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Direct mass spectrometry approaches to characterize polyphenol composition of complex samples

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ABSTRACT

Lower molecular weight polyphenols including proanthocyanidin oligomers can be analyzed after HPLC separation on either reversed-phase or normal phase columns. However, these techniques are time consuming and can have poor resolution as polymer chain length and structural diversity increase. The detection of higher molecular weight compounds, as well as the determination of molecular weight distributions, remain major challenges in polyphenol analysis.

Approaches based on direct mass spectrometry (MS) analysis that are proposed to help overcome these problems are reviewed. Thus, direct flow injection electrospray ionization mass spectrometry analysis can be used to establish polyphenol fingerprints of complex extracts such as in wine. This technique enabled discrimination of samples on the basis of their phenolic (i.e. anthocyanin, phenolic acid and flavan-3-ol) compositions, but larger oligomers and polymers were poorly detectable. Detection of higher molecular weight proanthocyanidins was also restricted with matrix-assisted laser desorption ionization (MALDI) MS, suggesting that they are difficult to desorb as gas-phase ions. The mass distribution of polymeric fractions could, however, be determined by analyzing the mass distributions of bovine serum albumin/proanthocyanidin complexes using MALDI-TOF-MS.

1. Introduction

Phenolic compounds are usually analyzed by reversed-phase high performance liquid chromatography (HPLC) coupled to diode array detection (DAD) and/or mass spectrometry (MS). The former enables the distinction of the various classes (e.g. anthocyanins, hydroxycinnamic acids and flavonols, flavan-3-ols) on the basis of their characteristic UV–visible spectrum, whereas the latter gives access to intact molecular ions and fragment ions from which both the molecular weight of the compound and information on its structural features can be established. However, HPLC separation is restricted to rather simple compounds that represent only a small proportion of phenolics, whereas polymers are generally eluted as unresolved broad peak(s). In particular, resolution of the chromatographic profiles becomes increasingly poor and analysis of proanthocyanidins (i.e. flavan-3-ol oligomers and polymers) becomes increasingly difficult as their molecular weight increases. This is due to the larger number of possible regio- and stereoisomers, and smaller amounts of each individual compound.

Proanthocyanidins are usually analyzed after their acid-catalyzed depolymerization in the presence of a nucleophilic reagent and conversion of their original extension and upper constitutive units into the corresponding derivatives. The reaction products obtained by this reaction (referred to as thiolysis when toluene α-thiol is used, Thompson et al., 1972; Foo and Porter, 1978) can be quantified by HPLC analyses (Rigaud et al., 1991; Prieur et al., 1994). This allows determination of the average composition of the proanthocyanidin chains and the opportunity to calculate their average degree of polymerization (aDP). On the other hand, it does not provide any information on the molecular mass distribution of a complex proanthocyanidin fraction. To overcome this problem, strategies relying upon direct flow injection mass spectrometry profiling, without prior separation, have been developed.

Mass spectrometers use the difference in mass-to-charge ratio (m/z) of ionized molecules to separate one from another. This requires first that the various molecular species of interest have been charged (often by loss or addition of protons) and transferred into the gas phase, and that they are then separated as a function of their m/z values. These two steps are achieved by the mass spectrometer source and analyser, respectively. Various instruments based on different sources and analysers are available, and the

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various combinations made from them provide a large range of analytical possibilities more or less suitable for a given application according to the specificity of the source and analyser.

Sources used to analyze phenolic compounds can be listed chronologically as follows: fast atom bombardment (FAB) (Self et al., 1986; Barofsky, 1988; Ohnishi-Kameyama et al., 1997), electrospray ionization (ESI) (Cheynier et al., 1997; Guyot et al., 1997; Le Roux et al., 1998; Fulcrand et al., 1999a,b; Revilla et al., 1999; Foo et al., 2000; Yang and Chien, 2000; Taylor et al., 2003), atmospheric pressure ionization (API) including atmospheric pressure chemical ionization (APCI) (Revilla et al., 1999) and atmospheric pressure photo-ionization (APPI) (Gomez-Ariza et al., 2006), and in parallel to the advent of electrospray advent, matrix-assisted laser desorption ionization (MALDI) (Ohnishi-Kameyama et al., 1997; Yanagida et al., 1999, 2000; Krueger et al., 2000; Foo et al., 2000; Yang and Chien, 2000). Thermospray analysis (TSP) has also been proposed but has proven to be unsuitable for the analysis of oligomers and polymers, due to thermal degradation (Gabetta et al., 2000). However, pyrolysis, a very drastic ionization method involving thermally assisted hydrolysis–methylation of the sample with tetramethylammonium hydroxide, coupled with GC–MS analysis, has proven useful for characterization of the highly degraded phenolic materials present in archeological samples, that proved to be problematic to other types of analysis (Garnier et al., 2003).

Samples are injected in solution into the ESI, APCI and TSP sources, which can thus be coupled to HPLC systems, whereas the use of MALDI and FAB require mixing of the sample with a matrix solution. In MALDI, a mixed solution of the analyte and a solid matrix, often an aromatic organic acid, is crystallized on the MALDI target by solvent evaporation. This target is then irradiated with a laser beam to vaporise the matrix, desorb the analytes into the gas phase and ionize, usually cationize them, by attachment of either protons or metal ions. In FAB, the sample mixed with a liquid matrix is applied to a probe tip and bombarded with a fast atom (or ion) beam. In these two methods, the sample preparation process is thus crucial to ensure the success of the experiment.

