Study of Wine Tannin Oligomers by On-Line Liquid Chromatography Electrospray Ionization Mass Spectrometry

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Thiolysis of a wine tannin fraction yielded trihydroxylated flavanol units (as previously observed in grape skins) in addition to the well-known procyanidins (dihydroxylated units), usually described in the literature for grape condensed tannins. To determine how they occur in condensed tannins, the wine fraction was analyzed by liquid chromatography coupled to electrospray ionization mass spectrometry. Thus, various series of ion peaks containing a variable number of trihydroxylated units were detected as monocharged ions from dimers up to pentamers. From pentamers, oligomers were found as doubly charged ions. Heptamer species corresponded to the highest mass detected. These results showed that wine condensed tannins consist of, besides procyanidins, mixed tri- and dihydroxylated flavanol units and also of pure trihydroxylated flavanol units. These new data should be taken into account to interpret organoleptic properties of wines.

Keywords: *Wine tannins; trihydroxylated flavanol units; proanthocyanidins; procyanidins; prodelphinidins; ESI-MS; LC/MS*

INTRODUCTION

Polyphenols largely contribute to organoleptic properties of fruits and derived products. In particular, anthocyanin pigments are responsible for reddish colors (Markakis, 1982), whereas condensed tannins are usually associated with astringent perception (Singleton, 1972; Lea and Arnold, 1978; Haslam and Lilley, 1988; Somers and Verette, 1988; Clifford, 1997). Nevertheless, only a few studies have chemically investigated the correlation between the latter property and the structure of condensed tannins. Condensed tannins are oligomers and polymers of flavan-3-ols, also named proanthocyanidins because their upper units yield anthocyanidins (colored cations) when heated in acidic medium. Among them, several classes can be distinguished on the basis of their hydroxylation pattern. These include, in particular, procyanidins consisting of catechin units [(+)-catechin and (−)-epicatechin] (Figure 1, R = H) and prodelphinidins, based on gallocatechin units [(+)-gallocatechin and (−)-epigallocatechin] (Figure 1, R = OH). In fact, the lack of chemical studies is probably due to the difficulty in achieving tannin analysis, given their polymeric nature and large structural diversity. Structural investigations have been performed by thiolysis, mass spectrometry, and NMR analyses. Thiolysis is generally applied to a crude fraction of condensed tannins, and it allows the determination of both the average composition and the mean degree of polymerization (mDP) (Thompson et al., 1972; Rigaud et al., 1991; Prieur et al., 1994; Matthews et al., 1997). Electrospray ionization occurring at atmospheric pressure can be therefore easily interfaced with a liquid chromatograph, allowing analysis of each constituent within a mixture. Thus, the technique has been largely used to analyze polar molecules such as polyphenols (Baldi et al., 1995; Fulcrand et al., 1996; Nawwar et al., 1997). Applications to tannins isolated from apples allow one to detect B-type procyanidins with polymerization degrees up to 17 (Guyot et al., 1997). Besides, similar analyses of lychee pericarp extracts revealed the presence of A-type linkages along with the most common B-type linkages within the tannin skeleton (Le Roux, 1998). Other particularities may occur in the structure of some condensed tannins, as in the case of grapes. Besides (+)-catechin and (−)-epicatechin residues, (−)-epicatechin-*3*-*O*-gallate (more abundant in seeds than in skins) and (−)-epigallocatechin (only in skins), which is trihydroxylated on the B ring, are released by thiolysis of grape tannin extracts (Rigaud et al., 1991; Prieur et al., 1994; Souquet et al., 1996). The corresponding structures are displayed in Figure 2. Thus, grape seed tannins are partly galloylated procyanidins, whereas grape skin tannins contain both prodelphinidin and procyanidin units. Although (−)-epigallocatechin usually accounts for 20−30% of tannin units in grape

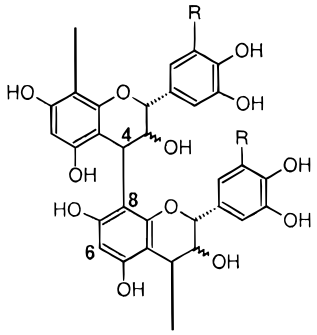

Figure 1. Structure of condensed tannins.

