Contributions of mass spectrometry in the Australian Wine Research Institute to advances in knowledge of grape and wine constituents

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Abstract

Since 1971 mass spectrometry (MS) has made a significant contribution to wine research at the Australian Wine Research Institute (AWRI). In the past decade (1995–2004), MS has been involved in an expanded range of studies and now accounts for approximately 40% of AWRI publications appearing in peer-reviewed scientific journals. Studies involving MS include the analysis of grape-derived and fermentation-derived volatiles, oak volatiles, taint compounds, proteins, pigments and tannins. We discuss the contribution MS has made to wine research at the AWRI and the significant advances made by key scientists in this area. In particular, this review focuses on three main areas of analysis of compounds important to wine quality – volatile aroma and off-flavour compounds, involatile larger molecules such as proteins and tannins, and investigations into taint problems.

Abbreviations

4-EP 4-ethylphenol; **APCI** atmospheric pressure chemical ionisation; **CI** chemical ionisation; **ECD** electron capture detector; **EI** electron impact ionisation; **ESI** electrospray ionisation; **ESI-MS** electrospray ionisation mass spectrometry; **FID** flame ionisation detector; **GC** gas chromatography; **GC-MS** gas chromatography-mass spectrometry; **GC-sniff** GC-olfactory detection; **HPLC** high performance liquid chromatography; **LC-MS** high performance liquid chromatography-electrospray ionisation-mass spectrometry; **LC** liquid chromatography; **MALDI** matrix assisted laser desorption ionisation; **MLCCC** multi layer coil countercurrent chromatography; **MS** mass spectrometry; **MSD** mass selective detector; **MS/MS** tandem mass spectrometry; **PDA** photo-diode array detector; **PDMS** polydimethylsiloxane; **PR** pathogenesis related; **SBSE** stir bar sorptive extraction; **SIDA** stable isotope dilution analysis; **SPME** solid-phase microextraction; **TCA** 2,4,6-trichloroanisole; **TDN** 1,1,6-trimethyl-1,2-dihydronaphthalene; **TPB** (E)-1-(2,3,6-trimethylphenyl) buta-1,3-diene; **Trap-MS** protein trap-mass spectrometry

Keywords: *mass spectrometry, GC-MS, LC-MS, grape and wine aroma compounds, wine pigments, anthocyanins, grape and wine proteins, off-flavours, taint*

Introduction

The use of mass spectrometry (MS) for grape and wine research at the Australian Wine Research Institute (AWRI) started in 1971, when a specialised analytical apparatus, a gas chromatograph-mass spectrometer (GC-MS), was coupled for studies into the chemical composition of Australian brandies and fortified spirits. This system, one of the first in Australia, would have cost about $30 000, which translates to an investment of millions of dollars in today's terms. This commitment gives an indication of how important the AWRI considered mass spectrometry would be. Soon after, GC-MS was proven to be an excellent tool for aiding the wine industry in identifying off-aromas and promoting the advancement of better techniques in many aspects of wine and spirit production. In those days, the instruments were temperamental and fragile, clumsy to operate and difficult to maintain. With the operation and data processing relying much on the 'magic' hands of the MS experts, analytical productivity was low. Therefore, the MS and GC-MS techniques were complicated and time-consuming and thus more suitable for research investigations than routine analyses. Now, due to major improvements in versatility, sensitivity, specificity and applicability, the instruments have become much more robust, automated, reliable, upgradeable, affordable and user-friendly.

The importance of MS for research and development at the AWRI has also substantially increased along with the improvements and advances in mass spectrometry and its hyphenated techniques. Figure 1 is a timeline, showing the history of MS and related instrumentation in the AWRI from 1965 until 2004. The acquisition of each new instrument was followed by the development of novel analytical methodologies, which in turn led to

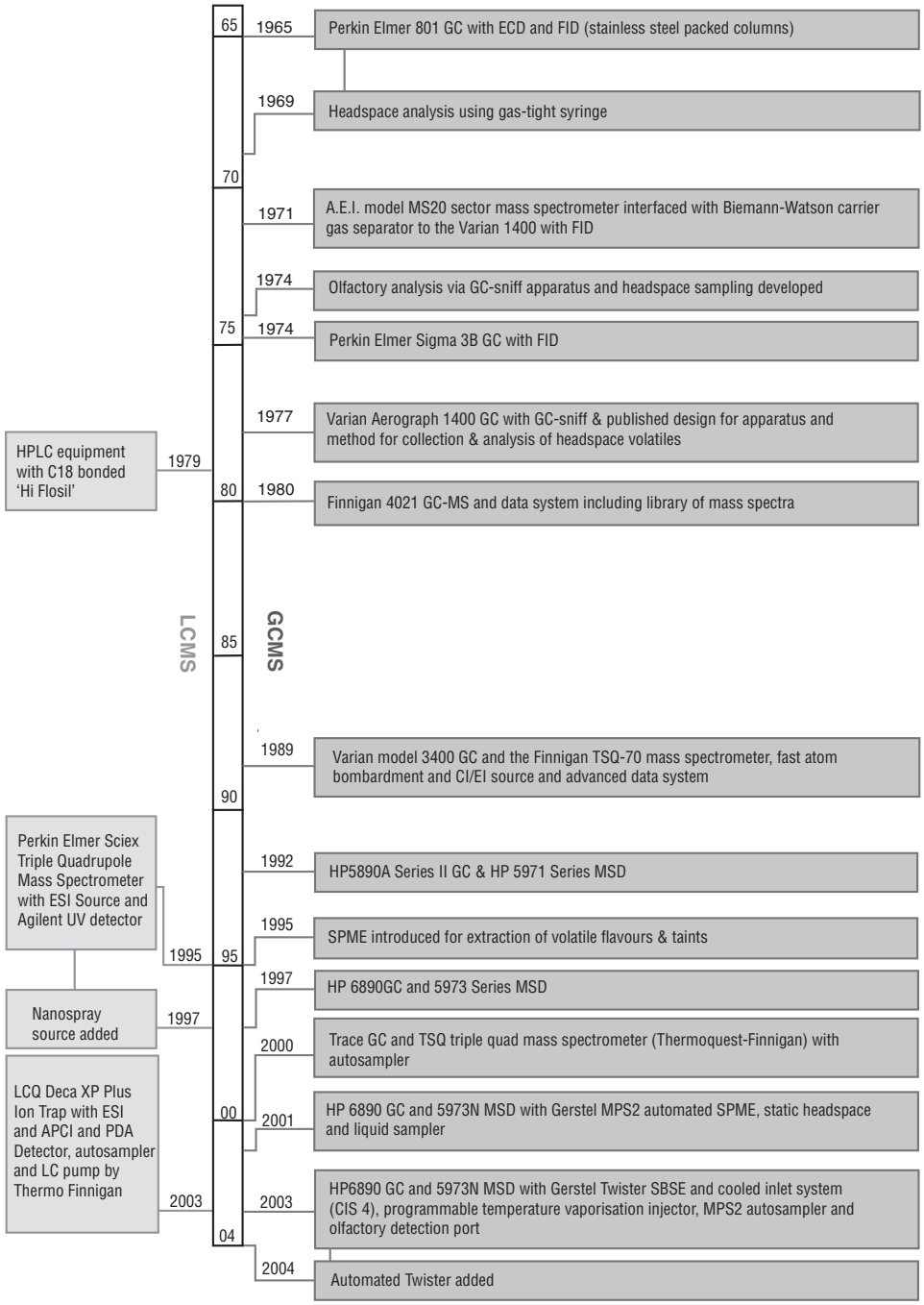


Figure 1. A time record of the introduction of mass spectrometers and associated instrumentation into the AWRI from 1965 to 2004.

valuable applied research outcomes.

From the founding of the AWRI in 1955 the research groups in the Institute have responded to Australia's rapidly expanding wine and spirit industry by producing breakthrough research in a variety of disciplines. Mass spectrometry has played a role in these research advances from the time of the purchase by the Institute of the first instrument in 1971. In this period, the role played by MS in the research output evolved in two phases that can be conveniently distinguished as falling into two time intervals. In the interval following the acquisition of the first instrument and up to the purchase of the third mass spectrometer in 1989, AWRI scientists employed MS particularly in studies on the volatile flavour compounds of wines and spirits. Table 1 highlights some of the pioneering work of this first interval, which was published, predominantly in refereed journals. Nevertheless, much work of this early period went unpublished and is preserved only in client, industry and AWRI annual reports, as well as in progress bulletins and articles in Technical Review.