Other major differences between the various sources are the type of signals generated. Thus, FAB gives access to the molecular weight and specific fragments (Self et al., 1986; Barofsky, 1988), while ESI and APCI (Favretto and Flamini, 2000) as well as MALDI have been reported to yield cationic species with little or no fragmentation. However, an increase in either cone voltage (ESI) or laser energy (MALDI) increases the propensity toward fragmentation (Taylor et al., 2003).

ESI, APCI, FAB, and MALDI are operated in both the negative and positive ion modes, generating anions such as $[M-H]^-$ and $[M+Cl]^-$ or cations such as $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$, respectively; ESI yields multiply charged ions ($z = 1, 2, 3 \ldots$), while MALDI and FAB yield mostly singly charged ions ($z = 1$).

Analyzers that have been used to analyze phenolic compounds are quadrupole (Q), magnetic sector (B), ion-trap (IT), time-of-flight (TOF), and Fourier-transform ion cyclotron resonance (FT-ICR) that differ, among other factors, by the available mass range and resolution. TOF gives access to a theoretically unlimited mass range and is thus well suited for analysis of high molecular weight polymers, and also provides high resolution, giving access to accurate mass determination. FT-ICR provides the highest mass resolution and most accurate mass determination, making it theoretically possible to assign molecular formula unambiguously for smaller oligomers. Further information on the molecular structures can be gained by multiple step mass spectrometry (MS/MS or MS^n) experiments. This consists in isolating specific ions for fragmentation in a first stage of mass analysis and then inducing their dissociation by collision with inert gas molecules so as to analyze the fragments thereby yielded, in the second stage of mass analysis. Tandem mass spectrometry (MS/MS) experiments can be achieved by mass spectrometers combining two analyzers in series (e.g. triple quadupole, Q-TOF, and Q-IT). Some of them allow monitoring of selective fragments which differ by a predetermined amount (neutral loss) and can be used for identification of particular derivatives, e.g. 162 for glucosyl derivatives (Hayasaka and Asenstorfer, 2002) and for quantification. The ion-trap enables successive fragmentations of selected precursor ions (MS^n) which is very helpful in the identification of unknown compounds.

ESI and APCI sources can be coupled to various analyzers, including quadrupole (Q), ion-trap (IT), time-of-flight (TOF), and Fourier-transform ion cyclotron resonance (FT-ICR); additionally, MALDI is coupled primarily to the last two, whereas FAB has been generally used in conjunction with magnetic sector instruments. Although both ESI-TOF and MALDI-TOF are capable of detecting intact molecular ions with high masses, ESI is poorly suited for characterization of polydisperse polymers due to the signal complexity arising from the formation of multiply charged ions. MALDI-TOF MS usually produces only a single molecular ion for each parent molecule and allows detection of high mass ions with excellent precision when delayed extraction and reflection ion mirrors are employed (Montaudo et al., 2002). It has been used to determine the molecular weight distributions of polymers in complex samples, the amount of each species being determined directly from ion abundances in the mass spectrum (Hanton, 2001). The number-average molecular weight (M_n), and the weight-average molecular weight (M_w) values obtained, on one hand from the MALDI-TOF mass spectra, on the other hand from gel permeation chromatography (GPC) analysis, are comparable for various types of polymers such as polystyrenes. However, MALDI-TOF measurements provide correct average M_w's only for samples with narrow molecular weight distributions ($M_w/M_n < 1.2$) so that much more accurate values can be obtained after GPC fractionation (Montaudo et al., 1995).

Moreover, MALDI-TOF shows decreased sensitivity with increasing molecular weight. Investigations performed on DNA mixtures demonstrated that instrumental effects, namely ion detection efficiency, detector saturation, and ion beam divergence, rather than effects due to the chemical behaviours of the analytes, such as fragmentation and variations in ionization efficiency or suppression effects, were responsible for the fall off of signal intensity with increasing mass (Chen et al., 2003).

Further information on mass spectrometry (Dass, 2002; Downard, 2004; Siuzdak, 2004) and its application to polymer analysis (Hanton, 2001) can be found in general reviews. The present paper describes applications of MS techniques, for both profiling phenolic composition of plant extracts and determination of proanthocyanidin molecular weight distributions.

2. Results and discussion

2.1. Phenolic profiling by mass spectrometry

Phenolic compounds can be detected by mass spectrometry, either in the positive or in the negative ion mode. The positive ion mode is very well suited for the detection of anthocyanins which are present in acidic extracts as their cationic forms (i.e. flavylium ions). The negative ion mode, by contrast, has proven more efficient for anthocyanin derivatives bearing a carboxylic acid group (Fulcrand et al., 1998). Both modes have been successfully used to analyze uncharged flavonoids such as flavan-3-ols which have been detected as deprotonated $[M-H]^-$ species (Cheynier et al., 1997; Guyot et al., 1997; Le Roux et al., 1998; Fulcrand et al., 1999a), and as positive $[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$ ions (Gabetta et al., 2000; Taylor et al., 2003; Mané et al., 2007).