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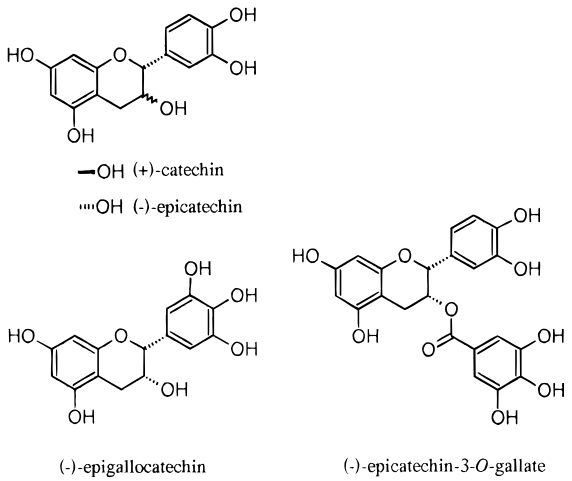

Figure 2. Constitutive units of grape condensed tannins.

skins, the only oligomeric tannins detected so far in grape and wine are partly galloylated procyanidins (Czochanska, 1979; Boukharta et al., 1988; Ricardo Da Silva, 1991; Escribano-Bailon, 1992). Therefore, the question was to determine the origin of trihydroxylated units detected by thiolysis. Do polymers consisting of pure trihydroxylated units (prodelphinidins) exist, or are the latter mixed with dihydroxylated units (procyanidins) as shown earlier in barley (Outtrup, 1981; Mulkay et al., 1981)? The results of electrospray mass spectrometry analysis of a wine tannin fraction are presented herein.

EXPERIMENTAL PROCEDURES

Sample. The wine selected is a *Vitis vinifera* var. Cabernet Sauvignon wine, made at Arzens (southern France), vintage 1994.

Fractionation Procedure. A wine aliquot (5 mL) was dealcoholized by concentration under vacuum prior to fractionation. The concentrate was then deposited on a Fractogel (Toyopearl TSK gel HW-50 (F), bed 12 × 120 mm) column. A water wash (25 mL) was performed to discard eventual residual sugars. Simple polyphenols were eluted with a mixture (50 mL) of ethanol, water, and trifluoroacetic acid (55:45:0.005; v/v). The polymeric fraction was recovered by elution with 60% acetone in water (50 mL). After concentration under vacuum, the aqueous solution containing wine polymeric tannins was freeze-dried. The powder thus obtained was analyzed by HPLC-DAD as follows and stored in a desiccator under vacuum.

LC-DAD Analysis. The polyphenolic composition of the wine fractions (F1 and F2) was determined by HPLC with a diode array detector (DAD). The apparatus is a Waters-Millipore system including two M510 pumps, a U6K manual injector, an automated gradient controller, and a 990 DAD. The elution conditions were as follows: flow rate, 1 mL/min; temperature, 30 °C; column, reversed-phase Licrospher 100-RP18 (5 μm, 250 × 4 mm) protected with a guard column of the same material (10 × 4 mm); solvent A, 2% HCOOH in H_2O; solvent B, $CH_3CN/H_2O/HCOOH$ (80:18:2, v/v/v); isocratic gradient, 2% B in 3 min; linear gradients from 2 to 10% B in 2 min, from 10 to 30% B in 20 min, from 30 to 50% B in 10 min, from 50 to 60% B in 5 min, and from 60 to 90% B in 5 min, followed by a return to initial conditions in 5 min and by re-equilibrating the column. UV−visible spectra were recorded on-line from 250 to 600 nm, and the system was calibrated with caftaric acid (at 310 nm) and catechin (at 280 nm) for quantification of hydroxycinnamic acids and flavanols, respectively. As well, concentrations of anthocyanins were calculated from the peak area of malvidin-3-*O*-monoglucoside at 515 nm, and those of flavonols were evaluated from the peak area of quercetin at 365 nm.

