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Evidence for galloylated type-A procyanidins in grape seeds

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Abstract

In this work, procyanidins were extracted with methanol from the seeds of white and red grape varieties, and fractionated using graded methanol/chloroform precipitation in order to obtain the oligomers of lower molecular weight. These were analyzed by electrospray ionization-mass spectrometry (ESI-MS and ESI-MS/MS) in the positive mode. Protonated molecules, $[M+H]^+$, of procyanidin species of nongalloylated and monogalloylated type-A and type-B oligomers, with degree of polymerisation 2–5, and digalloylated oligomers, with degree of polymerisation 2–3, were observed in the ESI-MS spectra. Type-A procyanidin abundance accounted for 60–80% of the abundance of the corresponding type-B species. Independent of the interflavanic linkage, the abundance of monogalloylated dimers accounted for 20% of the abundance of the corresponding nongalloylated ones. For the higher degrees of polymerisation, the abundance of galloylated oligomers was shown to reach up to 60% of the abundance of the corresponding nongalloylated oligomers. Thiolytic analyses showed that the type-A interflavanic linkages were present in the terminal units whereas the type-B interflavanic linkages were present as extension units. Although many reports are already available regarding the analyses of procyanidin polymers and grape seed procyanidins, this is the first report for the occurrence of type-A galloylated procyanidins. The similarity of the relative abundances and structural features observed in the samples of both white and red grape varieties, shows that these are characteristic of grape seeds.
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Keywords: Grape seed; Tannins; Gallic acid; Interflavanic linkages; Type-A procyanidins; Type-B procyanidins; Galloylation; Thiolysis; ESI-MS/MS

1. Introduction

Procyanidins are mixtures of flavan-3-ol units, (−)-epicatechin and/or (+)-catechin. They are linked mainly through C4–C8 bonds (Fig. 1a), although C4–C6 linkages can also, alternatively, occur. Both structures are defined as type-B procyanidins. Type-B procyanidins can occur esterified with gallic acid, forming 3-*O*-gallates through the (−)-epicatechin units (Santos-Buelga, Francia-Aricha, & Escribano-Bailón, 1995). In addition to the C4–C8 bond, the flavan-3-ol units can also be doubly linked by a C2–C7 ether bond, giving origin to type-A structures (Fig. 1b) (Gu et al., 2003a), resultant from an oxidative intramolecular reaction (Kondo et al., 2000).

Health promoting benefits such as antioxidant, anticarcinogenic, and anti-inflammatory effects have been reported for procyanidins from grape seeds (Kondo et al., 2000). The pharmacological properties of selected grape seed procyanidins are related to an increase of tonicity and resistance of capillary blood vessels, as well as to the decrement of susceptibility of healthy cells to toxic

Abbreviations: ESI, electrospray ionization; MS/MS, tandem mass spectrometry; LC, high performance liquid chromatography; PCE, procyanidin crude extract; WM-F2.5, fraction 2.5 extracted with methanol from white grape seeds; WM-F2.6, fraction 2.6 extracted with methanol from white grape seeds; RM-F2.2, fraction 2.2 extracted with methanol from red grape seeds; DP, degree of polymerisation; P_nG_m, polymer with a total of n monomeric units of which m are epicatechin-*O*-gallate monomeric units; QM, quinone-methide fragmentation path; HRF, heterocyclic-ring-fission fragmentation path; RDA, retro-Diels–Alder fragmentation path.

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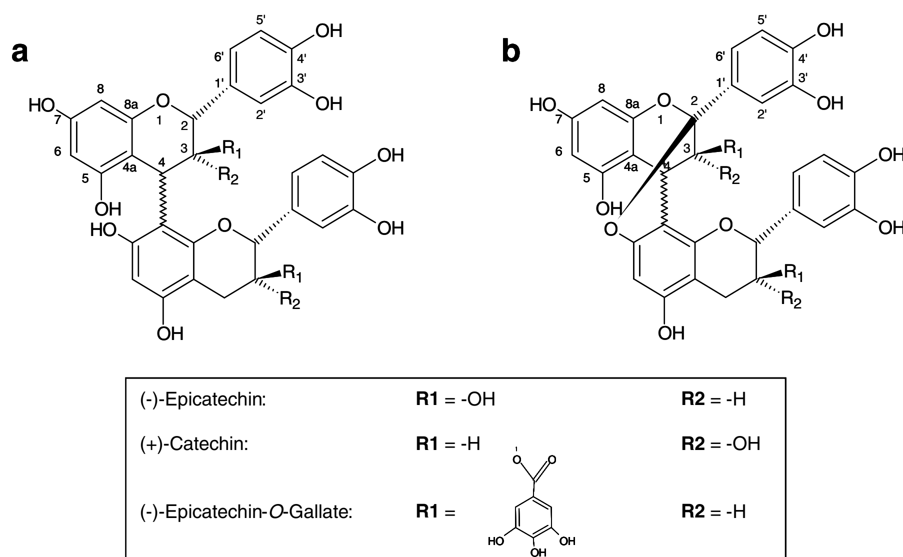


Fig. 1. Structure of procyanidin dimers: (a) type-B, containing (C4–C8) interflavanic linkage and (b) type-A, containing (C4–C8) and (C2–C7) interflavanic linkages.

and carcinogenic agents (Kondo et al., 2000; Maffei et al., 1998). The procyanidins esterified with gallic acid, whose anti-inflammatory and antiallergenic properties have been reported to be higher than those of nongalloylated ones, are of special interest (Escribano-Bailón, Gutiérrez-Fernández, Rivas-Gonçalo, & Santos-Buelga, 1992). Although type-A procyanidins have lower antioxidative activity when compared with type-B (Kondo et al., 2000), they present a higher antiviral potential activity against herpes simplex virus (HSV) and human immunodeficiency virus (HIV), and complement inhibition (De Bruyne et al., 1999).

Grape seeds are known as an abundant source of procyanidins consisting of both (+)-catechin and (−)-epicatechin forming units that, in the particular case of (−)-epicatechin, can appear galloylated or not (De Freitas, Glories, Bourgeois, & Vitry, 1998; Escribano-Bailón et al., 1992; Flamini, 2003; Fuleki & Ricardo da Silva, 1997; Gabetta et al., 2000; Hayasaka, Waters, Cheynier, Herderich, & Vidal, 2003; Krueger, Dopke, Treichel, Folts, & Reed, 2000; Monagas, Gómez-Cordovés, Bartolomé, Laureano, & Ricardo da Silva, 2003; Peng et al., 2001; Reed, Krueger, & Vestling, 2005; Santos-Buelga et al., 1995; Saucier, Mirabel, Daviaud, Longieras, & Glories, 2001; Vivas et al., 2004; Yang & Chien, 2000).