APCI and ESI sources have been compared for phenolic analysis after HPLC separations. The APCI source showed poor sensitivity for anthocyanins which were best analyzed by ESI-MS (Revilla et al., 1999). Direct flow injection analysis, i.e. without prior HPLC separation, using a Q-IT mass spectrometer equipped with an ESI interface, has been successfully applied to establish grape anthocyanin profiles (Favretto and Flamini, 2000). Structural identification of anthocyanins was based on the molecular ion detected in the positive ion mode M with fragmentation patterns obtained by means of multiple step mass spectrometry. Thus anthocyanins, which are glycosylated and often further acylated, yield a characteristic anthocyanin aglycone fragment ion. The presence of isobaric compounds in the mixtures was shown by MS^n experiments, as fragment ions corresponding to more than one aglycone were generated. This approach was unable to differentiate between isobaric compounds based on the same aglycones which yielded identical fragment ions (e.g. malvidin- 3,5-*O*-diglucoside and malvidin 3-(6-*O*-caffeoyl)monoglucoside, both with *m*/*z* 655, and aglycone ion signal at *m*/*z* 331). This was, however, achieved by deuterium exchange experiments, leading to different mass shifts in agreement with the respective numbers of exchangeable protons in the molecules. The relative abundances of M^+ species in the ESI mass spectrum, with respect to the intensity of the malvidin 3-glucoside signal (at *m*/*z* 493), were used as a semi-quantitative estimation of anthocyanin composition for rapid comparison of grape varieties. Although suppression effects, i.e. suppression of ionization towards some components of a mixture by that of others, are classically described, they were considered minimal in the case of anthocyanins that were present as cations in the original acidic solution.

More recently, APPI and ESI both coupled to a Q-TOF mass analyser were applied to establish anthocyanin profiles as fingerprints of red wines (Gomez-Ariza et al., 2006). Both methods allowed detection of a large number of pigments in wine, including the grape anthocyanins and products formed from them in the course of winemaking. However, after suitable optimisation of the analysis conditions (solvent, temperature, declustering, and focusing potentials), APPI proved to produce less chemical noise background from solvent molecules, thus improving the signal-to-noise ratio, compared to ESI. However, ions corresponding to anthocyanin aglycones also showed very high intensities in the wine full-scan mass spectra, meaning that some fragmentation took place under these optimised conditions. Nevertheless, both procedures show potential as high sample throughput methods for red wine classification.

Another study aiming at establishing wine fingerprints without any prior purification step compared positive and negative-ion ESI combined with Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometry (Cooper and Marshall, 2001). Identification of these compounds was based on accurate mass determinations provided by the FT-MS instrument. The positive-ion mass spectra of red wines were dominated by anthocyanins but also showed proanthocyanidin dimers, flavonols and dihydroflavonols (as their protonated forms and potassium adducts). Most of these compounds were previously known as wine constituents, but identification of some methoxylated flavan-3-ol and flavanonol derivatives that are reported for the first time requires confirmation. Elemental compositions were assigned for most of the peaks in the negative ion spectra but, surprisingly, none of them were attributed to phenolic compounds. Although the negative ion spectra showed higher discriminating potential, the positive ion mass spectra thus appeared preferable for phenolic fingerprinting.

Direct flow injection in either an ESI source or an APCI source coupled on line with the TOF analyzer were used to analyze, in both the negative and positive ion modes, a series of red wines from two different varietal and geographical origins (namely Gamay Beaujolais wines from France and Dornfelder wines from Germany). Four mass spectra were thus recorded from each of the 122 wine samples studied (Preys, 2006).

The major ion signals detected in the positive ion mode (Fig. 1a) could be easily attributed to anthocyanins (Fig. 2, top). Thus, the signals at *m*/*z* = 493, 535, and 639 correspond, respectively, to malvidin-3-glucoside, and its acetylated and *p*-coumaroylated derivatives, with the signal at *m*/*z* = 331 corresponding to their aglycone fragment, and that at *m*/*z*= 619 to the aglycone of the flavan-3-ol-malvidin 3-glucoside dimer previously shown to occur in wine (Salas et al., 2004). The mass spectra in the negative ion mode were much more complex and showed a number of ions that could be interpreted as proanthocyanidins (e.g. *m*/*z* = 577) and fragment ions arising from cleavage of their interflavanyl bonds (upper units at *m*/*z* = 287, 575 and 591; lower units at *m*/*z* = 289 and 577) as illustrated in Fig. 2, along with molecular ions of hydroxycinnamic acids (at *m*/*z* = 295 and 311, for *p*-coumaroyl and caffeoyl tartaric esters) and of anthocyanins, detected as the $[M-2H]^-$ species at *m*/*z* = 329 and 491 (Fig. 1b). Thus, most of the major ions detected in all four mass spectra could be interpreted as phenolic signals so that these mass spectra, obtained from direct flow injection analysis of wines, can be considered as a phenolic fingerprint.