Thiolysis. The tannin powder was dissolved in methanol (1 mg/mL) and introduced into a glass vial together with an equal volume of a 5% solution of toluene-α-thiol in methanol containing HCl (0.2 M). After sealing and heating at 90 °C for 2 min, the thiolyzed solution was analyzed directly by HPLC. This procedure was performed twice, and the results presented herein are an average of the two determinations. The apparatus was an HPLC system (Kontron, Milan, Italy) including a model 430 dual-wavelength detector, a model 460 autosampler, and a model 325 low-pressure pump. The elution conditions were as follows: flow rate, 0.8 mL/min; temperature, 30 °C; column, Nucleosil 120-3 μm−C18 (125 × 4 mm); solvent A, 2% HCOOH in H_2O; solvent B, $CH_3CN/H_2O/HCOOH$ (80:18:2, v/v/v; linear gradients from 15 to 75% B in 15 min and from 75 to 100% B in 5 min, followed by washing and re-equilibrating the column; detection, UV 280 nm. Identification and calibration (based on peak areas at 280 nm) were performed using flavan-3-ol and benzylthioether standards prepared as described earlier (Prieur et al., 1994).

LC/MS Analysis. The MS apparatus coupled to the chromatographic system was a Sciex API *I Plus* simple quadrupole mass spectrometer with a mass range of 2400 mass units, equipped with an electrospray ion source (Sciex, Thornhill, Ontario, Canada). The mass spectrometer was operated in the negative-ion mode. Ion spray voltage was set at −4000 V and orifice voltage at −60 V. HPLC separation was carried out on a narrow-bore reversed-phase column (Superspher 100-RP18, 3 μm packing, 125 × 2 mm i.d., Merck, Darmstadt, Germany) with an ABI 140B solvent delivery system (Applied Biosystems, Weiterstadt, Germany). The column was connected with the ES interface via a fused-silica capillary (length 100 cm, 100 μm i.d.). The sample was injected with a rotary valve (Rheodyne model 8125) fitted with a 20 μL sample loop. Elution was achieved with solvents A (2% formic acid in water) and B (acetonitrile/water/formic acid, 80:18:2, v/v) by using a two-step linear gradient at a flow rate of 200 μL/min. The conditions were as follows: from 5 to 30% of solvent B in 20 min and from 30 to 50% B in 10 min, followed by washing and reconditioning of the column. The absorbance at 280 nm was monitored by an ABI 785A programmable absorbance detector. The flow was split after UV detection so that 50 μL/min went to the electrospray source.

RESULTS AND DISCUSSION

Wine analysis is currently performed with an LC system coupled to a DAD either directly or after wine fractionation on a Fractogel column. The fractionation procedure allows separation of wine constituents between simple polyphenols and polymeric material (Derdelinckx and Jerumanis, 1984; Ricardo Da Silva et al., 1991; Prieur-Delorme, 1994). Thus, the first fraction contains mainly phenolic acids, flavonols, anthocyanins, and flavanol monomers [(+)-catechin, (−)-epicatechin, (−)-epicatechin-3-*O*-gallate]. The second fraction collected after flavanol monomer elution, consists of oligomeric and polymeric compounds. These are, in part, unchanged condensed tannins and, for the rest, polymers resulting from different reactions with other wine constituents occurring in the course of wine-making and wine maturation (Jurd, 1969; Haslam, 1980; Cheynier et al., 1997a). HPLC analysis of the first fraction allows one to assign easily the different types of compounds according to their specific absorbance spectra and their retention times, whereas HPLC analysis of the second fraction does not allow one to distinguish the various polymers. Considering only the condensed tannins, the diversity of monomer units and their potential sites of linkages [C-6 or C-8 on one side and C-4 (*R*) or (*S*) on the other side] lead to a great number of isomers for

Table 1. Polyphenolic Composition of F1 and F2 Fractions Isolated from a Cabernet Sauvignon Wine

composition	monomeric fraction (F1) (mg/L)	polymeric fraction (F2) (mg/L)
anthocyanins	140	undetected
flavonols	30	4.8
phenolic acids	111.5	undetected
flavanol monomers	60	17
tannins (estimated by thiolysis)		871

each polymer length, the whole eluting as a hump under unresolved peaks. For this reason, thiolysis is an alternative method to achieve some information on tannin structure, as mentioned in the Introduction. Nevertheless, when applied to a crude fraction, it does not allow one to determine the molecular weight of each tannin species or their composition, unlike electrospray ionization mass spectrometry.