Grape seed procyanidins upto of tetramers have been fully separated and identified (Fuleki & Ricardo da Silva, 1997), although procyanidins with higher degrees of polymerisation have also been reported (Monagas et al., 2003).

Mass spectrometry (MS) is a very effective and highly sensitive method for characterizing procyanidins from complex matrices (Flamini, 2003; Reed et al., 2005). In recent years, electrospray ionization (ESI) has been shown to be suitable for the analysis of polar compounds in aqueous solutions without any previous sample derivatisation (Gaskell, 1997). ESI permits the identification of the molecular weight of procyanidins with different degrees of polymerisation. In addition, tandem mass spectrometry (MS/MS) can give even more information about the structural details of the different molecules. These techniques, due to their high accuracy and higher sensitivity, have been used for the conclusive identification of type-A procyanidins in a broad range of materials, such as fruits, cereals, nuts and spices (Gu et al., 2003a).

In order to have a deeper knowledge of the structural features of grape seed procyanidins, the compounds with lower molecular weights extracted from white and red grape varieties were analyzed by ESI-MS and ESI-MS/MS. The derivatives generated by thiolysis were also separated and analyzed by LC–MS.

2. Experimental

2.1. Reagents

Methanol, ethyl acetate, *n*-hexane and acetone, from Sigma–Aldrich Co. (St. Louis, MO, USA), were of analytical grade. Water from Merck (Darmstadt, Germany), acetic acid and acetonitrile from Sigma–Aldrich Co. (St. Louis, MO, USA), were of chromatographic grade quality. (+)-Catechin, (−)-epicatechin, (−)-epicatechin-*O*-gallate, procyanidin B2 and benzyl mercaptan, were purchased from Fluka Sigma–Aldrich Co. (St. Louis, MO, USA). Other reagents were of analytical grade or higher available purity.

2.2. Plant material

Seeds were collected from grapes (*Vitis vinifera* L.) of the white variety 'Chardonnay' at technological maturity, in

Bairrada Appellation, from an experimental vineyard (Estação Vitivinícola da Bairrada, Anadia, Portugal), during transfer of the musts for wine fermentation. A mixture of red grape varieties 'Touriga Nacional', 'Touriga Francesa' and 'Tinta Roriz', were also provided by wine producers of Adega Cooperativa de Pinhel (Portugal). The remaining pulp and skins were separated from the seeds by decantation and sieving (pore size < 2.8 mm diameter). The seeds were then submitted to several washes with water (200 g/L) under gentle stirring with a magnetic bar at 4 °C during a minimum of 3 days, with two water exchanges a day, until a minimum turbidity was constantly observed, assuring that no remaining adherent tissues were present. The purified seeds were then washed with ethanol, air dried at room temperature, and stored at 4 °C until use.

2.3. Procyanidin crude extract (PCE)

Seeds were immersed into liquid nitrogen, milled in a domestic coffee mill and sieved (pore size < 0.75 mm diameter). The extraction methodology was adapted from Guyot, Marnet, and Drilleau (2001), as described by Cardoso et al. (2005). Seed powder was extracted three times with *n*-hexane to remove the lipids. It was then treated three times with methanol containing 5% acetic acid to extract the phenolic compounds. The phenolic extracts were combined and filtered through a G3 sintered glass filter, and concentrated under vacuum at 40 °C, with several additions of water to assure the complete removal of methanol and acetic acid. The resultant concentrated aqueous solutions were frozen and freeze-dried.

2.4. Procyanidin fractionation

The methanol PCE from white and red grape seeds was fractionated according to the methanol/chloroform graded precipitation proposed by Saucier et al. (2001). The PCE powder (10 g/L) was dissolved in 5% acetic acid. The undissolved material was removed by centrifugation (Centrifuge 3K30, Sigma, St. Louis, MO, USA) and the supernatant was submitted three times to a liquid–liquid extraction with ethyl acetate, using a water/ethyl acetate ratio of 6:4 (v/v). The aqueous phase was evaporated to dryness and redissolved in methanol (10 g/L). The undissolved material was removed by centrifugation and the supernatant was submitted to successive additions of chloroform. The white grape seed extracted material that precipitated between 73% and 79% (v/v) chloroform (WM-F2.5) and between 79% and 84% (WM-F2.6), and the red grape seed extracted material that precipitated between 43% and 60% (RM-F2.2) were used for analysis.

2.5. Thiolysis and HPLC–UV analysis

Thiolysis was carried out according to the methodology described by Ferreira et al. (2002). HPLC analysis followed the conditions described by Peng et al. (2001). The HPLC apparatus used was from Perkin Elmer (series 200), with UV–vis Detector (785A UV–VIS Detector). Samples were loaded at 30 °C into a C_{18} column (LichroCart 250-4 Superspher 100 RP-18) equipped with a C_{18} guard cartridge with the same packing material equilibrated with 0.2% (v/v) formic acid (eluent A). Phenolic compounds were eluted by a gradient with 82% (v/v) acetonitrile and 0.04% (v/v) formic acid (eluent B) from 0% to 15% eluent B in the first 15 min, from 15% to 16% from 15 to 40 min, from 16% to 17% from 40 to 45 min, from 17% to 43% from 45 to 48 min, from 43% to 52% from 48 to 49 min, held isocratic at 52% from 49 to 56 min, reduced from 52% to 43% from 56 to 57 min, reduced from 43% to 17% from 57 to 58 min, and reduced from 17% to 0% from 58 to 60 min. Samples were loaded, at least, in duplicate. Peaks were detected at 280 nm and the monomers and procyanidin B2 dimer were identified by comparison of their retention times with standards. The (−)-epicatechin thioderivative was identified by comparison with the retention time of the products of the procyanidin B2 dimer after thiolysis; the (+)-catechin thioderivative was identified by its retention time and abundance relative to the (−)-epicatechin thioderivative; and the (−)-epicatechin-*O*-gallate thioderivative was identified by its retention time and abundance relative to the (−)-epicatechin thioderivative. All thioderivatives, as well as type-A procyanidins, were identified by analysis of their MS spectra. The average degree of polymerisation was calculated as the ratio of all the flavan-3-ols units areas (thioether adducts plus terminal units) to the sum of the areas of (+)-catechin, (−)-epicatechin, (−)-epicatechin-*O*-gallate, and type-A procyanidins, corresponding to terminal units. The calibration curves for estimation of phenolic compounds were obtained using (+)-catechin, (−)-epicatechin, (−)-epicatechin-*O*-gallate, and the procyanidin B2 dimer, in the range of concentration of 0.005–0.5 g/L. Due to the lack of standard type-A dimers the calibration curve for procyanidin B2 was used. The quantification of phenolics in the fractions was made by comparison of the chromatographic area after thiolytic degradation of the samples with the respective calibration curve. In the absence of standards of the thioderivatives, considering the fact that the thiolytic derivatives were shown to have similar response factors as the correspondent monomeric units (Vivas et al., 2004), they were calculated from the respective monomer calibration curves.