Principal component analysis of the MS data permitted distinguishing Beaujolais from Dornfelder wines along the first principal component (PC1, accounting for 46% of the variance) and wines from different vintages along the second one (PC2, 11% of the variance), as illustrated in the case of negative ion mode ESI-TOF signals (Fig. 3a). Anthocyanin signals were positively correlated to the first principal component, while ions corresponding to hydroxycinnamic acids and fragments characteristic of proanthocyanidins were negatively correlated to this first principal component (Fig. 3b). A very good congruence was observed with the similarity map obtained from phenolic composition data determined by HPLC analysis in the plane defined by the first two principal components (Preys et al., 2006). This confirms that most of the information contained in the mass spectra was related to phenolic composition.

2.2. Determination of proanthocyanidin molecular weight distribution by mass spectrometry

Mass spectrometry has been used to analyze proanthocyanidins from various plant sources.

FAB-MS gives access to the molecular weight and some specific fragments, arising from cleavage of the interflavanyl bonds, that can be attributed respectively to the lower units (initially substituted in 8 or 6 position) and to upper and extension units, detected as the corresponding quinone methide (Barofsky, 1988). In FAB, the most common matrix is glycerol but the "magic bullet" matrix, consisting of dithiothreitol–dithioerythritol (5/1) is superior to glycerol for FAB-MS analysis of procyanidin oligomers (Barofsky, 1988). FAB is mostly used for identification of isolated molecules but can also be applied directly on complex mixtures. This allowed the detection of a series of procyanidin oligomers up to the undecamer in apple extracts, with similar intensities in both the positive and negative ion modes (Ohnishi-Kameyama et al., 1997). However, the method is unsuitable for determination of oligomer distributions, due to fragmentation of proanthocyanidins, generating ions with signals identical to those of lower molecular weight species (Ohnishi-Kameyama et al., 1997).

Flow injection ESI-MS and MALDI-TOF-MS have been successfully applied for direct analyses of proanthocyanidin extracts. Among the various matrices available, *trans*-3-indoleacetic acid (IAA) (Ohnishi-Kameyama et al., 1997; Krueger et al., 2000) and 2,5-dihydroxybenzoic acid (DHB) (Yang and Chien, 2000) were reported to be the best matrices for proanthocyanidin analysis by

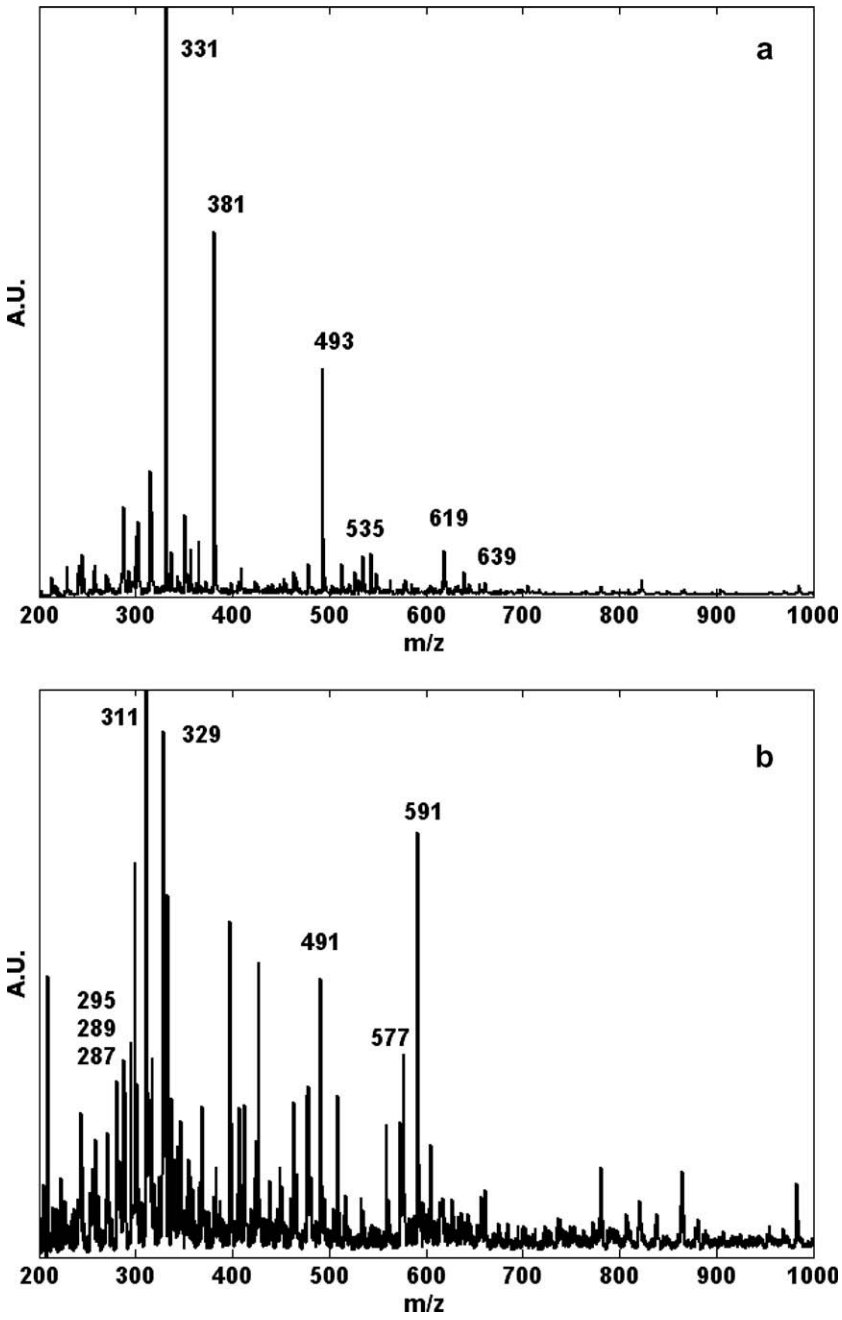

Fig. 1. Average ESI-TOF mass spectra of the 122 wine samples in the positive (a) and negative (b) ion modes.