To determine the distribution of trihydroxylated units in wine tannins, the polymeric fraction (F2) of a Cabernet Sauvignon wine was isolated according to the fractionation procedure described above. The monomeric fraction (F1) and the powder obtained after freeze-drying (F2) were analyzed by LC coupled to a DAD. Qualitative and quantitative results of both fractions are reported in Table 1. The presence of flavonols and flavanol monomers in the polymeric fraction suggested that F1 and F2 were not totally resolved or, more probably, that some degradations of tannin polymers occurred in the course of concentration and freeze-drying steps, restoring flavanol units. In any case, these compounds do not hinder LC/MS analysis. F2 was also thiolyzed to determine the monomeric composition and the mean degree of polymerization (mDP) of the tannin fraction. Thus, the thiolysis method allows the distinction between extension units (released as benzylthioether derivatives) and terminal units (recovered as nonderivated units). Consequently, mDP can be calculated as the ratio between the total number of units (extension plus terminal units) and the number of terminal units. That estimated for the F2 fraction from two runs was ~6.4, which is similar to values reported earlier in wine (Prieur-Delorme, 1994; Sarni-Manchado et al., 1999). The percentage of galloylated units was estimated at 3.3%, and that of trihydroxylated units was found at 17.9%. To go further in the determination of these trihydroxylated compounds, the sample was analyzed by on-line LC/electrospray mass spectrometry. Although HPLC separation requires acidic conditions, mass detection was performed in the negative ion mode, meaning that compounds were detected under their deprotonated forms $(M - H)^-$. Thus, it was found, as a general rule, that the response of polyphenols (except for anthocyanins) is better in the negative ion mode than in the positive one (Fulcrand et al., 1996). In this way, four ion peaks were detected at $m/z = 577.2$, as shown on the part of 3D map mass chromatogram presented in Figure 3. The occurrence of several spots for one m/z value indicated the presence of at least as many isomers. The value at 577.2 has been previously attributed to the mass of a procyanidin dimer (Self et al., 1986; Vivas et al., 1996; Cheynier et al., 1997b). Then, three ion peaks were detected at 593.2 and, in lower amounts, two others at 609.1 as testified by the corresponding spots on the part of the 3D map. The mass differences with 577.2 are 16 and 2 × 16 respectively, indicating the substitution of one hydrogen atom by one hydroxyl group for the first one and two substitutions for the second one. Therefore, the three series of ion peaks were attributed to the monocharged ions $(M - H)^-$ of dimers consisting of two catechin units, one catechin and one gallocatechin unit, and two gallocatechin units, respectively. Assignments of these pseudo-molecular ion peaks were confirmed by the presence simultaneously of cluster ion peaks, corresponding to addition of solvent molecules such as formate (M + 45) and trifluoroacetate (M + 113) salts. For the procyanidin dimers (M = 578), there were two other masses at 623.2 and 691.2 (noted

