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RESEARCH ARTICLE

Disambiguation of Isomeric Procyanidins with Cyclic B-Type and Non-cyclic A-Type Structures from Wine and Peanut Skin with HPLC-HDX-HRMS/MS

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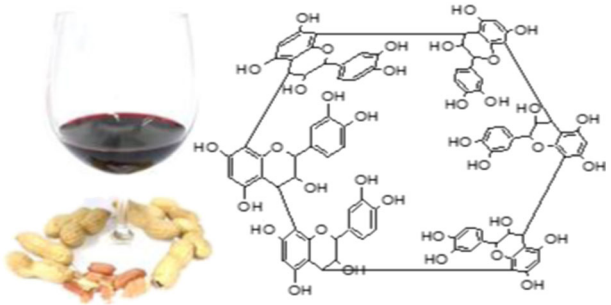

Abstract. Hydrogen/deuterium exchange coupled with high-resolution mass spectrometry was successfully applied for the identification of A-type tetrameric, pentameric, and hexameric procyanidins in peanut skin. This extended a previous study on isomeric cyclic B-type unconventional tetramer, pentamer, and hexamer procyanidins found in wine and cranberries. Not only had the method successfully identified the procyanidins with a single A-linkage (e.g., tetrameric *m/z* 1153.2608) by means of distinguishing them from their isomeric cyclic B-type analogues, but this method also worked for procyanidins with two or more A-linkages (such as the tetrameric *m/z* 1151.2452). As a further consequence, B-type cyclic pentamers and hexamers in wine have been elucidated with hydrogen/deuterium exchange (HDX) for the first time.

Keywords: Isotopic exchange, Cyclic procyanidins, Wine, Peanut skin, A-type procyanidins

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Introduction

Proanthocyanidins are a class of oligomeric flavonoids widespread in plants [1–8]. Thus, they are particularly abundant in derived food products and beverages, where they play a major role as antioxidants with consequent beneficial effects on shelf-life and health [9–13]. Their molecular structures are composed of linked flavan-3-ol monomers. Procyanidins are an important subclass composed only of (+)-catechin and (−)-epicatechin. A series of recent reports extended this chemical group: uncommon macro-cyclic (named also *crown*) tetrameric and pentameric procyanidins were proposed in red wines from the Bordeaux region [14, 15]. Ongoing investigation on the presence of oligomers in wines from this and other geographical regions continues to uncover new species such as the recently reported cyclic hexameric procyanidin [16]. The parallel identification of the same cyclic procyanidins in cranberries was also disclosed [16], and the selective binding of the new class of compounds with potassium and calcium was studied [17].

It was pointed out that another class of procyanidins containing one A-type linkage cannot be distinguished by classical high-resolution mass spectrometry alone. In fact, these two classes (non-cyclic A-type procyanidins and cyclic B-type procyanidins) are isomeric since they possess identical elemental compositions. It was noted, however, that these classes are distinct with respect to the number of phenolic protons they bear, and these are labile towards solvent exchange. An example of each of the different procyanidin classes (cyclic and non-cyclic, A- and B-types) investigated in this work is reported in Fig. 1. The labile phenolic protons are indicated by circles; the positions that cannot undergo solvent exchange because of the presence of an A-linkage are also indicated by arrows. Hence, a hydrogen/deuterium exchange (HDX) HPLC-HRMS/MS