MALDI-TOF-MS. IAA gave $[M-H]^-$ signals in the negative ion mode, whereas alkali metal adducts $[M+Na]^+$ and $[M+K]^+$ appeared in the positive-ion mode. When silver trifluoracetic acid solution was added to the IAA matrix, only silver ion adducts were formed, resulting in improved sensitivity.

Analysis of proanthocyanidin extracts from grapes (Cheynier et al., 1997; Hayasaka et al., 2003), apple (Guyot et al., 1997), and litchi (Le Roux et al., 1998) by direct flow injection ESI-MS in the negative ion mode enabled detection of signals corresponding to $[M-H]^-$ ions of procyanidin oligomers (up to the pentamer) but also to doubly charged ($[M-2H]^{2-}$) ions for higher molecular weight species (DP > 4) and triply charged ions ($[M-3H]^{3-}$) for DP > 10. For each, detected proanthocyanidin signal, this allows the determination of the number of hydroxyl groups in constitutive units (Fulcrand et al., 1999b), the degree of polymerization (DP), the presence of galloyl (Cheynier et al., 1997; Hayasaka et al., 2003) or glucosyl (Rodrigues et al., 2007) substituents, and the number of A or B-type linkages (Le Roux et al., 1998).

Interpretation of mass signals arising from both singly- and multiply-charged species is problematic as singly charged signals of DP *n* species overlap with doubly charged signals of DP 2*n* species (and triply charged ions of DP 3*n* species) as shown in Fig. 4. However, those corresponding to doubly charged ions of species with an uneven DP can be unambiguously defined. Polymers up to DP 22 were detected (as triply charged ions) by ESI-Q-MS in litchi skin (Le Roux et al., 1998). The charge state of a given signal can be determined from the distance between the peaks making up its natural isotopic distribution. Thus, the spacing between the isotopic peaks is equal to one mass unit for singly charged ions, 0.5 mass unit for doubly charged ions, 0.33 mass unit for triply charged ions, and so on. Interpretation of the charge states in a single mass signal is limited by the resolution of the mass spectrom-

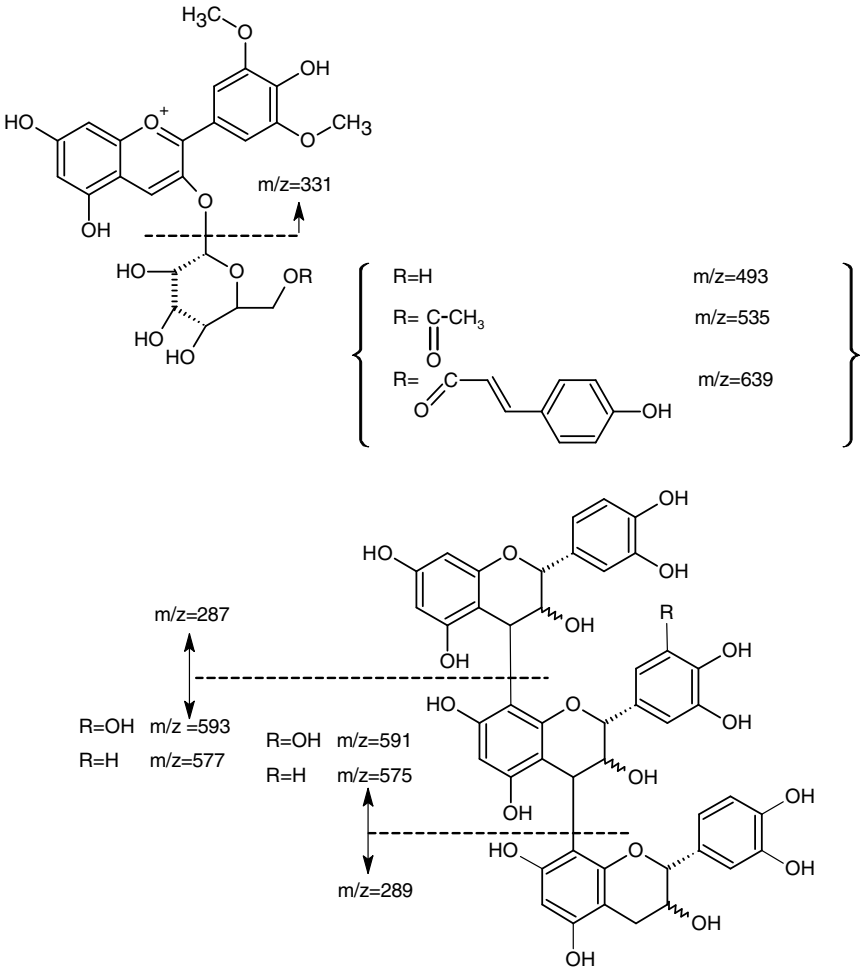

Fig. 2. Structures and fragments of malvidin 3-glucoside derivatives (top) and proanthocyanidins (bottom).

eter and can be much improved by the use of ESI-FT-MS instrumentation as illustrated in Fig. 5.