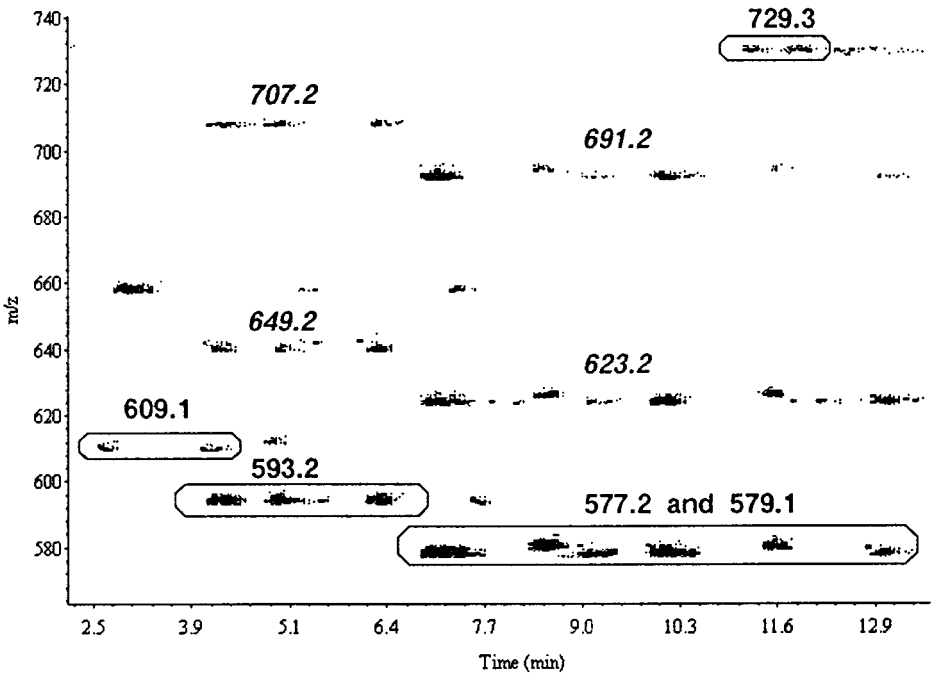

Figure 3. Plot of a part of the 3D map obtained from the mass chromatogram of a wine fraction: $m/z = 577.2$, mass of procyanidin dimers; $m/z = 593.2$, mass of dimers containing one catechin unit and one gallocatechin unit; $m/z = 609.1$, mass of dimers containing two gallocatechin units. Italic numbers refer to formate (M + 45) and trifluoroacetate (M + 113) adducts.

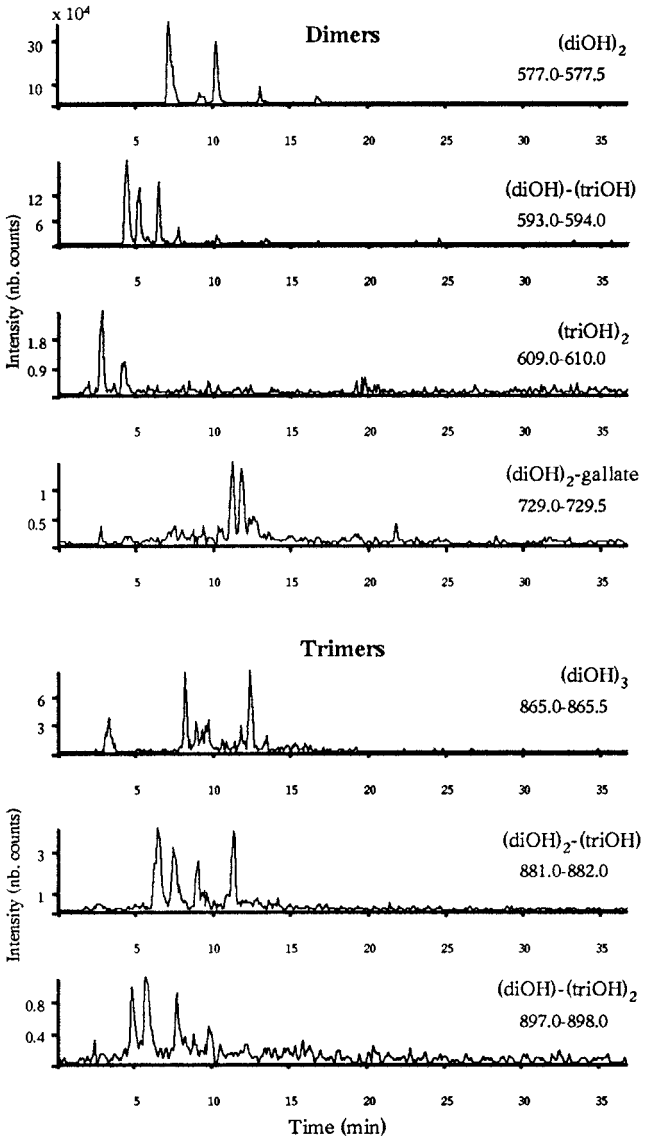

Figure 4. Plot of mass chromatograms extracted from the TIC trace recorded from wine analysis. (diOH) refers to catechin unit; (triOH) refers to gallocatechin unit.

in italics in Figure 3) corresponding to their formate and trifluoroacetate adducts, respectively. As well, the mass detected at 593.2 was associated with signals found at 649.2 and 707.2, corresponding to solvent adducts.