2.6. SPE-C_{18}

The C_{18} solid-phase-extraction column (SPE, Supelco-Discovery – 5 g) was used to exclude free sugars from procyanidin-rich extracts, prior to the ESI-MS analysis (Cardoso et al., 2005). The SPE column was preconditioned with 20 mL of methanol followed by 20 mL of water and 20 mL of 2% acetic acid. Each sample (20 mg) was dissolved in 20 mL of 2% acetic acid and loaded onto the preconditioned SPE column. The column was then washed with 60 mL of 2% acetic acid followed by 120 mL

diethylether. The procyanidins for ESI-MS analysis were eluted with 60 mL methanol, monitored at 280 nm, and dried by centrifugal evaporation (Univapo 100 ECH, Uni-Equip, Munich, Germany).

2.7. ESI-MS analysis

Electrospray analysis was performed by dissolving the dried samples (0.5 mg/mL) in MilliQ high purity water with 2.0% (v/v) formic acid. This solution was further diluted by a factor of 100 in $MeOH/H_2O$ (1:1, v/v) solution with 1.0% (v/v) formic acid. Samples were introduced into the mass spectrometer using a flow rate of 10 μL/min. Positive ion mode ESI-MS and MS/MS spectra were acquired using a Q-TOF 2 instrument (Micromass, Manchester, UK), setting the needle voltage at 3000 V with the ion source at 80 °C and desolvation temperature at 150 °C maintaining the cone voltage at 35 V (Reis, Domingues, Domingues, Ferrer-Correia, & Coimbra, 2003). Tandem mass spectra (MS/MS) of molecular ions were obtained using collision induced dissociation (CID), using argon as the collision gas and varying collision energy between 20 and 35 eV (Reis et al., 2003). Each spectrum was produced by accumulating data during 1–2 min. In MS and MS/MS experiments TOF resolution was set to approximately 9000 (FWHM).

2.8. LC–MS analysis

A HPLC system (Waters Aliance 2690) equipped with a UV detector (Knauer K-2500) set at $\lambda = 280$ nm was used. Samples (10 μL) were loaded at 20 °C into a SPE, Supelco-Discovery® BIO Wide Pore C_{18} HPLC column (10 cm, 0.32 mm i.d., 5.0 μm bead diameter). The mobile phase consisted of 10% (v/v) acetonitrile and 0.5% (v/v) formic acid (eluent A) and 100% (v/v) acetonitrile (eluent B). The eluent gradient was programmed as follows: 10–50% of eluent B in the first 40 min, held isocratic at 50% from 40 to 50 min, reduced from 50% to 10% from 50 to 65 min at 20 mL/min. After the UV detector, the flow was redirected into the MS interface with a 1:20 home-made split. MS was preformed as previously described.

3. Results and discussion

3.1. Procyanidin crude extract (PCE)

The PCE extracted with methanol represented 20.7% of the initial raw material of white grape seeds. An additional 4.6% of material was recovered by extraction with aqueous acetone (data not shown). For the red grape seeds, 6.6% of the initial material was obtained as PCE in the methanol extract. An additional 2.6% was recovered by extraction with aqueous acetone (data not shown).

In order to estimate the amount of procyanidins in each one of the methanol extracts, the samples were submitted to a thiolytic degradation process followed by HPLC separation and UV detection. Fig. 2 shows the chromatograms that were obtained before and after thiolysis. The comparison of the two chromatograms shows that the area of peak 2 (catechin) does not increase by thiolysis whereas the sharp peak 4 (epicatechin) shows an increase. Also, a broad peak 5 (type-A dimer) appears after thiolysis. These results allow to infer the occurrence of terminally linked epicatechin and terminally linked type-A dimers in the procyanidins backbone. Interestingly, their amount seems to be of the same order of magnitude. The amount of monomeric (+)-catechin, (−)-epicatechin and (−)-epicatechin-*O*-gallate was calculated using standard calibration curves. For the estimation of the amount of procyanidins present in each PCE, the sum of the amount of each monomer, given by its peak area, plus their corresponding thioderivatives, was used. The white grape seed extracts accounted for 20.1% of the estimated procyanidins. In the case of red grape seeds, PCE contained 10.8% of procyanidins. These low values are due to the high amount of sugars present in these extracts (data not shown). The average degree of polymerisation (DP) of procyanidins was estimated by thiolysis as 4.0 for the white and 6.5 for the red grape seeds. These results are in accordance with the values reported in literature for the average DP of grape seed procyanidins (Le Bourvellec, Picot, & Renard, 2006; Peng et al., 2001; Reed et al., 2005).

In order to obtain fractions containing the polymeric procyanidins with the lower DP, suitable for the ESI-MS

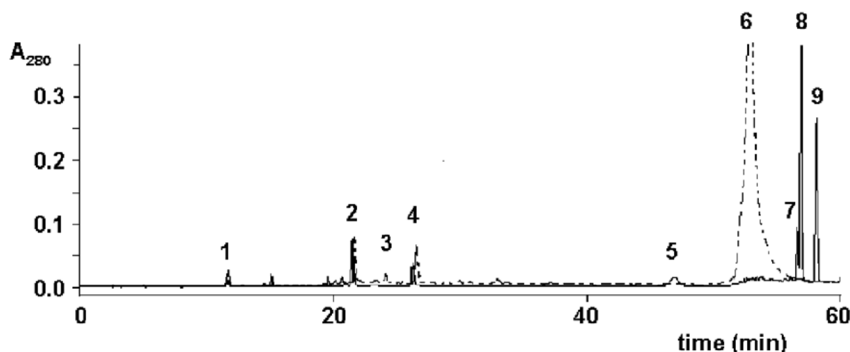


Fig. 2. Reversed-phase HPLC–UV chromatogram of methanol PCE obtained before (dashed line) and after (full line) thiolysis: (1) gallic acid; (2) (+)-catechin; (3) procyanidin B2; (4) (−)-epicatechin; (5) procyanidins A; (6) polymeric procyanidins; (7) (+)-catechin thioderivative; (8) (−)-epicatechin thioderivative and (9) (−)-epicatechin-*O*-gallate thioderivative.

analysis, the PCEs were dissolved in water and were submitted to a liquid–liquid extraction with ethyl acetate. The aqueous fraction obtained was redissolved in methanol and precipitated with chloroform. The fractions that precipitated between 73% and 79% chloroform (WM-F2.5) and between 79% and 84% (WM-F2.6) from the white grape seeds, and the fraction that precipitated between 43% and 60% chloroform from the red grape seeds (RM-F2.2), were collected to be analyzed by ESI-MS. Fraction WM-F2.5 accounted for 5% of WM-PCE, with a procyanidin content estimated as 22.4%, and an average DP of 6.9; fraction WM-F2.6 accounted also for 5% of WM-PCE, and the procyanidin content was estimated as 22.8%, with an average DP of 5.3. Fraction RM-F2.2 accounted for 7% of RM-PCE, and contained a procyanidin content estimated as 10.3%, and an average DP of 6.8. These fractions were used because of their abundance, having low and comparable DP ranges. Because of the presence of sugars, these samples were purified by passage through a C_{18} solid-phase column prior to ESI-MS analyses.