In the second interval, and particularly in the past decade (1995–2004), MS has been involved in an increasing number of more diverse studies and figured prominently in approximately 40% of all AWRI publications to peer-reviewed scientific journals. Subjects of these studies have involved the analysis of (a) volatile compounds derived from grapes, from primary and secondary fermentations, and from oak wood; also of taint compounds; (b) involatile compounds including, most recently, proteins, pigments and tannins. The growing list of publications involving mass spectrometry highlights the fact that MS is crucial to the investigation of an expanding range of vine and wine science research topics.

MS is an analytical technique for the identification of

Table 1. Topics of research work involving mass spectrometry done in the Australian Wine Research Institute from 1971 to 1989.

Distillation of spirits	Connell and Strauss (1974); Williams (1976); Strauss and Williams (1983); Williams and Strauss (1975, 1978a,b,e)
Flor sherry volatiles	Williams and Strauss (1978c,d)
Method development in distillation	Williams and Strauss (1976); Williams et al.(1981a)
Method development in wine aroma compounds	Williams et al. (1976); Williams and Strauss (1977); Simpson (1979b); Williams (1982)
Wine aroma compounds	Simpson et al. (1977); Simpson (1978a,b, 1979a,c, 1980); Simpson and Miller (1983, 1984)
Off flavours in wine	Craig and Heresztyn (1984); Strauss and Heresztyn (1984); Strauss et al. (1985a,d); Heresztyn (1986a); Simpson et al. (1986); Amon et al. (1987, 1989)
Grape flavour	Williams et al. (1980a,b,c, 1981b, 1982a,b, 1983a,b, 1985a,b); Dimitriadis and Williams (1984a,b); Wilson et al. (1984a,b, 1986); Strauss et al. (1985b,c, 1986, 1987a,b, 1988); Sefton et al. (1989); Williams (1989)

unknown compounds, the detection and quantification of known compounds, and the elucidation of the chemical properties of molecules. Although sensitivity depends upon the nature of the compound analysed and the type of instrumentation used, the detection of a charged species can usually be accomplished with minute quantities: as little as 10^{-12}g (i.e. picograms) or, under favourable circumstances, even less.

The basis of MS is the determination of the masses of molecules. These molecules are detected as ions by a mass analyser. How the sample gets into the MS (sample introduction) is also important. Figure 2 shows schematically the core process of mass analysis, in this case with a quadrupole mass analyser, and how sample introduction can be accomplished for gas chromatography-mass spectrometry (GC-MS) and high performance liquid chromatography-electrospray ionisation-mass spectrometry (LC-MS). Nowadays, the following techniques are generally used for the formation of ionised molecules.

For volatile compounds, usually analysed by GC-MS (top half of Figure 2), an overview of the process is given in the following steps.

1. The sample is injected. Typically, the sample in a liquid solvent solution is volatilised into the gas phase in the hot injector. The gaseous mixture passes into and through the GC column transported by a carrier gas (typically helium or hydrogen).
2. The sample mixture travels through the capillary GC column to the MS. On the way, component compounds of the mixture are separated according to a combination of their boiling points and polarities, depending on the characteristics of column chosen. The separated component compounds each exit from the end of column and sequentially enter the MS.
3. In the MS, charged molecules are produced in the gas phase by bombardment with electrons (electron impact ionisation: EI) or ion-molecule reactions (chemical ionisation: CI).
4. The resulting ions, molecular ions and/or their fragment ions, are subsequently introduced into a mass analyser to separate them according to their mass to charge ratio (*m/z*), before they are detected and recorded as a mass spectrum.

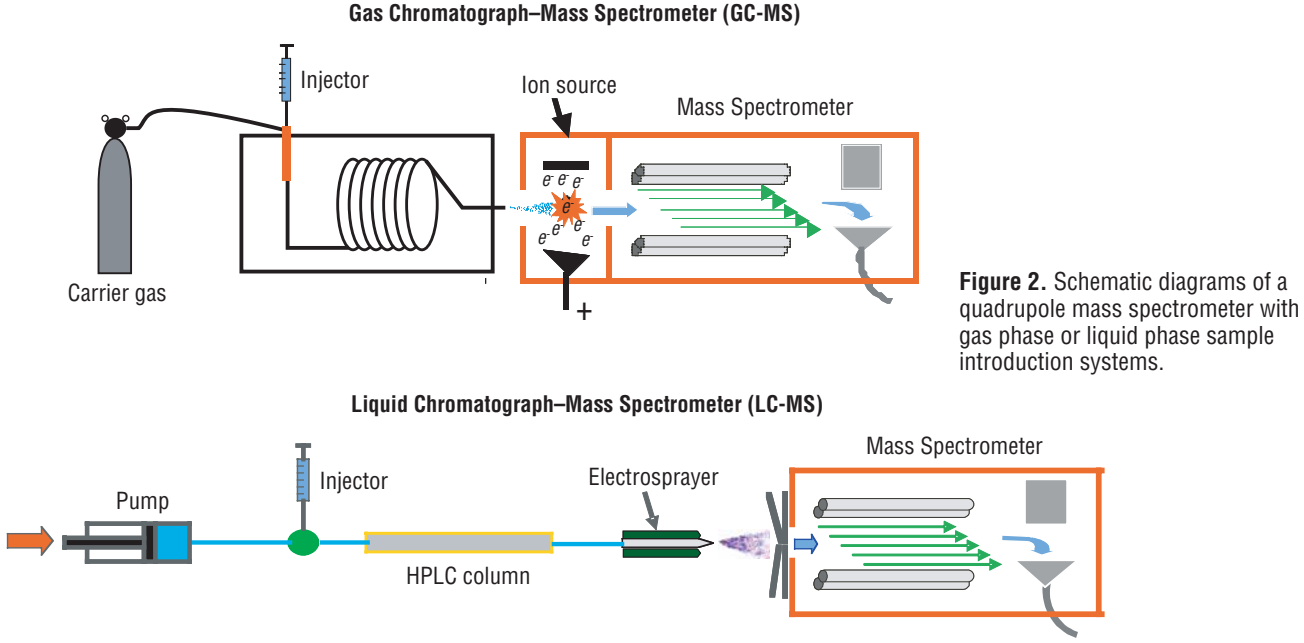


Figure 2. Schematic diagrams of a quadrupole mass spectrometer with gas phase or liquid phase sample introduction systems.

For involatile molecules, typically analysed by LC-MS (bottom half of Figure 2) an overview of the process is given in the following steps.

1. The liquid sample is injected, and pumped into and through a high performance liquid chromatography (HPLC) column by means of a mobile phase (a solvent or mixture of solvents).
2. The sample mixture travels through the packed HPLC column to the MS. On the way, component compounds of the mixture are separated according to their polarities (i.e. relative affinity for the HPLC column chosen).
3. Each separated component compound eluting sequentially from the HPLC column is sprayed by the electrosprayer (as in Figure 2) in order to transfer ion species from solution to the gas phase by solvent evaporation and Coulombic repulsion (electrospray: ESI) (Gaskell 1997, Kebarle 2000).
4. The resulting ions, molecular ions and/or their fragment ions, are subsequently introduced into a mass analyser to separate them according to their mass to charge ratio (*m/z*), before they are detected and recorded as a mass spectrum.

At the time of writing, five benchtop GC-MS systems and one GC-MS/MS (tandem mass spectrometer) owned by the AWRI, and two electrospray ionisation mass spectrometers (ESI-MS) (one each owned by the University of Adelaide and Provisor) are operated at the AWRI to the benefit of the wine industry and associated industries. The timeline for the acquisition of these instruments (by the AWRI and partner institutes) is shown in Figure 1. These mass spectrometers are used for strategic and applied research projects and to solve problems the industry is facing from vineyard to bottling.