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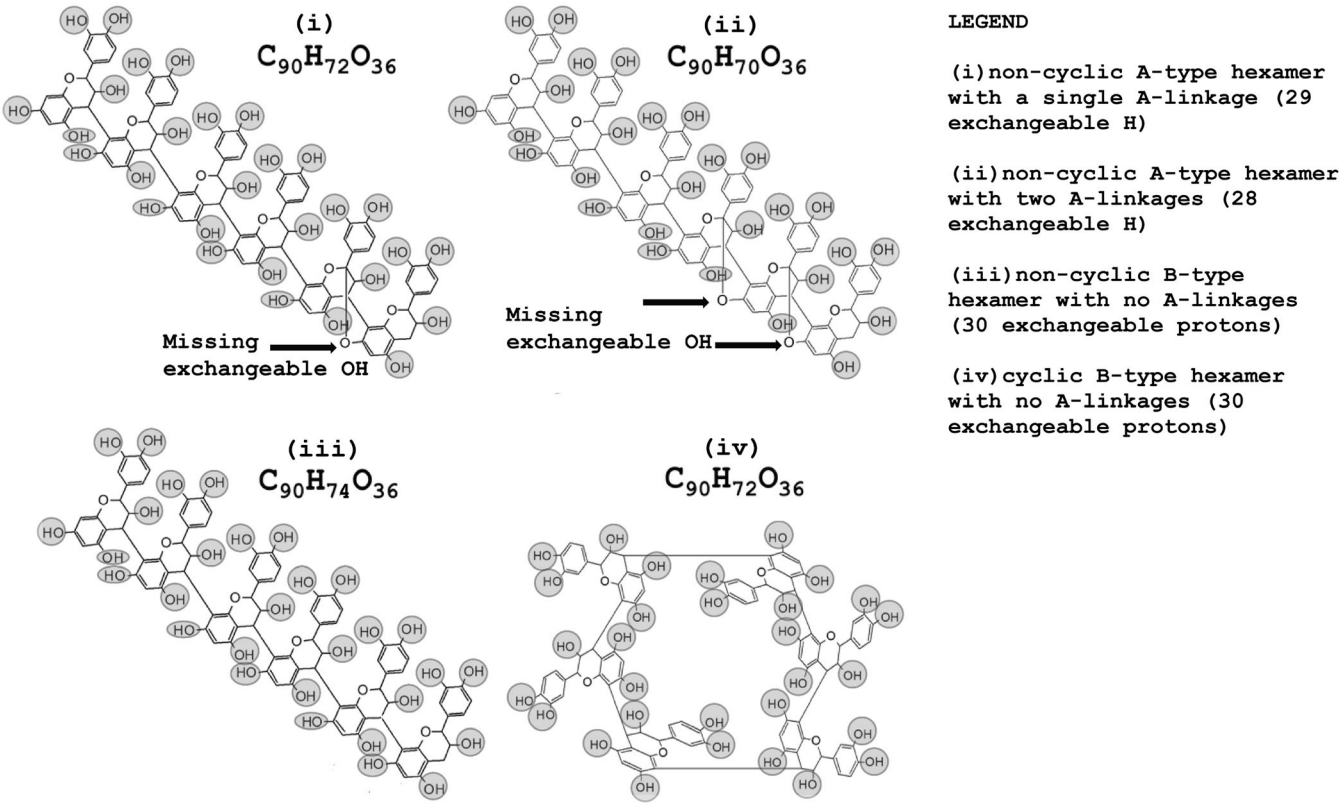

Figure 1. Models for the hexameric procyanidins studied in this work. (*i*) Non-cyclic A-type hexameric procyanidin with one A-linkage (isomeric form a); (*ii*) non-cyclic A-type hexameric procyanidin with two A-linkages; (*iii*) non-cyclic B-type hexameric procyanidin; (*iv*) cyclic B-type hexameric procyanidin with one head-tail B-linkage (isomeric form b). Neither the configurations of the stereogenic centers nor the C4-C6 inter-monomeric linkages preferences were resolved; the configurations shown are just examples

approach was devised in order to allow a fast assessment of the presence of cyclic procyanidins *n*-mers by counting the number of hydrogens exchanged with deuterium. Details of the proposed effects of HDX on these compounds were provided [16]. Briefly, by taking advantage of the labile, exchangeable phenolic –OH protons, it was shown that B-type species exchanged one H more than their correspondent A-type procyanidin analogues. Hence, these species are not isobaric after HDX, and they can be differentiated by mass spectrometric detection alone, without resorting to NMR or other structural approaches. While HDX was successfully applied to the cyclic tetrameric candidate with the substitution of five positions *per* (epi)catechin monomer (*m/z* 1153.2608 {H_2O} became *m/z* 1174.3926 {D_2O}), no evidence of its effect for the known A-type procyanidins could be obtained due to their scarcity in the analyzed matrices, with an exception for some traces found in a cranberry (*Vaccinium* sp.) extract. This scarcity in cranberries was unexpected since earlier works had proposed the presence of the A-type procyanidin tetramer as a major component [6]. Other differences were noticed, namely the distribution of the retention times of the cyclic oligomers (eluting in HPLC earlier than their non-cyclic analogues) and their MS^2 spectra. During the MS/MS experiments, these cyclic oligomers showed much lower degrees of fragmentation than their linear analogues. Moreover, the cyclic analogues displayed a much more limited isomeric distribution, with only one main isomer in contrast to the ample variability of their linear analogues. This was already demonstrated by NMR studies on the tetramer [14]. It was argued that strict requirements may be needed for the cyclization of the non-cyclic precursor, and this may cause the observed limitation in the distribution of cyclic isomers. The macro-molecular conformations depend on the specific stereogenic configurations of the carbon atoms (i.e., number and relative bindings of (+)-catechins and (−)-epicatechins) as well as on the specific interflavan-3-ol binding preferences (C4-C6 or C4-C8). Thus, only specific arrangements of monomers may lead to cyclization. It was also confirmed that in general, the cyclic analogues elute at lower retention times in reversed-phase LC, indicating that they possess a higher polarity than most of their non-cyclic analogues. Also, this effect may be a consequence of the macromolecular conformation of these species.

