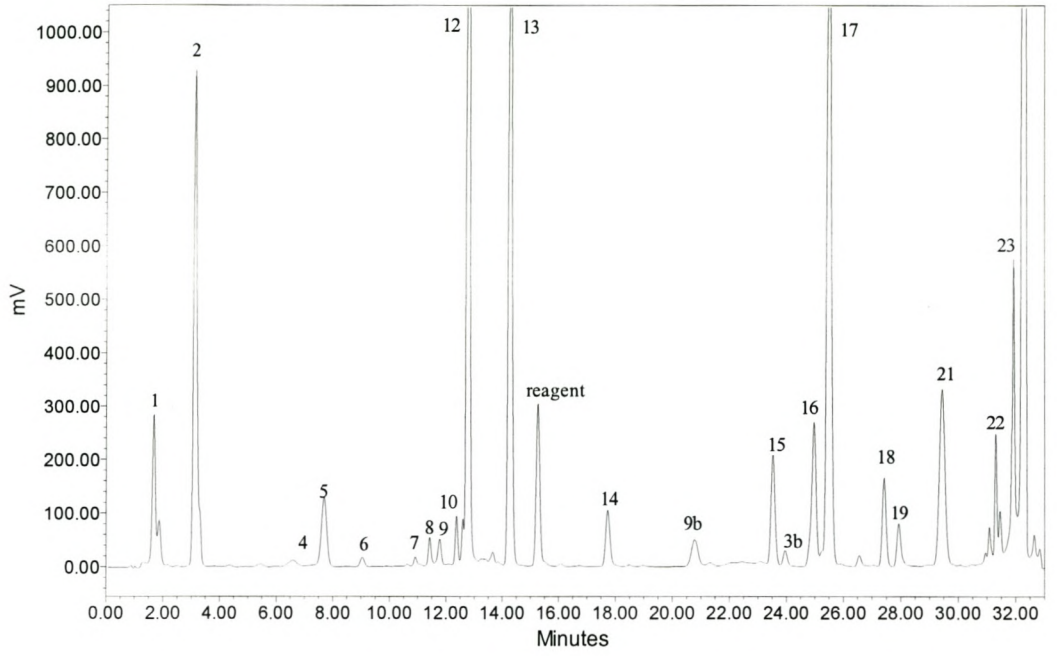

Figure A17: Wine Sample 17 = 2002 Chardonnay.

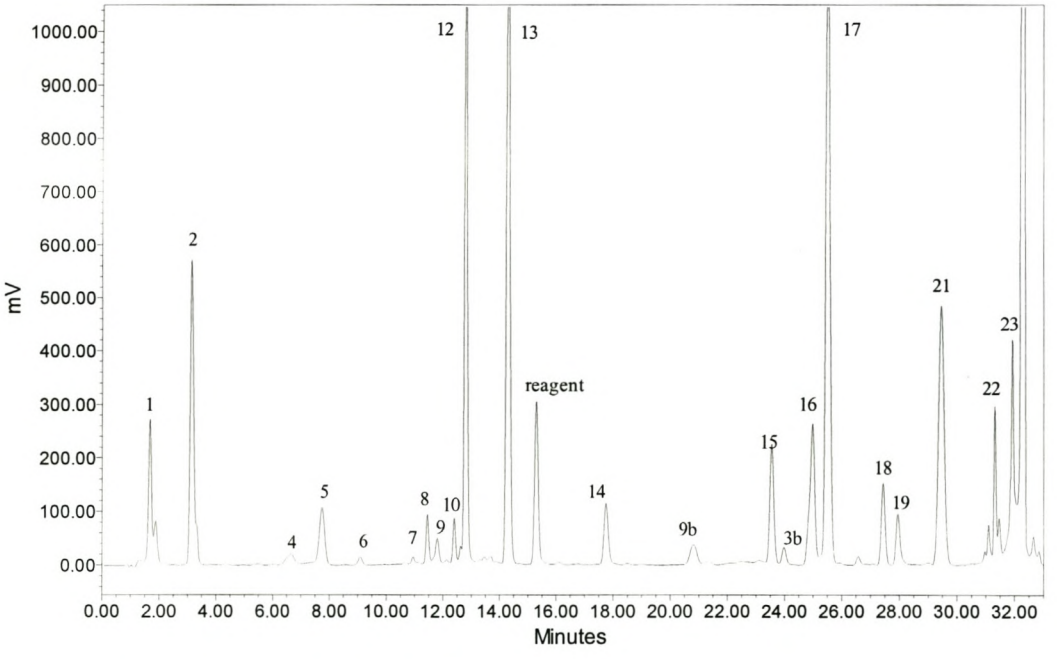

Figure A18: Wine Sample 18 = 2002 Sauvignon Blanc.