In this paper, we summarise the contribution MS has made to wine research at the AWRI, and the significant advances made by scientists in this area. In particular, this review focuses on three main areas of compositional analysis of compounds important to wine quality: volatile aroma and off-flavour compounds, involatile biopolymers such as proteins and tannins, and investigations into taint problems.

The analysis of volatile aroma compounds in grapes and wines by gas chromatography-mass spectrometry (GC-MS) and related techniques

Of all alcoholic beverages, wine displays perhaps the greatest variation in aroma and flavour. Subtle nuances create a unique character for each wine. Several hundred volatile components have been identified in grapes and wine and many of these are important to wine aroma and flavour (e.g. Schreier 1979, Rapp and Pretorius 1989, Maarse and Visscher 1994, Guth 1997a). There are several classes of compounds affecting the aroma and flavour of wine, and these have different origins, e.g. from grapes, from primary fermentation, from bacterial action at secondary fermentation (oak wood contact may be involved with either or both of these fermentation processes), and from external sources during bottling and storage.

Accurate quantification of these important volatile components in wine remains a challenge. In general, gas chromatography (GC) is the technique of choice for the analysis of volatile compounds in wine. Rankine (1967) appreciated the value of GC in wine research and realised the future potential of GC to study many trace volatile constituents of wine. This was quite visionary because, at the time, GC had about the same resolving power as thin layer chromatography (Connell and Strauss 1972). Today, capillary GC has greater resolving power (i.e. many more theoretical plates) than HPLC. GC offers a choice of injection techniques (e.g. split, splitless/split, pressure-pulsed splitless/split, on-column, liquid, headspace) that can be combined with sample extraction and enrichment techniques (e.g. solid-phase microextraction (SPME), stir bar sorptive extraction (SBSE)) and can be coupled to a range of detectors. A variety of GC columns are available, which allow tailoring of analytical methods to resolve and quantify compounds of interest, taking advantage of the affinity of the analyte (or lack thereof) to a bonded phase, or the boiling points of the compounds, or a combination of both. Chiral separations can be achieved by the use of shape-selective cyclodextrin derivative columns (commonly referred to as chiral columns) (e.g. Guth 1996, Shao and Marriott 2003). By their very nature, volatile compounds will be vaporised in the GC injector, leaving less volatile wine components (e.g. polyphenols, tannins, and pigments) behind. Experienced GC operators also take advantage of the injector block of the GC, optimising parameters (including injection temperature, speed, duration, pressure programming, and solvent focusing on the column) to suit the analytical application. Common detectors for GC include the flame ionisation detector (FID) or mass spectrometer (MS), but other detectors can also be used; these are of varying sensitivity and selectivity, e.g. electron capture detector (ECD), polyhalogenated hydrocarbon chemiluminescence, atomic emission, chemiluminescence, flame photometric, nitrogen phosphorous, photoionisation, and thermal conductivity detectors, or the human nose.

Overall, mass spectrometry is a superior detector for GC as it has a good linear range, low level of detection, and an unrivalled capacity to confirm the identity of compounds being analysed. This latter follows from the fact that under EI conditions, in particular, each compound has its own fragmentation pattern or spectral fingerprint. Schreier (1979), citing 67 references, listed 318 neutral volatile compounds identified and/or quantified in wine, of which 237 were analysed by GC (using various detectors) with 203 by GC-MS. Of the more than 780 AWRI publications listed on the AWRI website at 2 November 2004, 159 of these are based on a major contribution of GC analyses, and 133 include significant work done by GC-MS. For quantitative analysis, 31 of these papers rely on GC-MS coupled with stable isotope dilution analyses (SIDA), published from 1996 onwards.

Stable isotope dilution analyses (SIDA)

SIDA is generally the most accurate, precise and robust method to determine the levels of important compounds

in difficult matrices (e.g. wine), although it is not a 'magic bullet' and care has to be taken to not form artefacts as a result of the analysis itself (Sejer-Pedersen et al. 2003, Pollnitz et al. 2004a). In any analysis, the internal standard (IS) used should be as similar as possible to the analyte measured, without being present originally in the matrix. The isotopically labelled standards used in SIDA are ideal as they have virtually identical physical and chemical properties to their unlabelled analogues, but can be discriminated from the analyte by mass spectrometry. As the labelled internal standard is virtually identical chemically to the substrate being assayed, the accuracy of the analysis is not reduced by inefficiency in isolation or by analyte decomposition. An advantage of this is that complete extraction of the analyte of interest from the matrix is no longer a necessity. Another advantage is that methods can be streamlined so that several components can be determined in a single extraction and GC-MS run (Pollnitz et al. 2000a,b,c, 2004a, Sejer-Pedersen et al. 2003, Siebert et al. 2005), even though the compounds have different chemical properties.

The principles, advantages and applications to wine of SIDA have been discussed extensively, and SIDA has been used for well over a decade to determine the concentration of methoxypyrazines in grapes and in wine (Harris et al. 1987, Allen and Lacey 1993, 1998, Allen et al. 1994, 1995, 1996, Kotseridis et al. 1998, 1999a). Recently, SIDA techniques have been described for the quantitation in wine of trichloroanisole (Pollnitz et al. 1996), vanillin (Spillman et al. 1997), ethyl dihydrocinnamate, ethyl cinnamate, methyl anthranilate and ethyl anthranilate (Aubry et al. 1997), damascenone, α-ionone and β-ionone (Kotseridis et al. 1998, 1999b,c), diacetyl (Hayasaka and Bartowsky 1999), oak lactone (Pollnitz et al. 1999, 2000c), 4-ethylphenol (Pollnitz et al. 2000a), linalool, geraniol, nerol and α-terpineol (Sejer-Pedersen et al. 2003) and guaiacol, 4-methylguaiacol and other oak volatiles (Pollnitz et al. 2004a). In a landmark paper, Guth (1997b) described the analysis and odour contribution of 44 volatile compounds in white wine, 41 of which were analysed using isotopically labelled standards, on three different capillary columns, with up to 20 compounds processed in one extraction and analysis. But in this study Guth (1997b) was constrained to using relatively time consuming extraction and concentration procedures in order to achieve the low detection limits for some of the analytes. In a recent paper (Siebert et al. 2005) we report a novel method for the determination of a range of 31 fermentation-derived fatty acids, alcohols, acetates and ethyl esters by SIDA in combination with headspace SPME and GC-MS. Sample preparation takes just a few minutes, requires only a small sample volume (1 mL) and gives good precision and accuracy. The method is a valuable research tool with many relevant applications under way (e.g. Smyth et al. 2003, Pollnitz et al. 2004b).

4-Ethylphenol (4-EP) is detrimental to the quality of wine, especially at higher concentrations (e.g. Chatonnet et al. 1992, 1995, Pollnitz et al. 2000a,b,c, Coulter et al. 2003). In 2000, we published details of the first rapid, precise and accurate analytical protocol for 4-ethylphenol,

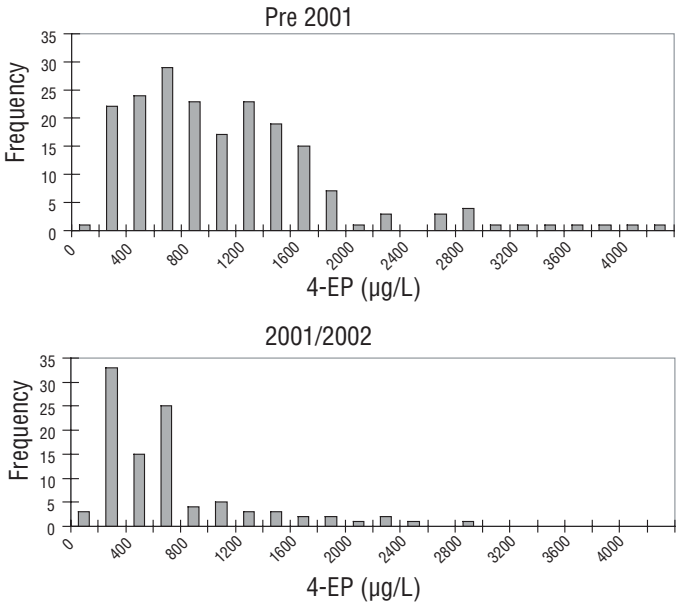


Figure 3. Frequency of the number of red wines analysed and found to contain 4-EP at concentrations between the values shown. Top: concentrations of 4-EP found in wines vinified prior to 2001. Bottom: concentrations of 4-EP found in wines vinified in 2001 and 2002. Figure adapted from data published by Coulter et al. (2003).

and a survey of single varietal Australian red wines (Pollnitz et al. 2000a,b,c). This analysis, which is also offered as a commercial service, has provided the data that were essential for developing winemaking strategies that have helped to reduce the concentration of 4-EP in Australian wines. Thus, Figure 3 (Coulter et al. 2003) shows the decreasing incidence of red wines with 4-EP concentrations above 800 µg/L after 2001.