Moreover, two additional spots at $m/z = 579.1$ were eluted at 8.45 and 11.53 min along with the ion series detected at $m/z = 577.2$, corresponding to the retention times of (+)-catechin and (−)-epicatechin, respectively. These signals reflected the formation of noncovalent dimers by staking of two (+)-catechin or two (−)-epicatechin molecules, one of them being deprotonated ($[M + (M - H)]^-$). This phenomenon is currently observed by electrospray at relatively high concentration and referred to as self-association in the literature. Finally, the dimer series (starting from pure trihydroxylated up to pure dihydroxylated) eluted in decreasing polarity order, as could be expected on a reversed phase column. Then, other ion series corresponding to the masses of different trimers overlapped with those of dimers, as shown by the traces of their extracted ion chromatograms (Figure 4). Values detected at $m/z =$ 865.2, 881.2, and in trace amount at 897.2 were assigned to trimers containing from zero to two trihydroxylated units, respectively. Again, their formate and trifluoroacetate adducts were well detected. An ion peak series containing mainly two spots followed very closely those of dimers and trimers with a value at $m/z = 729.2$ (Figure 4). It corresponds to monogalloylated procyanidin dimers, as already observed in grape seeds (Czochanska et al., 1979; Boukharta et al., 1988; Ricardo Da Silva, 1991; Escribano-Bailon, 1992) and skins (Souquet et al., 1996). Note that $m/z = 881.2$ also corresponds to the molecular weight of a digalloylated procyanidin dimer, but the retention times of the ion peaks detected in this analysis are not consistent with those normally expected for such digalloylated procyanidin dimers (Ricardo Da Silva, 1991). The mass spectrum obtained from the total ion current (TIC) chromatogram between 6 and 15 min (Figure 5) showed m/z values corresponding to tetra- and pentamer molecular weights. Thus, 1153.4, 1169.5, 1185.4, and 1201.5 were assigned to tetramers containing from zero to three trihydroxylated units, respectively. However, some of the ion peaks detected at 1169.5 could be digalloylated trimer species eluting along with the tetramers, although such species are expected to be in very low amounts given the proportion of galloylated units in this wine fraction (3%). The value at $m/z = 1155.1$ (Figure 5) corresponded to the stacking of the procyanidin dimers eluting at 7.05 and 10.03 min. Formate adducts related to the first two masses of this tetramer series appeared at 1199.4 and 1215.6. Among monogalloylated tetramer series, the pure dihydroxylated type was found at 1305.4 and that containing one trihydroxylated unit was detected at 1321.6, nearing noise level. Values detected at $m/z = 1441.6$, 1457.4, 1473.4, and 1489.6 appearing in the mass spectrum (Figure 5) were assigned to pentamers containing from zero to three trihydroxylated units, although the value found at $m/z = 1457.4$ may include ions corresponding to digalloylated tetramer species.

It can be noted that signal intensity within the various ion peak series decreases as the number of trihydroxylated units increases as expected, given the average tannin composition (di-OH/tri-OH ratio is approximately 4:1). In particular, the probability that pure trihydroxylated oligomers occur quickly drops with the polymerization degree, explaining why such species were not detected beyond the dimer series.

Finally, pentamers were the highest m/z values that could be detected in this wine fraction. In a general way, signal intensity decreased as the polymerization degree increased. Thus, the potential number of trihydroxylated units and isomers becomes larger and larger, extending continuously the polymer distribution. Moreover, beyond tetramers, oligomers mostly occur as multicharged ions, meaning that the probability to find them as monocharged ions is lower. Actually, doubly charged ion peaks of pentamers ($m/z = 720.4$, 728.2, 736.2, and 760.0) were detected in the spectrum simultaneously with the corresponding monocharged ions in trace amounts, as well as some doubly charged ions of hexa- and heptamers. Retention times of such oligomers spread out from the beginning of the chromatogram up to the middle (not to the end), as previously observed (Lea, 1982). In fact, the different chain lengths seem randomly separated on the reversed phase column, whereas, for a given DP, the various species are eluted later as the number of hydroxyl groups decreases.