3.2. Analysis of procyanidins by ESI-MS

The positive electrospray mass spectrum of fraction WM-F2.5 shows the presence of ions between *m/z* 440 and 2000 (Fig. 3). These ions were identified as the protonated molecules, $[M+H]^+$, of different procyanidins. The major ions observed at *m/z* 579, 867, 1155, 1443, and 1731, showing a series with a mass difference of 288 Da, can be attributed to the $[M+H]^+$ type-B procyanidin nongalloylated species, with DP between 2 and 6 ($P_{2–6}$). The ion at *m/z* 577, with 2 mass units less than the corresponding P_2, can be attributed to a $[M+H]^+$ type-A nongalloylated procyanidin (P_2^*). The ions at *m/z* 443, 731, 1019, 1307, 1595, and 1883 also belong to a series with a mass difference of 288 Da. These ions can correspond to the $[M+H]^+$ ions of type-B procyanidin monogalloylated species, $P_{1–6}G_1$. Sodium and potassium adducts of type-B galloylated and nongalloylated procyanidins, as well as type-A nongalloylated procyanidins, were also observed, although in very low abundance. When only methanol was used as the solvent, the sodium and potassium adducts were the major ions (data not shown). However, the use of methanol/water acidic conditions diminished these to less than 10%. Other ions in the spectrum correspond to different combinations of doubly charged procyanidin ions with H^+, Na^+ and K^+ over their DP ranges. This observation shows that, although the chloroform precipitated samples contained a large percentage on nonprocyanidin material, their purification by passage through the C_{18} solid-phase column resulted in fractions almost constituted by procyanidin material, which allows their structural analysis by ESI-MS.

Table 1 summarizes the protonated single charged ions observed in the ESI-MS spectrum of Fig. 3, their probable identification, and their abundances in relation to the type-B dimer. The ions observed at *m/z* 883, 1171, 1459, and 1747, showing a mass difference of 288 Da, and having 152 mass units higher than the correspondent $[M+H]^+$ of type-B procyanidin monogalloylated species, can be attributed to the $[M+H]^+$ ions of type-B digalloylated procyanidin series, $P_{2–5}G_2$.

The ions observed at *m/z* 577, 865, 1153, 1441, having 2 mass units less than the corresponding $[M+H]^+$ ion of type-B procyanidin nongalloylated series, can be attributed to the $[M+H]^+$ ions of type-A nongalloylated procyanidins, $P^*_{2–5}$. Furthermore, the ions at *m/z* 729, 1017, 1305 and 1593 can be attributed to the $[M+H]^+$ ions of type-A galloylated procyanidins ($P_{2–5}G_1^*$), whereas the ions at *m/z* 881 and 1169 can be attributed to the $[M+H]^+$ ions of type-A digalloylated procyanidins ($P_{2–3}G_2^*$). Table 1 resumes these series of type-A procyanidins.

All the ions observed in the ESI-MS spectrum of fraction WM-F2.5 were also observed in fraction WM-F2.6, from the same white grape seeds, and fraction RM-F2.2, from red grape seeds (Table 1). Comparing the most abundant species, very similar relative abundances are observed between the samples from white and red grape varieties,

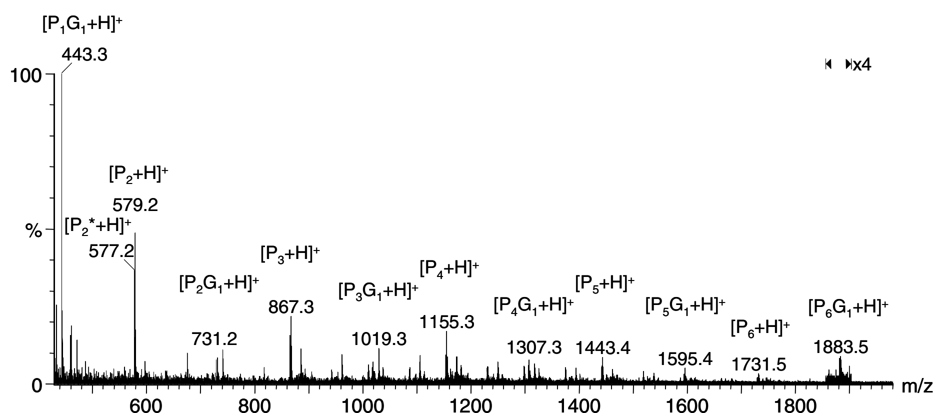


Fig. 3. ESI-MS spectrum of fraction FW2.5 from white grape seeds. Type-B nongaloyllated procyanidin series, $[P_n + H]^+$, $m/z = 290 + 288 * (n - 1) + 1$, ($n = 2–6$); type-B monogaloyllated procyanidin series, $[P_nG_1 + H]^+$, $m/z = 290 + 288 * (n - 1) + 152 + 1$, ($n = 1–6$); type-A nongaloyllated procyanidin, $[P_2^* + H]^+$, *m/z* 577.