While GC-MS is the analytical method of choice for the analysis of volatile aroma compounds in wine (and related matrices), sample preparation and introduction techniques are also of crucial importance. Some of the more successful approaches to sample preparation and introduction are briefly discussed below.

Analysis of the headspace above wine: solid-phase microextraction (SPME)

GC analysis of the headspace above wine is particularly inviting because of the ability of this sampling method to give results in which artefacts resulting from extraction and concentration are kept to a minimum (e.g. Williams and Strauss 1975, 1977, 1978d, Francis et al. 1993, Leino et al. 1993). This is a particular advantage when analysing volatile aroma compounds in a complex matrix, such as wine, because the most volatile compounds, including those of interest, can be separated from those of low volatility. This can increase the signal-to-noise ratio in instrumental analyses (Simpson 1978b). Unfortunately, conventional headspace concentration procedures can present various problems, especially with repeatability, although in the early days (Rankine et al. 1969) static headspace extraction facilitated quicker and more accurate diacetyl determination than the corresponding chemical method, and Williams and Strauss (1977) demonstrated precise determination of volatiles in the headspace of

wine and spirits. More recently, we have used static headspace with some success for the analysis of volatile compounds linked to the spicy 'black pepper' aroma of Shiraz (Pollnitz et al. 2004b) and in the sensitive quantitative analysis of low molecular weight sulfur compounds (Siebert et al., manuscript in preparation). Conventional headspace analysis can be easily combined with inside-out chromatography or SPME. Developed by J. Pawliszyn in 1989 SPME has been commercially available since 1993, thus opening the door for research applications, especially when combined with GC (e.g. Hayasaka and Bartowsky 1999, Pollnitz et al. 2004a,b, Siebert et al. 2005). The SPME device consists of a *c.* 10 mm long tube of fused silica with an adsorbent material matrix (e.g. polydimethylsiloxane or Carbowax/divinylbenzene) bonded to the outside. This fused silica is attached to the end of a metal fibre such that the entire assembly can be retracted into a hollow metal needle of slightly larger diameter than a standard GC syringe. The headspace above the wine sample contained in a vial sealed with a rubber septum is collected by puncturing the septum with the metal needle, extending the fibre into the headspace above the wine and waiting for the analytes in the gas phase to concentrate on the fibre. Once this is completed (usually after 5–30 minutes) the fibre is retracted and the assembly functions like a GC syringe with the metal needle puncturing the septum of the GC injector port, followed by extension of the fibre in the injector and subsequent thermal desorption of the headspace volatiles onto the GC column. SPME has the advantage of concentrating the headspace volatiles onto the fibre *in situ*, unlike more time consuming liquid/liquid extraction procedures (e.g. Simpson et al. 1986, Sefton et al. 1993a,b, Spillman et al. 1998a,b, 2004a,b), solid-phase extraction or traditional static or purge and trap headspace techniques (e.g. Francis et al. 1993, Leino et al. 1993) where concentration steps are often required prior to sample analysis. Unlike static headspace sampling where volatility of the components is the major factor determining the amount of each individual component in the headspace sample, with SPME the selectivity of the fibre is the dominant influence. The nature of the bonded phase and the time of extraction affect the absolute and relative concentrations of the captured volatiles. This is both an advantage and a disadvantage. On the positive side, SPME can be more selective than static headspace sampling, in that fibres and extraction conditions can be manipulated to favour the collection of analytes of interest over other volatiles present in the matrix. The disadvantage is that a distorted picture of the relative concentrations of different volatiles might be observed, unless the analyst uses reliable internal standards and/or thoroughly calibrates the method over a variety of conditions. Successful headspace analysis, whether by SPME or conventional methods, relies on (1) an effective headspace injector design on the GC, (2) reproducible agitation of the sample, (3) temperature control of the sample and headspace volume and (4) the time of extraction. Ionic strength and the levels of polymeric materials (e.g. polysaccharides and tannins) are always variables in wines and these significantly effect the headspace concentration of volatiles. It is seldom easy to achieve reproducible sample matrix, agitation, temperature and time control during headspace extraction, even with automated extractors, but the use of isotopically labelled standards for SIDA solves this problem. This follows as the ratio of the analyte to its isotopically labelled internal standard will remain constant regardless of uncontrolled headspace extraction conditions. These details have been discussed recently (Pollnitz et al. 2004a, Siebert et al. 2005).

Stir bar sorptive extraction (SBSE)

SBSE uses a stir bar (typically 10 mm length) incorporated into a glass tube and coated with polydimethylsiloxane (PDMS). Upon stirring in a liquid sample matrix, the analytes are partitioned between the matrix and the PDMS phase on the stir bar according to their partitioning coefficients. Subsequently, the stir bar is transferred from the sample to a compact thermal desorption unit mounted on a Programmable Temperature Vaporisation injector of a gas chromatograph (GC) where the analytes are thermally desorbed and delivered to the GC column. The extraction properties of SBSE are the same as those for SPME with PDMS fibre coating, but the volume of the PDMS phase is significantly greater with typically 55 µL for SBSE (ranges from 25 to 125 µL) and 0.6 µL for SPME (100 µm fibre) (Baltussen et al. 1999, 2002, Bicchi et al. 2002, Hayasaka et al. 2003a). This affects directly the enrichment of analytes since their recoveries from a liquid sample increases with the volume ratio of the PDMS phase to the sample matrix.

We recently published a study of an evaluation of the capability of the SBSE technique to analyse flavour and off-flavour compounds as well as agrochemicals in wine (Hayasaka et al. 2003a). SBSE analysis was generally orders of magnitude more sensitive than conventional SPME or liquid-liquid extraction, and enabled confirmation of identity through full scan MS at levels of detection that were previously achievable only with selected ion monitoring. This enhanced sensitivity of SBSE proved useful for elucidating the structures of unknown compounds, as evidenced by the recent discovery (Janusz et al. 2003) of a potent grape-derived odorant, (E)-1-(2,3,6-trimethylphenyl) buta-1,3-diene (TPB). The newly discovered TPB is a thirteen carbon compound similar to TDN (1,1,6-trimethyl-1,2-dihydronaphthalene) the famous 'aged Riesling'/'kerosene' norisoprenoid aroma compound recognised by Simpson (1978a).

GC analysis using a combination of MS with an olfactory (sniff) detector

A valuable analytical tool for detecting and identifying new aroma compounds in wine is GC-sniff (e.g. Craig and Heresztyn 1984, Strauss and Heresztyn 1984, Heresztyn 1986a,b, Simpson et al. 1986, Amon et al. 1987, 1989, Miller et al. 1987, Lee and Simpson 1990, 1993, Simpson 1990, Herderich et al. 1995, Grbin et al. 1996, Pollnitz et al. 2004b), i.e. the coupling of gas chromatography (GC) with the human nose as a detector. GC-sniff is also known as GC-olfactory detection. Basically, the

effluent from the GC column is split into two streams at the detector end by the use of a Y-shaped zero dead volume splitter. One stream of the split GC column effluent is connected to a conventional detector, such as an FID or MS and the other stream goes to a heated sniffing cup, through which a stream of humidified air is passed. Extracts should ideally be run more than once and assessed by more than one sniffer because aroma thresholds and descriptors vary from person to person. GC with olfactory detection also can establish which aroma components are more important and which are less important. The main limitation of GC-sniff is that some compounds that are detected at the heated sniffing cup are not normally detected by the human nose. A feature of GC-sniff is that many compounds are not necessarily volatile in the matrices in which they occur (e.g. wine), but are typically ~90–100% vaporised in the injector block of the GC. In other words, GC-sniff can over-estimate the importance of an aroma compound and also change its perceived aroma character. For example, cyclotene and maltol have been labelled with burnt woody and toasty aroma descriptors by GC-sniff and it has been suggested that these two compounds are responsible for sweet, burnt woody and toasted wood aromas in alcoholic beverages (Nishimura et al. 1983). However, results from the AWRI show extremely high aroma detection thresholds for cyclotene (3100 µg/L) and maltol (11400 µg/L) in white wine (Spillman et al. 2004b) as these two compounds are highly water-soluble and presumably have low partial vapour pressures in wine. These data indicate cyclotene and maltol might not be at all significant to wine aroma, as their concentrations present in wine were far below these thresholds. As another example, ethyl dihydrocinnamate, ethyl cinnamate, methyl anthranilate and ethyl anthranilate have been assumed to be important in the aroma of Pinot Noir wines, based on GC-sniff assessments, but were actually present at levels less than half their sensory thresholds in water (Aubry et al. 1997).