After the confirmation for the cyclic B-type tetramer [16], a proper validation of the HDX method on a matrix rich in A-type procyanidins was desirable. However, no matrix rich in A-type procyanidins has been analyzed so far in order to describe the effect of HDX on this isobaric class. In the current work, peanut skin as a rich source of A-type procyanidins has been studied. In fact, peanut skin was known for containing dimeric, trimeric, tetrameric, and pentameric A-type procyanidins in

high proportions [2]. These results should provide final evidence of the applicability of this method as a fast and convenient tool complementary to NMR for identifying cyclic procyanidins and discriminating properly between these two classes of oligomeric polyphenols.

Experimental

LC-MS grade solvents (deuterium oxide 99.9%$_D$, acetonitrile, and methanol) and LC-MS additives (formic acid, formic acid-d_2, ammonium formate, and ammonium formate-d_5) were purchased from Sigma Aldrich Srl (Milan, Italy). Milli-Q water was employed and produced *in-house* (18.2 MΩ cm resistivity) by a dedicated device (Arium Mini, Sartorius Italy Srl, Viale A. Casati, 4, 20835 Muggiò, Italy). The wine (Lagrein, 2016 vintage) was donated by a local winery (Kellerei Bozen, Gries, Bolzano, Italy). Whole roasted peanuts were purchased from a local shop.

Peanut Skin Sample Preparation

Peanuts were opened and peeled by hand and 1 g of peanut skin was homogenized in hexane. The suspension was filtered and washed with another 10 mL of hexane. The residue was dried under N_2 and then extracted according to a modified published protocol [6]. Briefly, the residue obtained after hexane washing was extracted with 20 mL of acetone/water/formic acid (70;29.9:0.1 *v*/*v*). The mixture was sonicated (99 W at 50 °C) for 30 min and then filtered. The sample was concentrated at low pressure (9 mbar) followed by gentle N_2 flushing, then dissolved in 250 µL of a mixture of eluent A containing 5% of eluent B. When HDX was performed, the extracted sample was first dried under vacuum (9 mbar), then dispersed in pure deuterated eluent B before fluxing with N_2 to dryness. Finally, it was reconstituted with 250 µL of a mixture of deuterated eluent A containing 5% of deuterated eluent B.

SPE Purification of Wine

The SPE preparation of the wine sample was performed following a modified published report [18]. Briefly, wine was concentrated under a partial vacuum (9 mbar) as shown for peanut skin extract and as reported in an earlier report [16]. The residue was re-dissolved in water to reach one tenth of the initial wine volume. Then, three 6 mL/1 g SPE C18 cartridges (ASPEC C18, 40–63 µm, lot number 66597, Gilson, Inc., 3000 Parmenter Str., Middleton, 53562, WI, USA) were conditioned with 2 mL of methanol followed by equilibration with 2 mL of water, leaving the water level up to the top frit of the cartridges. The concentrated wine sample (2 mL/cartridge) was loaded under gravity into the cartridge. When all the wine was absorbed, the top water was gently dried with N_2. Each cartridge was eluted with 10 mL of 5%$_{aq}$ (*v*/*v*) formic acid in acetonitrile (fraction F1). Then, the cartridges were eluted with 0.1% (*v*/*v*) formic acid in methanol (fraction F2). Finally, 300 µL of pure formic acid was applied to each cartridge followed by 10 mL of 95% (*v*/*v*) methanol. Then, all fractions were evaporated at low pressure (9 mbar) at 30 °C, then washed and re-dried from gradient eluent B, followed by N_2 flushing. The dried fractions F1, F2, and F3 weighed respectively 93.6 mg, 20.1 mg, and 18.0 mg. Equal amounts were destined to analysis with normal or deuterated solvents. When HDX was performed, the samples were first dried and then reconstituted in pure deuterated eluent B before fluxing N_2 and reconstituting in 250 µL of 5% deuterated eluent B in deuterated eluent A.