In ESI-MS, negative ionization is more sensitive and selective for phenolic compounds than the positive ion mode, due to the gentle acidity of the hydroxyl groups (Rodrigues et al., 2007), but proanthocyanidins have also been analyzed in the positive ion mode using either ESI or MALDI mass spectrometry (Gabetta et al., 2000; Taylor et al., 2003; Perret et al., 2003; Mané et al., 2007). MALDI-TOF MS analysis yields mostly singly charged ions and enabled detection of signals corresponding to proanthocyanidin polymers up to DP 12 (Ohnishi-Kameyama et al., 1997) in apple, to DP 20 (Taylor et al., 2003) in hop, as the corresponding $[M+H]^+$ ions, and up to DP 30 as $[M+K]^+$ ions in soybean seed coat extracts (Takahata et al., 2001). ESI-MS analysis of the hop extract in the positive ion mode yielded $[M+H]^+$signals up to DP 5 and doubly charged $[M+2H]^{2+}$ signals for larger polymers (Taylor et al., 2003), which rendered its interpretation more complex, but failed to detect any species beyond DP 15. The MALDI mass spectra thus proved best suited for detecting larger molecular weight proanthocyanidins in complex mixtures, which is also well documented for other types of polymers (Hanton, 2001). Further improvement was achieved after fractionation of the extract, where higher molecular weight compounds were more easily detected after elimination of the smaller oligomers, as shown earlier for DNA mixtures (Chen et al., 2003).

It has been postulated that the intensity of each peak signal in the mass spectrum can be used as a measure of relative abundances of oligomers present in the original solution. The distribution of mass signals in the ESI mass spectra of fractions containing A-type procyanidin oligomers from litchi were consistent with their average degree of polymerization (aDP) estimated by thiolysis (aDP 5 and 7) (Le Roux et al., 1998). Similarly, the aDP calculated from MALDI-TOF-MS signals for a cranberry proanthocyanidin oligomer fraction (aDP 4.7) was consistent with that obtained by ^{13}C spectrometry (Foo et al., 2000). However, this hypothesis has proven invalid for higher molecular weight proanthocyanidins, which were not efficiently detected.

The discrepancy between the molecular weight estimated by thiolysis and by mass spectrometry increases with the aDP value (Taylor et al., 2003; Mané et al., 2007). Thus, the congruence between MALDI-TOF-MS spectra and the aDP determined by thiolysis was rather poor for polymeric fractions (aDP 9 and aDP 28, isolated from apple). The largest species detected in these two fractions corresponded to DP 13 and 15, respectively and aDP values around 5 and 6, respectively, were calculated from the distribution of intensity values of all proanthocyanidin peaks in the mass spectra (Mané et al., 2007). This may be due to instrumental effects such ion beam divergence, ion detection efficiency or detector saturation effects that were proven to be more pronounced with increasing mass. However, these instrumental effects cannot be the single cause of non-detection of higher molecular weight species as the fractionation of crude proanthocyanidin extracts prior to mass analysis greatly improves the detection of larger molecular weight species (Taylor et al., 2003). Nevertheless, the largest polymers within these purified fractions are still undectable by MS, likely due to their lower propensity to desorption. In fact, the mass val-