In some cases, a characteristic aroma cannot be found via GC-sniff in any one part of the gas chromatogram, but the familiar characteristic aroma results from the concerted effects of many compounds (Vitzthum 1976). Several studies (e.g. Wang et al. 1983, Lawless 1986, Laing 1987, Rothe 1988, Meilgaard 1989) report combinatory effects between two or more volatile components that can enhance or reduce the overall aroma impression and such synergistic phenomena cannot be detected by GC-sniff.

GC-sniff can, nevertheless, be useful for highlighting some compounds or regions of chromatograms for further study, as has been demonstrated by investigations into mousy compounds (Craig and Heresztyn 1984, Strauss and Heresztyn 1984, Heresztyn 1986b, Herderich et al. 1995, Grbin et al. 1996), into *Brettanomyces/Dekkera* metabolites (Heresztyn 1986a,b), the loss of aroma compounds through carbon dioxide efflux during white wine fermentation (Miller et al. 1987) and the study of musty compounds associated with cork and oak taint (Simpson et al. 1986, Amon et al. 1987, 1989, Lee and Simpson 1990, 1993, Simpson 1990).

Recently, GC-sniff has been re-applied to identify 2-methoxy-3,5-dimethylpyrazine, the compound responsible for 'fungal must' cork taint (Simpson et al. 2004), and was crucial to the discovery of TPB (Janusz et al. 2003) in conjunction with SBSE (as described above), and applied in the 'black pepper' Shiraz project (e.g. Pollnitz et al. 2004b).

The analysis of non-volatile and high molecular weight compounds in grapes and wines by electrospray ionisation mass spectrometry (ESI-MS)

In 2002, Professor John B. Fenn and Mr Koichi Tanaka were rewarded with the Nobel Prize in Chemistry for the development of soft ionisation methods, electrospray ionisation (ESI) by Fenn and matrix assisted laser desorption ionisation (MALDI) (Zenobi and Knochenmuss 1998) by Tanaka. The development of ESI and MALDI offered an increase in mass range and sensitivity that has opened a new era for the application of MS to the analysis of biomacromolecules. For example, the major advances in proteome research could not have been achieved without the development of ESI and MALDI (Larsen and Roepstorff 2000, Godovac-Zimmermann and Brown 2001, Aebersold 2003). The application of ESI is not limited to the analysis of biomacromolecules. The combination of HPLC as a versatile separation and sample introduction method and ESI-MS as a widely applicable ionisation and ion separation tool (LC-MS) have overcome many analytical problems in the characterisation of polar, thermally labile compounds of low volatility present in complex mixtures. These developments have benefited many scientific fields, including grape and wine research.

Apart from water and ethanol, the wine matrix contains only a small portion of volatile (including aroma) compounds (approximately 1 g/L) and the remaining portion chiefly consists of polar, less volatile and/or large molecules, many of which are thermally labile. The non-volatile compounds include pigments, tannins, carbohydrates including polysaccharides, amino acids, organic acids, glycosides and proteins. The use of MS for the analysis of non-volatile compounds in grapes and wines has been rapidly expanded to polyphenols (Flamini 2003, Hayasaka et al. 2003b), anthocyanins (Gläßgen and Seitz 1992, Favretto and Flamini 2000), proteins (Hayasaka et al. 2001, Kwon 2004), heterocyclic aromatic amines (Richling et al. 1997), stilbene derivatives (Baderschneider and Winterhalter 2000, Careri et al. 2004) and flavanol-peptide complex (Sarni-Manchado and Cheynier 2002).

Studying colour development of red wine by MS

Anthocyanins are one of the most important phenolic groups in red grapes and they greatly influence the perception of red wine colour and quality. *Vitis vinifera* red grapes contain five common anthocyanidins, i.e. malvidin, petunidin, delphinidin, peonidin and cyanidin, which exist as 3-glucosides and their acylated derivatives, i.e. esters of glucose with acetic acid, *p*-coumaric acid, or caffeic acid. Accordingly, the colour of red grape skins, with the exception of a few varieties like Pinot Noir which has no acylated anthocyanins, results from the super-

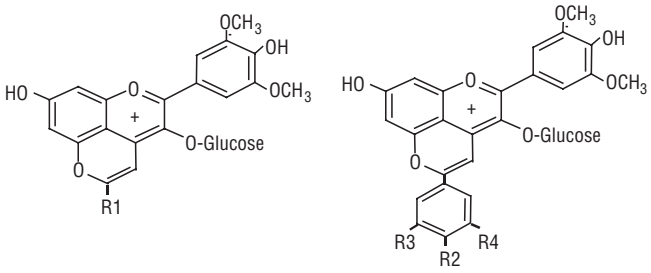
imposed spectra of at least 20 anthocyanins (five anthocyanidins in four different glucosyl isoforms).

Naturally, those grape-derived anthocyanins become the prime pigments contributing to the colour of young red wines. However, these pigments are short-lived, even though the wine exhibits long-lasting red colour. In five-year-old red wine there are almost no anthocyanins left. The decreasing level of the grape-derived anthocyanins is concomitant with the formation of new pigments, which are generated by the reaction of anthocyanins with wine constituents such as yeast metabolites and proanthocyanidins.

The formation of new pigments from anthocyanins occurs immediately after crushing, accelerates during vinification and continues during ageing. Seemingly, it is a never-ending reaction. As a result, the structural diversity of wine pigments is enormously broad and the characterisation of pigments in red wines remains an analytical challenge.

When characterising anthocyanins from cell culture and plant tissues of *Daucus carota* L., ESI-MS and LC-MS (and MS/MS) were proven to be powerful methods for the investigation of plant pigments (Gläßgen and Seitz 1992, Gläßgen et al. 1992). The positive ion mass spectra of anthocyanins obtained from ESI-MS were shown to be quite simple but informative, giving the molecular cation (M^+) together with the fragment ions corresponding to the aglycone (anthocyanidins) resulting from a neutral loss of the glycosyl moiety from the respective anthocyanins (Gläßgen and Seitz 1992). This method was adopted for grape and wine research; as a result, nineteen grape anthocyanins from *Vitis vinifera* Sangiovese and Colorino varieties were identified using LC-MS (Baldi et al. 1995). Since then, LC-MS combined with a diode array detector has become a common and indispensable analytical tool for the characterisation and identification of anthocyanins in grapes and wines (Giusti et al. 1999, Revilla et al. 1999). Anthocyanins in grape extracts from the hybrid grape varieties Clinton (*Vitis labrusca* × *Vitis riparia*) and Isabella (*Vitis vinifera* × *Vitis labrusca*), and from *Vitis vinifera* Cabernet Franc were investigated by direct infusion ESI-multiple tandem mass spectrometric analysis with an ion trap mass spectrometer (Favretto and Flamini 2000). By this method, a range of anthocyanins including isobaric compounds in crude grape extracts was rapidly identified and semi-quantified, without HPLC separation prior to mass spectrometric analysis.