Table 1
Identification of protonated grape seed procyanidins and their abundance in relation to the type-B dimer ($m/z = 579$)

Compound	Type	Molecular weight	Relative abundance in ESI-MS spectra		
			WM-F2.5	WM-F2.6	RM-F2.2
P_1	B	290	24	39	59
P_1G_1	B	442	229	126	346
P_2	B	578	100	100	100
P_2^*	A	576	73	71	90
P_2G_1	B	730	16	16	22
$P_2G_1^*$	A	728	15	16	16
P_2G_2	B	882	4	4	9
$P_2G_2^*$	A	880	6	5	7
P_3	B	866	51	70	26
P_3^*	A	864	30	35	19
P_3G_1	B	1018	15	19	10
$P_3G_1^*$	A	1016	12	13	9
P_3G_2	B	1170	4	6	4
$P_3G_2^*$	A	1168	tr	tr	tr
P_4	B	1154	33	49	10
P_4^*	A	1152	23	20	8
P_4G_1	B	1306	13	17	8
$P_4G_1^*$	A	1304	10	7	6
P_4G_2	B	1458	4	6	tr
P_5	B	1442	17	23	5
P_5^*	A	1440	10	8	5
P_5G_1	B	1594	10	9	tr
$P_5G_1^*$	A	1592	5	5	tr
P_5G_2	B	1746	tr	tr	tr
P_6	B	1730	6	8	tr
P_6G_1	B	1882	4	4	tr

P – number of monomeric units in the molecule; G – number of galloylated units in the molecule; *Type-A procyanidins; tr – traces (lower than 4%).

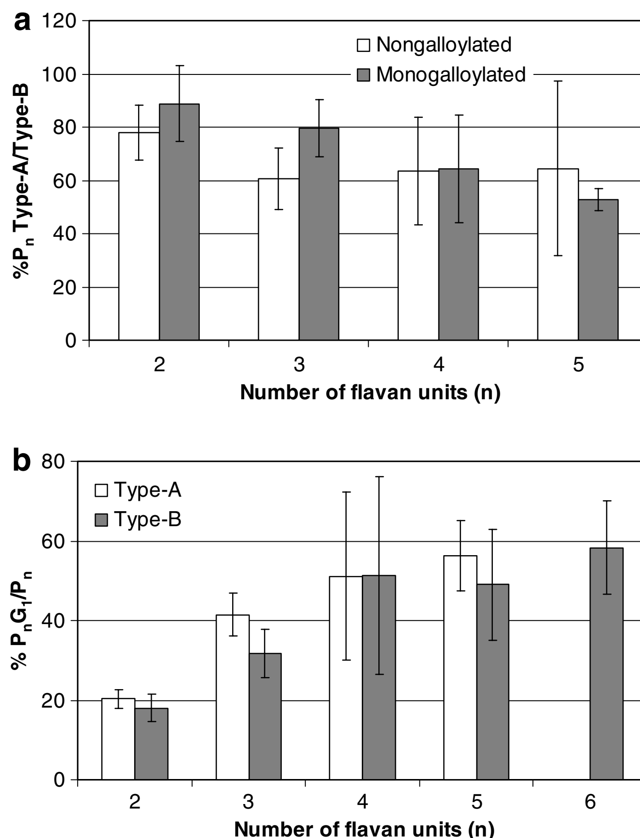


Fig. 4. Relative abundance of procyanidin oligomers present in fractions WM-F2.5, WM-F2.6, and RM-F2.2 (data from ESI-MS spectra shown in Table 1): (a) type-A/type-B; (b) monogalloylated/nongalloylated.

showing that the structural features reported are not a characteristic of a single sample or grape variety but can be attributed to grape seeds in general. Type-A procyanidins are present as nongalloyated, and mono and digalloyated molecules (P_nG_m, where $n = 2$–5 and $m = 0$–2). As the structural similarity of type-A and type-B procyanidins suggests similar ionization efficiency for families with the same degree of polymerisation and galloylation, their relative abundances were compared, showing that nongalloylated and monogalloylated type-A ions account for 60–80% of the corresponding type-B ions (Fig. 4a). This abundance seems to be independent of the occurrence or not of galloylation. Also, the abundance of type-A, in relation to type-B, tends to decrease as the degree of polymerisation of the oligomers increases. Independently of the interflavanic linkage, the monogalloylated dimers accounted for 20% of the corresponding nongalloylated dimers (Fig. 4b). The abundance of monogalloylated oligomers observed in the ESI-MS spectra showed a tendency to increase, reaching up to 60% of the abundance of the corresponding nongalloylated oligomers.

Previous analysis of grape seed procyanidins by ESI-MS in positive (Gabetta et al., 2000) or in negative modes (Flamini, 2003; Hayasaka et al., 2003) did not allow the occurrence of type-A procyanidins to be observed. This might be due to the sample complexity and instrumental conditions used by the other authors. In our work, the fractionation steps used could have been determinant for a higher homogeneity of the highly soluble chloroform fractions obtained from PCE, resulting in more informative spectra, and allowing us to report, for the first time, the occurrence of type-A procyanidins as components of grape seeds. This is also in accordance with the results presented by Krueger et al. (2000) that showed an unattributed $[M+Na]^+$ ion at m/z 1175, in a MALDI-TOF mass spectrum. This resulted from a purified grape seed extract and could correspond to a nongalloylated type-A procyanidin tetramer (P_4^*), lacking further analyses (e.g., MS/MS) to confirm its presence. In order to confirm the occurrence of type-A in grape seed procyanidins, as well as the occurrence of type-A galloylated procyanidins, which have also never been reported, tandem mass spectrometry of the identified ions was performed.

3.3. Tandem mass spectrometry (ESI-MS/MS)

The MS/MS spectrum of a trimer with a type-A interflavanic linkage, $[P_3^* + H]^+$ ion, at m/z 865, is shown in Fig. 5a. The major fragment, at m/z 577, should be formed by cleavage of the type-B interflavan bond through a quinone-methide (QM) cleavage (Scheme 1), with loss of a