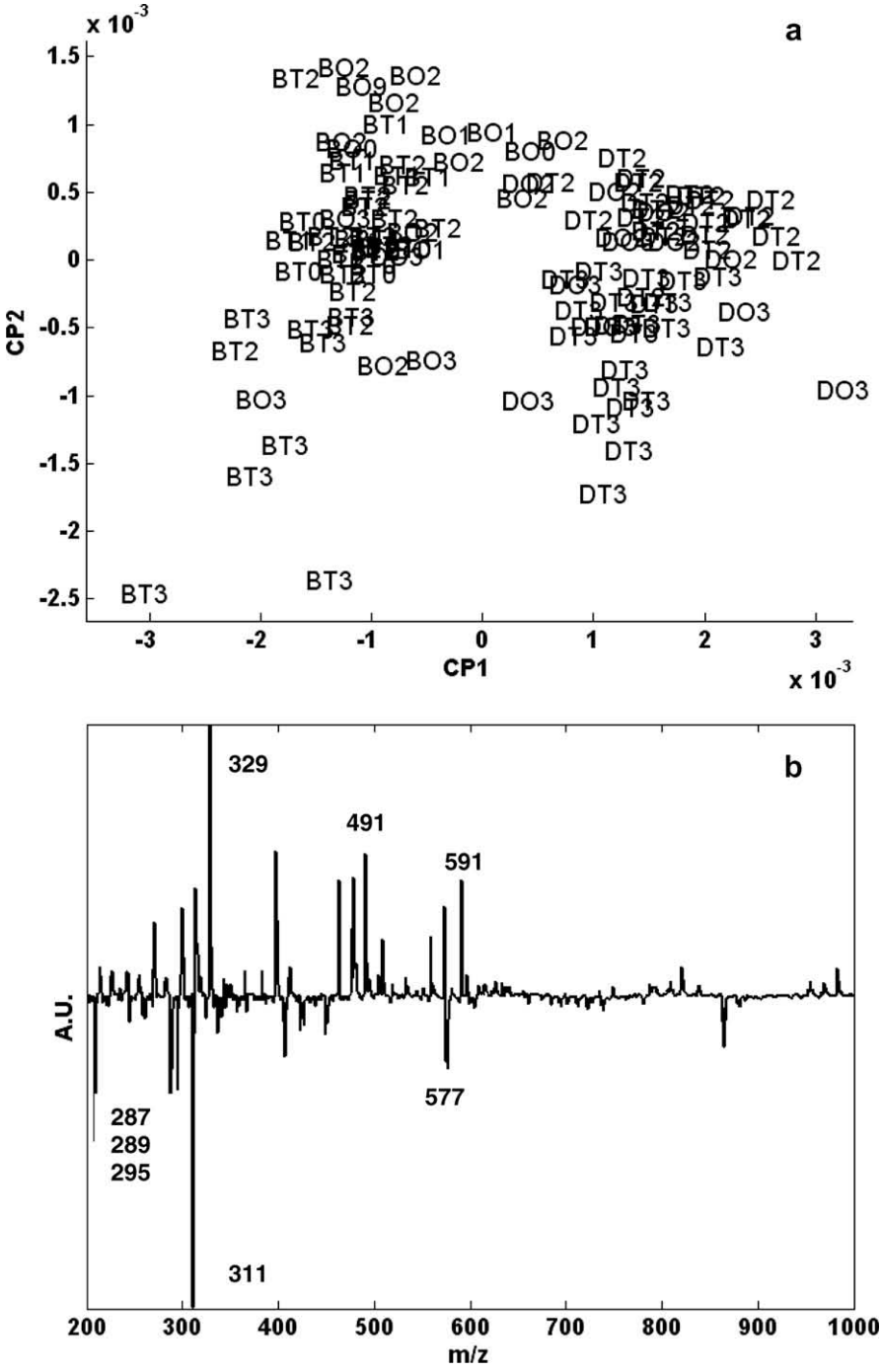

Fig. 3. Left: Projection of the wines of the first two principal components obtained from PCA of the ESI-MS data (negative ion mode) – B, D: Beaujolais and Dornfelder selections, respectively, 0, 1, 2, 3: 2000, 2001, 2002, 2003 vintages, respectively. Right: Loading of mass signal intensities on the first principal component.

ues of signals corresponding to the DP 10, 12, 15, and 20 proanthocyanidins in the MALDI mass spectrum of a hop extract were slightly different from the calculated expected masses (Taylor et al., 2003). This was explained by the formation of ion clusters by in source fragmentation of the polymers and non-covalent aggregation of the resulting fragment ions, as confirmed by FT-ICR-MS. While reducing the laser energy should theoretically limit fragmentation, this resulted in no detectable signals for this polymeric fraction (Taylor et al., 2003).

Thus, ESI and MALDI MS methods fail to provide a full picture of proanthocyanidin mass distributions, as the conditions required to detect higher molecular weight proanthocyanidins also force these compounds to fragment.

To overcome this problem and achieve better evaluation of mass distribution, an alternative strategy using protein complexation was developed (Mané et al., 2007). This approach is based on the assumption that the protein can both serve as a charge carrier, thus limiting differences in ionization efficiency among the various proanthocyanidin structures, and protect polymeric procyanidins from fragmentation. MALDI-TOF-MS analysis was selected as interpretation of the ESI-MS spectra would have been much more difficult, due to the occurrence of numerous charge states for each given protein or complex species. Among the proteins tested, only BSA yielded proanthocyanidin complexes that were detected by MALDI-TOF-MS. Several matrices and spotting methods were tested. The best signal-to-noise ratio was obtained with a-cyano-

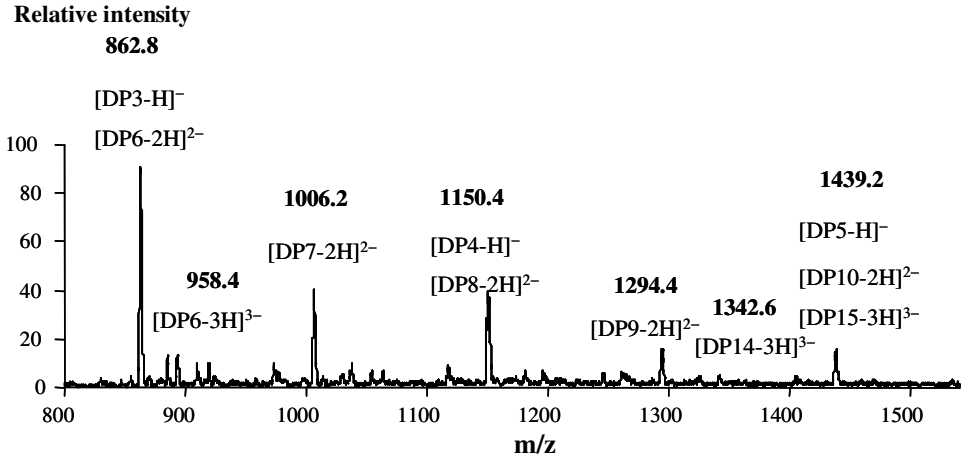

Fig. 4. Negative ion mode ESI-Q mass spectrum of a litchi skin extract (mass range: 800–1500).