As mentioned above, grape anthocyanins are continuously transformed from the time of crushing grapes into new pigments of wide structural diversity. ESI-MS has opened a new era to credibly explore the chemical nature of new pigments formed in wines and model wines. A number of new pigments commonly characterised by a vinyl linkage between C-4 and the hydroxyl group at C-5 of anthocyanin molecules (pyranoanthocyanins) have been proposed or identified with the aid of ESI-MS. They include anthocyanins condensed with pyruvic acid (Fulcrand et al. 1998, Schwarz et al. 2003a,c), acetaldehyde (Bakker and Timberlake 1997, Benabdeljalil et al. 2000), acetone (Benabdeljalil et al. 2000), 4-vinylphenol



(Adducts)	(R1)	(Adducts)	(R2)	(R3)	(R4)
Pyruvic acid	COOH	4-Vinylphenol	OH	H	H
Acetaldehyde	H	4-Vinylguaiacol	OH	OCH_3	H
Acetone	CH_3	4-Vinylcatechol	OH	OH	H
Catechin	Catechin	4-Vinylsyringol	OH	OCH_3	OCH_3

Figure 4. Structures of some malvidin 3-*O*-glucoside-derived pyranoanthocyanins found in red wine.

(Cameira dos Santos et al. 1996, Fulcrand et al. 1996), 4-vinylguaiacol (Asenstorfer et al. 2001) or vinyl(epi)catechin (Francia-Aricha et al. 1997, Asenstorfer et al. 2001) (Figure 4). In order to investigate the structural diversity of pyranoanthocyanins, a screening method for potential anthocyanin-derived pigments in red wine has been developed. Pyranoanthocyanins were separated from grape anthocyanins by bisulfite-mediated ion-exchange chromatography (Asenstorfer et al. 2001). This was followed by screening for new anthocyanin-derived pigments based on their fingerprint MS/MS mass spectra obtained by nanoelectrospray (nanoESI) combined with tandem mass spectrometry (MS/MS) (Hayasaka and Asenstorfer 2002). This method allowed the determination of the molecular mass and glycosyl isoform of the putative pyranoanthocyanins as well as the identification of the original anthocyanidin, which served as a precursor. In this study, thirteen different pyranoanthocyanidins were screened and tentatively identified in one three-year-old *Vitis vinifera* Shiraz wine. The majority of the pigments detected had been previously reported, and structures of malvidin 3-glucoside linked to 4-vinylcatechol or to 4-vinylsyringol were proposed for the first time (Figure 4) (Hayasaka and Asenstorfer 2002). The structure of the 4-vinylcatechol adduct was subsequently confirmed by two separate studies (Håkansson et al. 2003, Schwarz et al. 2003a). The team led by Peter Winterhalter clearly demonstrated that the vast majority of anthocyanin-vinylphenol pigments in red wines were formed from their corresponding free hydroxycinnamic acids (Schwarz et al. 2003b).

Pyranoanthocyanins have been proven to be chemically more stable than grape anthocyanins due to the resistance to nucleophilic attack of the former compounds (Sarni-Manchado et al. 1996, Bakker and Timberlake 1997, Håkansson et al. 2003). For instance, in a ten-year-old *Vitis vinifera* Cabernet Sauvignon wine, the pyruvic acid adduct of malvidin 3-glucoside persisted at a level of approximately 45% of its initial concentration, while malvidin 3-glucoside was no longer present (Schwarz et al. 2003c). However, the impact of individual pyranoanthocyanins on red wine colour in the aged wines remains a subject for further studies.

Somers (1971) reported that polymeric pigments are responsible for the major portion of colour in aged wine, accounting for as much as 50% and 85% in one-year-old and ten-year-old wines, respectively. Since then, the importance and contribution of polymeric pigments to wine colour has been the subject of many studies. As a result, the contribution of polymeric pigments to red wine colour was estimated to be 70% in 240-day-old Cabernet Sauvignon and Merlot wines (Nagel and Wulf 1979), over 20% in Carignane and Gamay wines after pressing (Bakker et al. 1986), 90% in two-year-old Roriz wine (Bakker et al. 1998), 90% in six-year-old Shiraz wine (Peng et al. 2002) and 70–90% in aged Pinotage and Cabernet wines (Schwarz et al. 2003c). Some of this variation is largely due to differences in the definition of polymeric pigments and the methods used for their quantitative estimation. Nevertheless, all studies indicated the importance of polymeric pigments for wine colour, even for young wines. However, their chemical structures remained largely speculative and uncharacterised. Indeed, it proved a very challenging task to characterise polymeric pigments since the combination of their structural diversity, wide range of molecular size distributions, and the low concentration of individual polymers impeded their isolation from the complex wine matrix. As potential components of polymeric pigments, direct (Jurd 1969, Somers 1971, Bishop and Nagel 1984) and acetaldehyde-mediated (Timberlake and Bridle 1976, Dallas et al. 1996, Eglinton et al. 2004) condensation products of anthocyanins with flavanols have been extensively studied. Hayasaka and Kennedy (2003, Kennedy and Hayasaka 2004) investigated isolated polymeric materials from a three-year-old Pinot Noir wine using ESI-MS and ESI-MS/MS. The ions related to polymeric pigments appeared in two series, one series starting at *m/z* 781 and the other at *m/z* 783. Each of the two series was observable up to *m/z* 2509/2511, with ions separated by a mass of 288. The ions with *m/z* 781 and 783 indicated the presence of direct condensation products (dimers) of malvidin-3-glucoside and (epi)catechin, with the anthocyanin bound to the flavanol via the A-ring of the anthocyanin (T-A type) and via the C-ring of the anthocyanin (A-T type), respectively (Figure 5a). The proposed structures were further supported by MS/MS spectral analysis. Subsequently, polymeric direct condensation products consistent with malvidin 3-glucoside linked to a proanthocyanidin containing up to seven sub-units could be observed by ESI-MS (Figure 5a). Based upon the postulated structures, the anthocyanin unit of T-A type polymer is in the flavylium form, therefore, this type of polymer is pigmented, while the A-T type conjugates are proposed to be colourless.

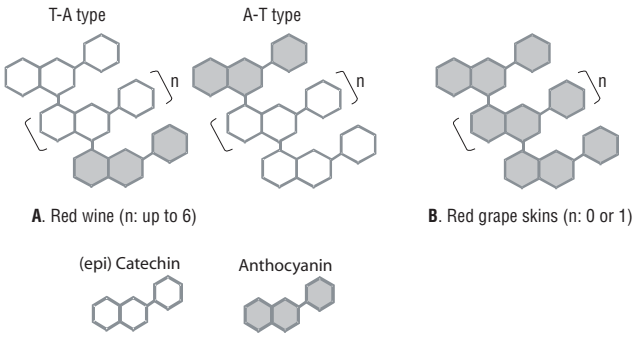


Figure 5. Proposed structures of pigmented oligomers found in (A) red wine and (B) red grape skin extracts based on studies with electrospray mass spectrometry. Figure adapted from data published by Hayasaka and Kennedy (2003) and Vidal et al. (2004a,b).

ESI-MS and LC-MS were also used for confirming the existence of pyranoanthocyanin oligomers in Shiraz grape marc and wine (Asenstorfer et al. 2001). These pigments were proposed to form from malvidin 3-*O*-glucoside through linking at the C4 position, via a vinyl linkage to either catechin/epicatechin or procyanidins (up to trimers).

On the other hand, relatively few studies have dealt with the presence of pigments other than monomeric anthocyanins in grapes. As a result, their presence and structures remain speculative. The existence of polymeric pigments in grape skin extracts was investigated by a combination of multilayer coil countercurrent chromatography (MLCCC) and ESI-MS (Vidal et al. 2004a,b). After the completion of the MLCCC separation of monomeric anthocyanins, pigmented materials still remained in the stationary phase and were almost devoid of monomeric anthocyanins. On the basis of the results of LC-MS analysis, colour-bleaching tests with sulfur dioxide and thiolysis, the remaining pigmented materials appeared to be almost exclusively polymeric. ESI-MS indicated that the remaining pigments were chiefly composed of direct condensation products of anthocyanins, extending up to trimers (Figure 5b). This is the first mass spectrometric evidence for the existence of anthocyanin oligomers in a grape skin extract. It will be interesting to investigate the formation of these anthocyanin oligomers, their diffusion into wine and stability in a wine matrix, for the assessment of their impact on wine colour and organoleptic properties. MS will doubtless have a central role in these future studies.