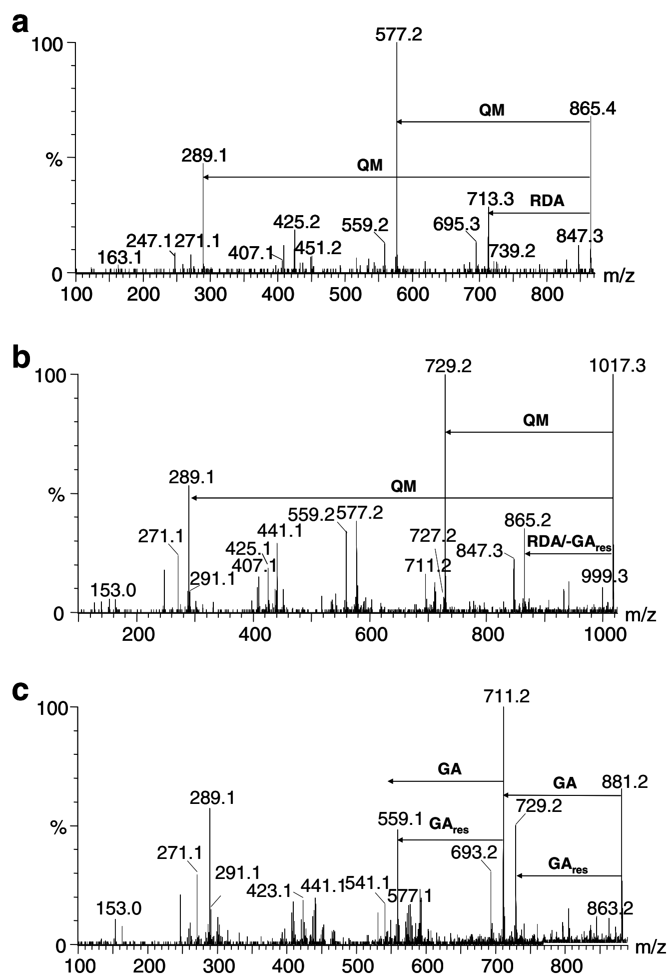
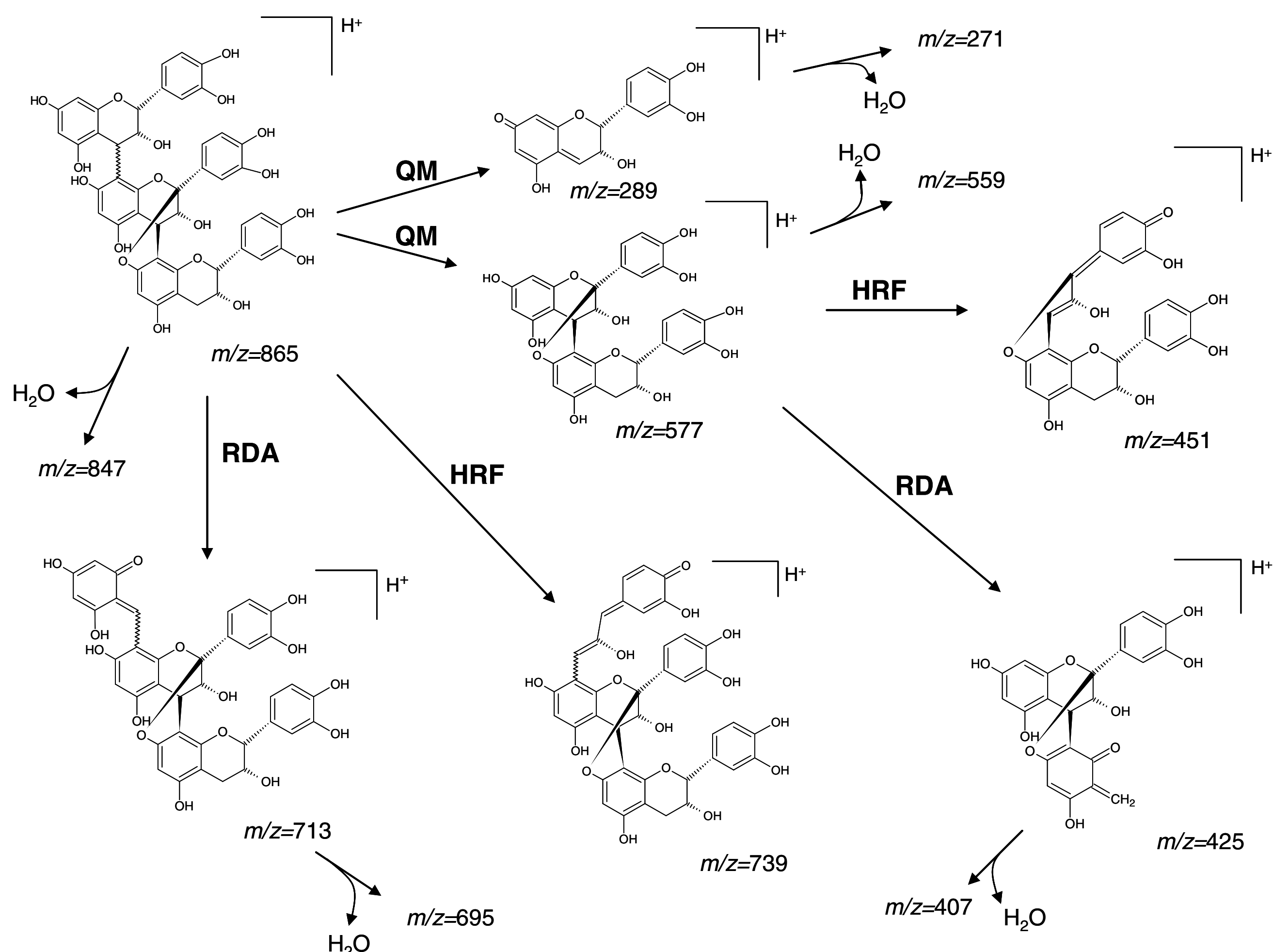


Fig. 5. ESI-MS/MS spectra of type-A procyanidin $[M+H]^+$ ions of (a) trimer (P_3^*); (b) monogalloylated trimer ($P_3G_1^*$) and (c) digalloylated dimer ($P_2G_2^*$). QM – quinone-methide fragmentation; RDA – retro-Diels–Alder fragmentation; -GA_{res} – loss of gallic acid residue.

neutral fragment with 288 Da, corresponding to the elimination of an extension unit (Flamini, 2003). The elimination of an extension unit gives rise to a fragment ion with a larger π–π hyperconjugated system than any of the other units, being more energetically favourable (Gu et al., 2003a). The complementary fragment ion at *m/z* 289, formed by the same QM fragmentation, with the charge retained in the extension unit (Scheme 1) is also observed. The absence, in this MS/MS spectrum, of a fragment ion at *m/z* 291 that, if present, would be resultant of a loss of the terminal unit, allows us to conclude that P_3^* is composed by a type-A procyanidin residue as a terminal unit and by a type-B procyanidin residue as the extension unit, as shown in Scheme 1. Retro-Diels–Alder (RDA) fragmentation (Gu et al., 2003b), as shown in Scheme 1, leads to the formation of the fragment ions at *m/z* 713, (−152 Da), and *m/z* 425, resultant from the ion at *m/z* 577. The fragment ion at *m/z* 739 (−126 Da) can be attributed to a heterocyclic-ring-fission (HRF). The HRF fragmentation can also explain the occurrence of the fragment ion at *m/z* 451, resultant from the fragment ion at *m/z* 577 (−126 Da) or/and resultant from the $[M+H]^+$ ion at *m/z* 865 by loss of 414 Da (occurrence of HRF on the second monomeric unit). Fragment ions at *m/z* 847, 695, 559, 407, and 271, result from loss of water from ions at *m/z* 865, 713, 577, 425, and 289 (Scheme 1). These results are in accordance with the fragment paths from $[M-H]^-$ proposed by Gu et al. (2002) for proanthocyanidins. When comparing the analysis of the type-A fragmentation paths, with exception to the part concerning the type-A double linkage, the backbone of the polymer produces the same type of fragmentation pattern that has already been assumed for the well characterized type-B molecules (Karchesy, Hemingway, Foo, Barofsky, & Barofsky, 1986).