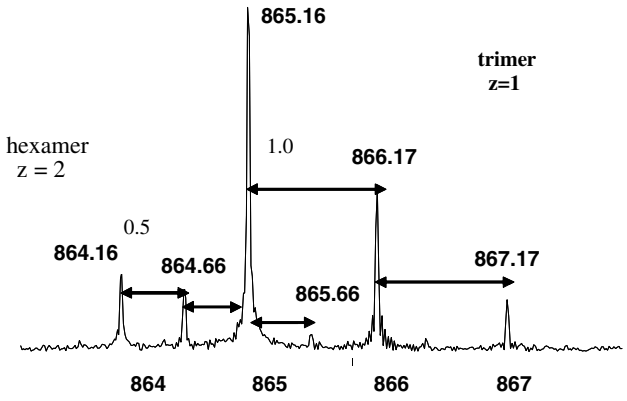

Fig. 5. Resolution of doubly-charged and monocharged ions of oligomeric procyanidin signals (DP 6 and DP 3, respectively) by ESI-FT-ICR-MS.

4-hydroxycinnamic acid as matrix with double droplet spotting. The protein affinity of flavan-3-ols has been shown to increase with their molecular weight (Sarni-Manchado and Cheynier, 2002), and binding of several flavan-3-ols to a single protein has been reported at higher polyphenol to protein ratios (Poncet-Legrand et al., 2006). To avoid biases due to differences in selectivity and overlapping of signals corresponding to different complex stoichiometries, conditions ensuring formation of 1/1 complexes were selected (BSA concentration: 1 mg L^{-1}, 1:1 stoichiometry, calculated using the average proanthocyanidin molecular weight determined by thiolysis). Under these conditions, the protein/procyanidin complexes were detected as a broad signal. The number-average molecular weight calculated from these signals ($M_n = \sum(m/z)_i * I_i)/\sum I_i$, with I = absolute intensity) for each of the proanthocyanidin fractions (namely aDP 3 from grape seeds, aDP 9 and aDP 27 from apple) was in good agreement with the average molecular weights calculated from thiolysis data, confirming that the mass distributions of detected BSA–procyanidin complexes reflect those of procyanidins. The distribution of procyanidin DP can be estimated by subtracting, for each discrete m/z value, the intensity of the BSA signal from that of the BSA/proanthocyanidin complex.

3. Conclusions

Mass spectrometry appears well suited to analyze the phenolic compositions of complex plant extracts such as wine, or in various applications such as authentication of food products, metabolic profiling of plant extracts, and process monitoring. However, larger molecular weight proanthocyanidins cannot get properly be detected, suggesting that ionization suppression and reduced desorption may occur in direct flow injection ESI-MS and MALDI-TOFMS analyses, respectively, as well as in source fragmentation under the conditions used both for direct flow injection ESI-MS and MALDI-TOFMS analyses. The conversion of proanthocyanidins into protein complexes appears to be a good approach for analyzing the distribution of proaznthocyanidin oligomers by MALDI-TOF-MS. That is, MALDI-TOF analysis of BSA–proanthocyanidin complexes enables determination of proanthocyanidin molecular weight distributions as the charge is provided by the protein and this protects the higher molecular weight polymers from fragmentation through complexation. This method represents a rapid alternative to methods involving depolymerization in the presence of a nucleophilic agent followed by HPLC separation for the determination of average molecular weight of polymeric polyphenol fractions, as well as size exclusion chromatography for providing information on polymeric distributions. Further developments will be aimed at evaluating its suitability for the determination of complex phenolic derivatives arising, for instance, from oxidation reactions.

4. Experimental

4.1. MS analyses

Wine samples (2 μL) were injected directly into a time of flight mass spectrometer (AccuTOF™ JMS-T100LC, Jeol, Japan) and analyzed using either atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI) in the positive and negative ion modes, yielding four mass spectra for each wine. The source voltage was set at −3500 V (negative APCI), 3394 V (positive APCI), −2000 V (negative ESI) and +2000 V (positive ESI), the orifice voltage at −30 V (negative APCI), +30 V (posistive APCI), −150 V (negative ESI) and +135 V (positive ESI), with the mass range being from 200 to 1000 Da.

Fourier-transform mass spectrometry experiments were performed on a grape seed fraction (aDP 9) isolated as described by Vidal et al. (2003) using a Bruker (Billerica, MA) Apex II 7.0 T Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometer. Electrospray current was maintained at 20–50 nA. Low flow electrospray (nanospray) was performed using sprayer tips purchased from New Objective (Woburn, MA). To reduce clogging, nanospray tips were broken to widen the aperture. For each solution, at least three mass spectra were acquired under the same conditions.

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