Pigmented polymers and grape and wine tannins are discussed in more detail in the review by Herderich and Smith (2005) elsewhere within this issue.

Characterisation of haze-forming proteins and the application of ESI-MS for varietal differentiation

Advances in grape and wine protein research, as well as the prediction and control of haze formation in white wines, are reviewed elsewhere in this issue (Waters et al. 2005). The scientific characterisation of haze-forming proteins began with the application of chromatographic techniques that led to the estimation of molecular mass (M_r) of wine proteins ranging from 18 kDa to 23 kDa (Bayly and Berg 1967) and from 11 kDa to 28 kDa (Yokotsuka et al. 1977). More recently, the M_r values of major wine proteins were estimated to be 24 kDa and 32 kDa and these proteins were found to be resistant to peptidases. In particular, 24 kDa proteins were identified as most important for haze formation (Waters et al. 1991, 1992). Furthermore Waters and co-workers identified the fact that haze-forming proteins of M_r 24 kDa and M_r 28 kDa had homology to plant thaumatin-like proteins and plant chitinases respectively. Therefore, the ubiquitous,

acid stable, proteolytically-resistant and troublesome proteins of wine were identified as grape pathogenesis-related proteins (PR-proteins) (Waters et al. 1996).

In the mid 1990s, ESI-MS was introduced to characterise haze-forming proteins. By the use of ESI-MS, the M_r of a major thaumatin-like protein in Muscat of Alexandria grapes was determined to be 21,272 Da (Tattersall et al. 1997) and identical to that isolated from Sauvignon Blanc wine (Peng et al. 1997). The latter study also found a minor thaumatin-like protein with M_r of 21,250 Da together with the major protein with M_r 21,272 Da. The composition of PR-proteins in Muscat of Alexandria grapes was further examined by ESI-MS. As a result, a minor thaumatin-like protein with M_r 21260 Da was confirmed along with the major protein with M_r of 21,272 Da and the molecular masses of the four major chitinases were determined to be 25,942, 25,588, 25,457 and 25,410 Da, respectively (Pocock et al. 2000). Chitinases isolated from Muscat of Alexandria wine were enzymatically digested and the resulting peptides were characterised by ESI-MS. The mass spectral and amino acid sequencing data demonstrated that the protein sequences of those chitinases were highly similar and the N-terminus of the three chitinases (M_r 25588, 25457 and 25410) had been modified by a pyroglutamate residue (Waters et al. 1998).

Since the early studies it has become clear that all grape cultivars synthesise a characteristic set of PR-proteins after veraison (Waters et al. 1996, Tattersall et al. 1997, Pocock et al. 2000) and that a number of isoforms of PR-proteins exist within individual varieties (Busam et al. 1997, Robinson et al. 1997, Waters et al. 1998, Jacobs et al. 1999). The use of ESI-MS for the analysis of PR-proteins clearly demonstrated the capability of this technique to differentiate the isoforms, despite their relatively small mass differences. This is clearly an advantage of ESI-MS, which provides a very high mass accuracy for M_r determination (within ±0.01%, equivalent to ±2 Da in 20 kDa) and a greater resolving power than any other technique currently available.

In order to investigate differences in the M_r of isoforms across varietal boundaries, a simple and rapid method for the M_r determination of PR-proteins was developed using ESI-MS combined with a protein trap cartridge (Trap-MS) (Hayasaka et al. 2001). Figure 6 shows the PR-protein M_r profiles obtained from Chardonnay, Semillon and Sultana juices when analysed by Trap-MS. The M_r profiles in these different juices exhibited obvious differences between the three varieties, and spurred further research on the use of ESI-MS for varietal differentiation of juice based on the M_r profile of PR-proteins. The Trap-MS method was applied to determine the M_r profiles of PR-proteins of juices obtained from berries from 20 different varieties (*Vitis vinifera*) harvested in at least two different seasons from seven different vineyards. As a result, the M_r profile of PR-proteins in the individual varietal juices showed significant differences and these differences were consistent regardless of where and when fruit had been grown. Based upon the detection of the indicative PR-proteins within four different M_r ranges of thaumatin-like proteins and 11 different M_r ranges of

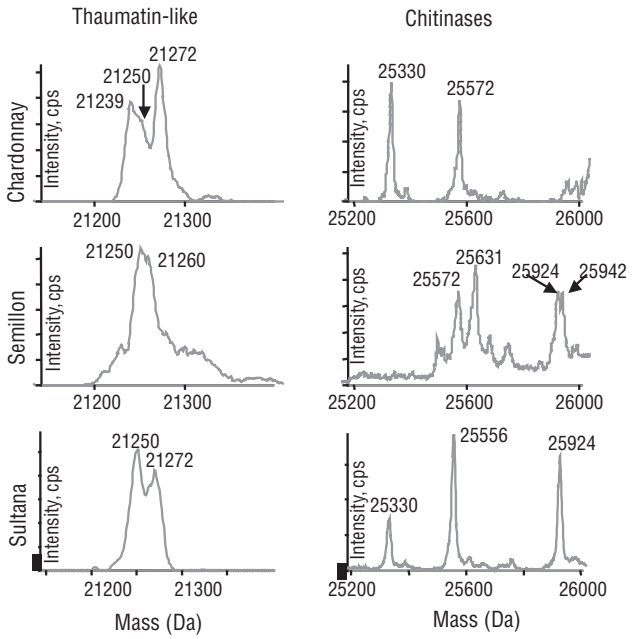


Figure 6. Mass profiles of pathogenesis-related (PR) proteins obtained from Chardonnay, Semillon and Sultana juices when analysed by Trap-MS. Figure adapted from data published by Hayasaka et al. (2001, 2003c).

chitinases, and with the aid of statistical analysis, the ESI-MS method developed is suitable for varietal differentiation of juice based on the M_r profile of PR-protein (Hayasaka et al. 2001, 2003c).

Taking advantage of ESI and MALDI to advance grape and wine research

ESI has made a significant impact on grape and wine research since the early 1990s. Numerous studies on polyphenols using ESI have been published (reviewed by Flamini 2003). Among the non-volatile constituents in wine, polyphenols have received the most attention from winemakers and scientists due to the importance of this class of compounds to the taste, colour and quality of wine.

For the control of desirable and undesirable aromas in the winemaking processes, the application of ESI for analysis of the precursors of aroma compounds, e.g. glycosidic (Winterhalter and Schreier 1994, Francis 1995) and S-cysteine conjugates (Tominaga et al. 1998, Peyrot des Gachons et al. 2000) in grapes and wines remains yet to be fully explored. These studies would ideally be made in conjunction with the accurate analysis of volatile aroma compounds by GC-MS.

Sensory studies on the interaction of non-volatile components and the perception of foods and beverages have recently been described. These include studies on taste-taste interactions (Keast and Breslin 2002), key wine components on mouth-feel perception in wine (Vidal et al. 2004c), interactions between food phenolics and aromatic flavour (Jung et al. 2000) and protein-flavour interactions (Heng et al. 2004). As soft ionisation techniques, ESI and MALDI can be expected to contribute to progress in the characterisation of not only non-volatile compounds

but also their non-covalently bound complexes, which may be related to the overall aroma and taste of wine.

Investigation into taint problems using mass spectrometry

MS as an analytical tool for taint investigation

Grape juice and wine might encounter contamination with chemicals at any stage of the grapegrowing and winemaking processes from vineyard to bottling. Contamination is not only a problem with respect to wine quality (off-flavour) and human health issues. It can also damage the credibility of the industry and cause irreversible economic loss. In general, taint spoilage due to chemical contamination is the result of accidental or unforeseen hazards. A survey of taints and off-flavours noted that minor spills or unseen leaks before or during production could go unnoticed, but nevertheless might impart a distinct taint to wine (Strauss et al. 1985a). It is one of the important roles of the AWRI to investigate the occurrence of contamination, as part of our problem solving activities in collaboration with the wine industry.

Mass spectrometry is the analytical technique of choice for taint investigations when identification and quantification of trace amounts of unknown contaminants is required. In fact, GC-MS has been used for this type of problem solving since the first instrument was installed at the AWRI in 1971. GC-MS is now employed on a regular basis for ensuring wine quality remains at a high standard.