Fig. 5b shows the MS/MS spectrum of a trimer with a monogalloylated type-A procyanidin, $[P_3G_1^* + H]^+$ ion, at *m/z* 1017. The fragment ions at *m/z* 729 and 289, resulting from losses of 288 (extension flavan unit) and 728 Da (terminal galloylated type-A dimer), can be attributed to the QM cleavage of the type-B interflavan bond, showing that the gallic acid residue occurs in the type-A moiety of the trimer. The loss of a gallic acid residue (−152 Da) and a gallic acid unit (−170 Da) from this type-A moiety (*m/z* 729) can produce the ions at *m/z* 577 and *m/z* 559, respectively. The fragment ions at *m/z* 577 and 441 can also be attributed to the QM cleavage of the type-B interflavan bond resulting from the loss of a procyanidin residue esterified with gallic acid, showing that the gallic acid residues can also occur in the type-B moiety of the molecule (Scheme 2a). These fragment ions denote that different galloylated isomers are present. Furthermore, the presence of fragment ions at *m/z* 291 and 727 allow to infer the existence of the type-A bound residues as extension units. Scheme 2b shows one of the two isomers that can be attributed to these type-A extension units. The other possible isomer is the galloylated residue in the other monomeric type-A unit. In accordance, and as reported by Gu et al. (2003b), the cleavage between the middle and the terminal unit is unique for type-A, as it converts the hydroxyl group on C5 of the middle unit into a quinone (Scheme 2b), contrasting to C7 as the preferred conversion site in all other cases (Scheme 2a). This generates a unique, complete monomeric nongalloylated unit at *m/z* 291 (Scheme 2b) or a galloylated unit at *m/z* 443, respectively, for the $[M+H]^+$ ions under study. However, the absence of the fragment ion at *m/z* 443 in the MS/MS spectrum of the $[P_3G_1^* + H]^+$ ion (Fig. 5b), allows the conclusion that this molecule is not composed by a type-B galloylated procyanidin residue as a terminal unit. The fragment ion at *m/z* 865 results from a loss of 152 Da and can be attributed to RDA fragmentation or to the loss of a gallic acid residue from the $[M+H]^+$ ion at *m/z* 1017. The fragment ion at *m/z* 425 can be attributed to a RDA fragmentation, with a loss of 152 Da, from the ion at *m/z* 577. Fragment ions at *m/z* 999, 847, 711, 559, 407 and 271, result from the loss of water from ions at *m/z* 1017, 865, 729, 577, 425 and 289.

The MS/MS spectrum of a dimer with a digalloylated type-A procyanidin, $[P_2G_2^* + H]^+$ ion, at *m/z* 881, is shown



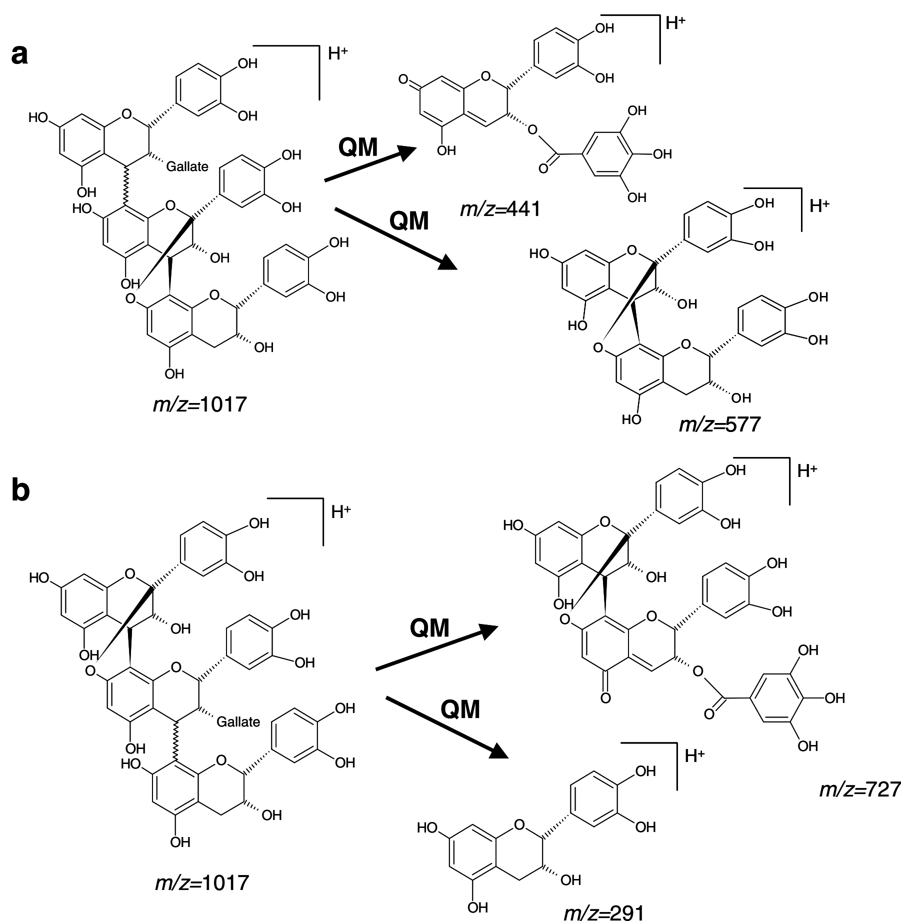
Scheme 1. ESI-MS/MS fragmentation pathways of a type-A procyanidin trimer $[P_3^* + H]^+$. QM – quinone-methide; RDA – retro-Diels–Alder; HRF – heterocyclic-ring-fission.

in Fig. 5c. The major fragment ion is observed at *m*/*z* 711, resultant from a loss of a gallic acid unit (−170 Da). The presence of the ion at *m*/*z* 729 can be attributed to a loss of a gallic acid residue (−152 Da). Loss of a second gallic acid residue/unit is also observed with formation of the ions at *m*/*z* 559/541. RDA fragmentation leads to the ion at *m*/*z* 577, by the loss of 304 Da (−152 + gallic acid residue). The opening of the (C4–C8)–(C2–C7) ring of the $P_2G_2^*$ dimer enables the occurrence of the fragment ions at *m*/*z* 441, due to loss of 440 Da, resultant from loss of the galloylated monomer from $[M+H]^+$, and the fragment ion at *m*/*z* 289, resultant from a loss of a gallic acid residue (152 Da) from the galloylated monomer fragment at *m*/*z* 441. Although type-A interflavan bonds do not undergo observable QM cleavage in the presence of a type-B interflavan bond, as stated by Gu et al. (2003b) and observed in this work for the presented trimer procyanidin structures (Fig. 5a and b), QM cleavage can be observed in type-A dimers (Fig. 5c), as also described by Karchesy et al. (1986). Fragment ions at *m*/*z* 863, 693, 541, 423, and 271, result from the loss of water from ions at *m*/*z* 881, 711, 559, 441, and 289, respectively.