HPLC-HRMS/MS Analysis

The HPLC-HRMS/MS method was adapted from a published report with slight modifications [16]. A Q Exactive HRAM mass spectrometer (Thermo Fisher Scientific, Rodano, Milan, Italy) coupled to an Agilent 1260 HPLC (Agilent Technologies Itala S.p.A., Cernusco sul Naviglio, Milan, Italy) with a 16-channel DAD detector was used. The chromatographic separation was carried out with a ODS Hypersyl C18 column (125 mm × 4.6 mm i.d., 5 µm, Thermo Fisher Scientific) protected with a column guard (ODS Hypersil, 5-µm-pore size, 10 × 4 mm drop-in guards, Thermo Fisher Scientific) at a flow rate of 1 mL min^{-1}. The mobile eluent consisted of a combination of eluent A (0.1% *v*/*v* formic acid in 0.02 mol L^{-1} ammonium formate in water or 0.1% *v*/*v* deuterated formic acid in 0.02 mol L^{-1} fully deuterated ammonium formate in deuterium oxide) and eluent B (0.1% *v*/*v* formic acid in saturated ammonium formate acetonitrile or 0.1% *v*/*v* deuterated formic acid in saturated fully deuterated ammonium formate acetonitrile, LC-MS grade). The gradient program was 0–21 min, 5–25% B; 22–27 min, 95% B; 28 min, 5% B followed by a 7-min re-equilibration at 5% B. The DAD spectra were recorded from 200 to 600 nm and provided real-time monitoring at 280 nm, 320 nm, 365 nm, 420 nm, 520 nm, and 620 nm (± 4 nm). A post-column flow splitter was used to feed both analyzers in parallel (DAD and HRMS) at a fixed ratio (Upchurch Scientific). HESI MS^1 analysis was performed in positive ion mode. Ion source and acquisition parameters were sheath gas = 20 (arbitrary units), auxiliary gas = 5 (arbitrary units), auxiliary gas temperature = 250 °C, spray voltage = + 3.8 kV (with water) or + 4.0 kV (with deuterium oxide), ion transfer tube temperature = 320 °C, and RF S-lens = 70 (arbitrary unit). Mass range = 500–2000 *m/z*; MS^1 working resolution = 70,000 (@200 *m/z*), AGC target = $3 \cdot 10^6$, max. injection time= 300 ms.

Parallel MS/MS experiments were performed only on 50 selected ions (manually specified in the inclusion list in the main instrument method): MS^1 parameters were kept as shown: MS/MS AGC target = 10^6, max. injection time = 175 ms, working resolution = 35,000 (@200 *m/z*), loop count = 5, isolation window = 2 *m/z*, normalized collision energy 15.0% "if idle" tool was set to "do not pick others". The mass detector was always externally calibrated before the analysis (Pierce LTQ ESI Positive ion Calibration Solution). In every case, lock masses were constantly applied to ensure accuracy (δ(ppm) < 2 ppm) across the MS^1

acquisition range throughout the experiments. Their selection was made on the basis of known elemental compositions of known contaminants and interferents [19].

When deuterium oxide was employed, the new theoretical values for the lock masses were calculated out of the HDX-forced shift observed and applied to the main instrument method. The MS data and results were analyzed by Xcalibur 3.1 software. Extracted ion chromatograms (EIC) are shown with a selected tolerance of 4 ppm unless differently indicated.

Results and Discussion

As mentioned in the "Introduction," cyclic B-type procyanidins were confirmed in wine by several groups [14–16]. However, despite several efforts, some key points have so far been left unresolved, namely the confirmation of the cyclic B-type structure for the *m/z* 1729.3876 hexamer in wine (that derived from several other considerations) and also the effect of HDX on the non-cyclic A-type isomers. A previous attempt to observe directly the effects of HDX on a non-cyclic A-type tetramer analogue (*m/z* 1153.2608) in cranberries failed since the isomeric cyclic B-type procyanidin (identical to that in wine) was unexpectedly found to be its major tetramer instead [16]. The lack of a proper application of HPLC-HDX-HRMS/MS to the A-type isomeric class is the rationale of this work. To achieve this goal, a peanut skin extract as a suitable source of A-type procyanidins was compared with wine [2].