The advances in GC-MS and related techniques that have been of benefit for the analysis of volatile aroma compounds (as described above) are equally applicable in the investigation of off-flavour problems. Individual compounds responsible for off-flavour of wines are often present at extremely low concentrations. For instance, the well known off-flavour compound, 2,4,6-trichloroanisole (TCA) has low olfactory thresholds ranging from 1.4 ng/L (Duerr 1985) to 4.6 ng/L in wine (Liacopoulos et al. 1999). Thus, just as for volatile aroma compounds, the analytical methods for detecting off-flavour compounds in juice or wine are required to be at least as sensitive as the human nose.

Cork taint

Volatile chemicals sometimes have a characteristic aroma, which might cause a deterioration of the original character of juice or wine. Chloroanisoles, in particular TCA, represent the most frequently found taint compounds in wine and have been a major concern in the wine industry since 1979. The use of GC-MS is crucial for the detection of chloroanisoles at their extremely low odour threshold levels in wine. Pollnitz et al. (1996) developed a method for the detection of TCA and other chloroanisoles in tainted wines and corks using GC-MS coupled with SIDA. The use of polydeuterated TCA as an internal standard significantly improved the method in terms of its accuracy, precision and robustness. The detection limit in wines and corks varied between 0.5 and 2 ng/L, therefore, the method was sufficiently sensitive (low signal-to-noise) to detect TCA at its odour threshold levels. This method was used for the provision of analytical evidence for rejecting tainted cork consignments as well as quality assurance programs and research. The method has been formally offered on a fee-for-service basis since 1998. In 2004, the introduction of a solvent-free headspace extraction technique, SPME dramatically improved the productivity of the analysis by reducing sample preparation time, while accuracy, precision and detection limits remained essentially the same (unpublished AWRI internal reports).

Taint from aromatic hydrocarbons

Depending upon the source, the aromatic hydrocarbons found usually include one or more of the following compounds: toluene, styrene, alkylbenzenes and alkylnaphthalenes. Sensory characteristics of taints resulting from these contaminants are generally disagreeable and their aroma descriptors vary, but usually include 'plastic', 'chemical', 'musty', 'medicinal', 'kerosene', 'petrol' and 'turpentine'.

Styrene contamination has occasionally been evident in wine in contact with synthetic materials such as synthetic closures (Godden et al. 2001) and plastic or fibreglass containers during storage or transport (unpublished AWRI internal reports). Other volatile substances, which can be associated with polystyrene materials, include toluene, ethylbenzene, propylbenzene, as well as other alkylbenzenes (e.g. Baner 2000). Container-related contamination might occur in storage or transit as a result of compounds leaching into the wine from unsuitable or faulty epoxy-lined or fibreglass tanks or plastic containers. Diesel oil has been found in previous studies to contain the highest concentration of aromatic hydrocarbons (Strauss et al. 1985a). Naphthalene and alkylnaphthalenes are major components of kerosene and diesel oil, although other sources have also been noted for naphthalene taints.

Wine quality could be affected when tens of µg/L of an aromatic hydrocarbon contaminant is present. While the overall frequency of taint spoilage resulting from petroleum-derived aromatic hydrocarbons has been very low it still represents the second most common type of volatile taint encountered, second only to chloroanisoles in the past decade (unpublished AWRI internal report). In 2004, a screening method for petroleum-derived aromatic hydrocarbons was developed using GC-MS combined with SIDA and SPME techniques. Toluene, styrene, alkylbenzenes and alkylnaphthalenes were targeted for screening. Those compounds were detected by monitoring their characteristic ions (selected ion monitoring) and subsequently quantified using their respective deuterated internal standards. With this screening method the individual compounds were detectable at levels as low as 1 µg/L in red or white wine (Baldock and Hayasaka 2004).

Taints from chlorophenols

In 1978, chlorophenols were confirmed using GC-MS to be contaminants of tainted wines described as having medicinal, plastic, and/or a phenolic off-flavour. It was concluded that the presence of chlorophenols was the result of an interaction between an epoxy paint containing phenols that had been applied as an internal coating to a juice holding tank and a sterilising agent containing

chlorine used in cleaning the tank. A similar incident occurred in 1982, and, consequently, the AWRI advised the Australian wine industry to select only phenol-free products as coatings for vintage and vinification equipment. Since then, chlorophenol taints have occurred sporadically. Investigations by the AWRI over many years have revealed diverse sources of chlorophenol contamination. The usual source of chlorophenols was found to be either chlorinated sanitiser applied in the presence of a plastic, rubber or epoxy compound, or contact with chlorinated biocides either in the forest, at some stage in cork processing or during transport or storage of containers (including barrels). In 2002, SPME was applied to the extraction of chlorophenols from wine and ^{13}C labelled analogues of mono-, di- and tri- chlorophenols were synthesised and used as internal standards for quantification utilising SIDA. GC-MS coupled with SPME and SIDA is currently employed providing a rapid, robust and reliable method allowing for the determination of chlorophenols (unpublished AWRI internal report).

Brine contamination

Temperature control is one of the most important and essential requirements of modern winemaking. Refrigeration is used in most stages of vinification including grape, must and juice cooling, fermentation control, cold stabilisation and storage. Wineries commonly use a secondary refrigeration system with alcohol or glycol as a so-called 'brine'. Accidental brine contamination of juice, must or wine might occur even when a refrigeration system is well maintained. Depending on the scale of production, large quantities of wine could be affected if this occurs.

Methods used for the investigation of brine contamination depend upon the type of brine employed, e.g. glycol-based brine or alcohol-based brine and what additives are contained in the brine, e.g. dye, antioxidant or anticorrosion agent. In all cases, MS is a vital tool for the detection, confirmation and quantitative estimation of brine contamination in juice or wine. Propylene glycol is commonly used as glycol-based brine and can be detected and quantified by GC-MS. In 2004, a method for the detection of propylene glycol as well as ethylene glycol and diethylene glycol was developed using GC-MS combined with SIDA and SPME (unpublished AWRI method). Propylene glycol is rapidly and reliably quantifiable in a range of 10 to 250 mg/L, therefore, this method is capable of detecting approximately 0.001% glycol in wine. However, propylene glycol has been reported to be a naturally occurring compound in wine (McCallum and Muirhead 1982). From GC analytical data obtained from 49 uncontaminated New Zealand white wines the usual levels of propylene glycol were reported to be less than 0.003% (approximately 30 mg/L) (McCallum and Muirhead 1982). Therefore, propylene glycol is unsuitable for use as an indicator for glycol-based brine contamination except in cases of gross contamination. Preliminary in-house data from a small survey of randomly selected different varietal Australian white wines suggests that the concentration of naturally occurring propylene glycol in Australian white wine approximates that of the New Zealand white wines. A more representative survey of propylene glycol levels in wines from Australia and other countries would provide valuable baseline data for investigation of future brine leaks of this type.

Contamination with alcohol-based brine can be monitored and quantitatively estimated by measuring additives such as rhodamine by LC-MS-MS in juice or wine (Hayasaka and Baldock 2005). Detection limits for brine in red wine and white wine were estimated to be 0.001% and 0.0001% respectively. However, the concentration of rhodamine in brine can vary between manufacturers and usage conditions. Therefore, confirmation of brine contamination requires a reference sample spiked with the same 'suspect' brine.

Conclusion

In the past 30 years MS, coupled with a range of chromatographic techniques, or used off-line with novel sample preparation and introduction methods, has played an expanding and now pivotal role in wine and vine science. Traditional applications for MS such as in the analysis of volatile aroma compounds (including those derived from grapes, fermentations, or oak wood), grape flavour precursor compounds as well as off-flavour compounds, have all proceeded apace. With new applications of MS in research on proteins, pigments and tannins, the analytical horizon in grape and wine science has been significantly elevated. We confidently predict that these developments will continue and that MS and its associated methodologies will provide more technological breakthroughs in addressing future challenges facing viticulture and oenology. To date the message is clear: the provision of 'state-of-the-art' technology in combination with the talents and dedication of industry personnel and AWRI scientists has contributed to the Australian wine industry continuing to produce wines of quality and character that are in demand throughout the world.

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