In order to study the position of the galloylated type-A in the backbone of grape seed procyanidins, the sample WM-F2.6 was subjected to thiolysis and the galloylated derivatives were separated and analyzed by HPLC–MS.

3.4. Analysis of galloylated procyanidin derivatives after thiolysis

Fraction WM-F2.6 was submitted to LC–MS. The LC–MS mass spectrum of the peak where the polymeric galloylated procyanidins and their thioderivatives eluted (correspondent to peak 9 in Fig. 2 for HPLC–UV) is shown in Fig. 6. The mass spectrum shows the presence of ions between *m*/*z* 100 and 1500. The main ion observed in the spectrum, with *m*/*z* 565, can be attributed to the thioderivative of epicatechin-*O*-gallate monomer (P_1G_1). The higher abundance of this ion, associated with the tiny abundance of $[P_2G_1 + \text{thiol} + H]^+$ dimer, with *m*/*z* 853, allows the inference that the thiolysis reaction performed had occurred with a high level of efficiency. Also, the occurrence of a small peak attributed to type-A procyanidin dimers (peak 5 in Fig. 2) and the absence of type-B procy-



Scheme 2. ESI-MS/MS quinone-methide fragmentation pathway of a monogalloylated type-A procyanidin trimer $[P_3G_1^* + H]^+$ containing a type-B unit: (a) terminal type-A unit and (b) extension type-A unit.

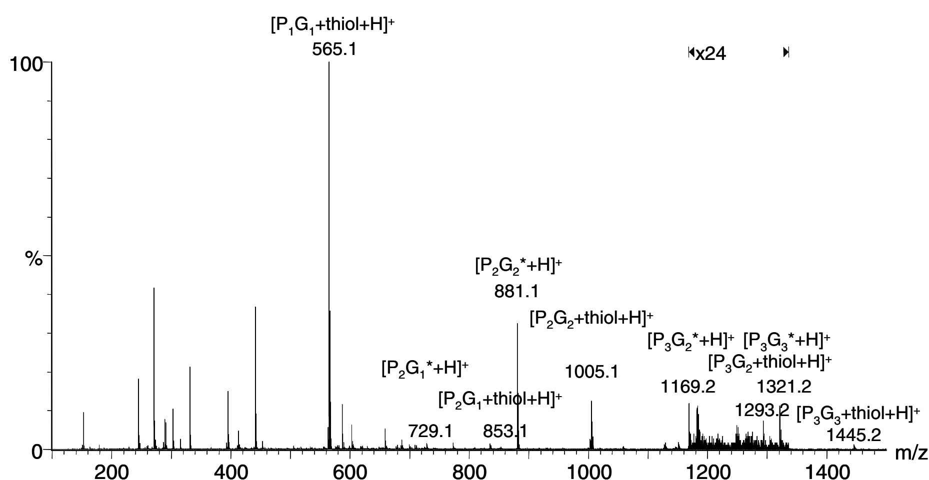


Fig. 6. LC–MS mass spectrum of galloylated procyanidin derivatives obtained after thiolythic degradation. *Type-A procyanidins.

anidin dimers observed after thiolysis in the LC–MS chromatogram (peak 3 in Fig. 2), confirm the resistance of type-A linkages to thiolytic degradation when compared to type-B. The presence of type-A interflavan linkages prevent the interflavanic cleavage due to the existence of the C2–C7 ether bond bridge. The type-A terminal units are released as dimers, whereas the type-A extension units are released as benzylthioether type-A dimers (Gu et al., 2003a).

The ions at *m*/*z* 881 and 1005 can be attributed to a type-A digalloylated procyanidin dimer ($P_2G_2^*$) and a type-B digalloylated procyanidin dimer associated with a thiol group (P_2G_2 + thiol), respectively. Although in very

low abundance, a type-A monogalloylated procyanidin dimer ($P_2G_1^*$) at m/z 729, a type-A digalloylated procyanidin trimer ($P_3G_2^*$) at m/z 1169 and a type-B digalloylated procyanidin trimer associated with a thiol group (P_3G_2 + thiol) at m/z 1293 were also observed. The ions at m/z 1321 and m/z 1445 can be attributed to the trigalloylated type-A trimer ($P_3G_3^*$) and the trigalloylated type-B trimer thioderivative (P_3G_3 + thiol), respectively. The presence of dimers and trimers with high levels of galloylation allows to infer that although the thiolysis reaction performed had occurred with a high level of efficiency, it does not cleave all these interflavanic linkages, which could possibly be due to a higher resistance to thiolysis conferred by the presence of the high level of gallic acid residues. This observation shows that the DP of highly galloylated procyanidins by thiolysis could be underestimated.

In this spectrum all type-B procyanidin ions occurred as thioderivatives whereas all type-A procyanidin ions occurred without the thiol group. These results were also observed in the nongalloylated procyanidins spectra separated by LC–MS (data not shown), which allows the conclusion that, in grape seeds, the type-A interflavanic linkages are present in the terminal flavan units whereas the type-B interflavanic linkages are present as extension units. This is in accordance with the observations made by MS/MS from the spectrum shown in Fig. 5a. The extension type-A interflavan linked units detected in the spectrum of Fig. 5b seem to occur as small features, not detected by thiolysis. The occurrence of type-A units only as terminal interflavan linkages has already been detected in plums, whereas the occurrence of type-A units only as extension interflavan linkage has been detected in avocado, curry and cinnamon, and cranberry and peanuts present both possibilities (Gu et al., 2003a).

3.5. Concluding remarks

The analysis of grape seed procyanidins by ESI-MS, ESI-MS/MS and LC–MS after thiolysis allowed a report, for the first time, of the occurrence of type-A galloylated procyanidins. These compounds seem to be a general characteristic of these tissues, as very similar relative abundances and structural features were observed between the samples from white and red grape varieties. In this essay, the abundance of type-A procyanidins was 60–80% of that of the correspondent type-B species and, independent of the interflavanic linkage, the abundance of monogalloylated species was 20–60% of the abundance of the correspondent nongalloylated ones. Type-A interflavanic linkages of grape seeds were found to be present mainly in the terminal units.

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