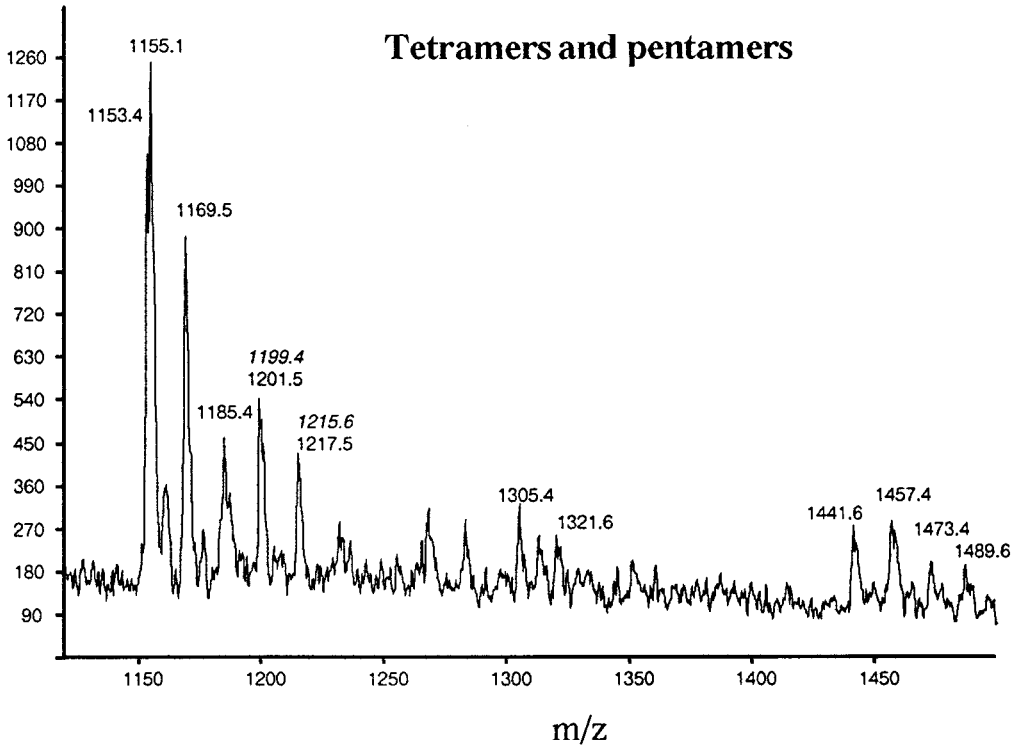

Figure 5. Plot of the mass spectrum obtained from the TIC chromatogram between 6 and 15 min. Italic numbers refer to formate adducts (M + 45).

An alternative method to increase signal intensity is to inject the sample continuously via a syringe with an infusion pump. Each mass is then detected under a single peak, representing the sum of all the corresponding isomer signals. However, analysis of the wine fraction in this way gave no better results. Again, this can be explained by a signal distribution between a larger number of molecular species and a greater number of multicharged ions when the DP increases. Moerover, interpretation also became increasingly difficult because overlapping occurred between multicharged signals.

To our knowledge, this is the first time that tannin oligomers containing both trihydroxylated and dihydroxylated units have been shown in wine, whereas such prodelphinidins have been already found in barley (Outtrup, 1981; Mulkay et al., 1981). Thus, wine proanthocyanidins are constituted, in addition to the well-known procyanidins, with mixed procyanidin−prodelphinidin structures and pure gallocatechin oligomers. However, the latter are more difficult to detect as the degree of polymerization increases. This indicates that trihydroxylated units, which represent ~20% of total tannin units in the wine extract studied, are randomly distributed in grape proanthocyanidin structures, as postulated by Haslam in 1980. The largest mass detected in this wine fraction by electrospray corresponds to the mass of heptamers, which is just above the value of the mDP (6.4) estimated by thiolysis. Nevertheless, larger polymers were expected to be detected as previously found in apple and lychee (Guyot et al., 1997; Le Roux, 1998). This can be explained by a greater homogeneity of monomers in apples and lychees in comparison to grapes. Finally, the presence of trihydroxylated tannins in wines constitutes a new element to take into account in their structural analysis as well as in the study of their organoleptic properties.

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