In wine, procyanidin oligomers were identified almost exclusively in the F3 fraction; therefore, the analysis of fractions F1 and F2 was not discussed further. Accordingly, the F3 wine fraction was compared to the peanut skin extract. The MS^1-extracted ion chromatograms and the MS^1 spectra for the tetramers are reported in Fig. 2. The MS/MS fragmentation spectra for the *m/z* 1153.2608 and *m/z* 1151.2452 tetramers in the peanut skin extract and F3 SPE fraction are shown in Fig. 3.

First, the major feature characterizing the wine extract with respect to the peanut skins is the presence of just one

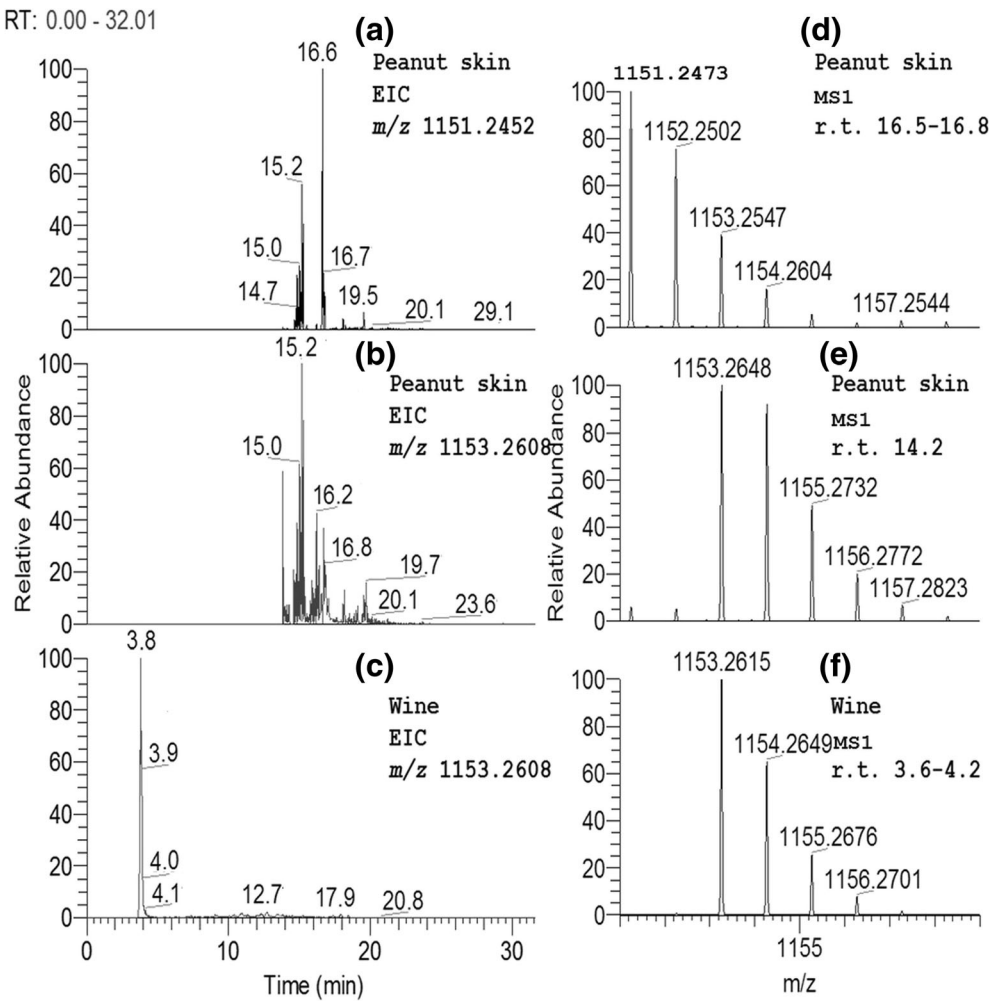

Figure 2. HPLC-HRMS analysis of tetramers. (**a**) Extracted ion chromatogram of *m/z* 1151.2452 (4 ppm filter applied) in peanut skin extract; (**b**) extracted ion chromatogram of *m/z* 1153.2608 (4 ppm filter applied) in peanut skin extract; (**c**) extracted ion chromatogram of *m/z* 1153.2608 (4 ppm filter applied) in wine extract; (**d**) MS^1 spectra of peanut skin (*m/z* 1151–1159 shown range) at 16.5–16.8 min; (**e**) MS^1 spectra of peanut skin (*m/z* 1151–1159 shown range) at 14.2 min; (**f**) MS^1 spectra of wine (*m/z* 1151–1159 shown range) at 3.6–4.2 min. The MS^1 spectra are shown in the *m/z* 1151–1159 mass range

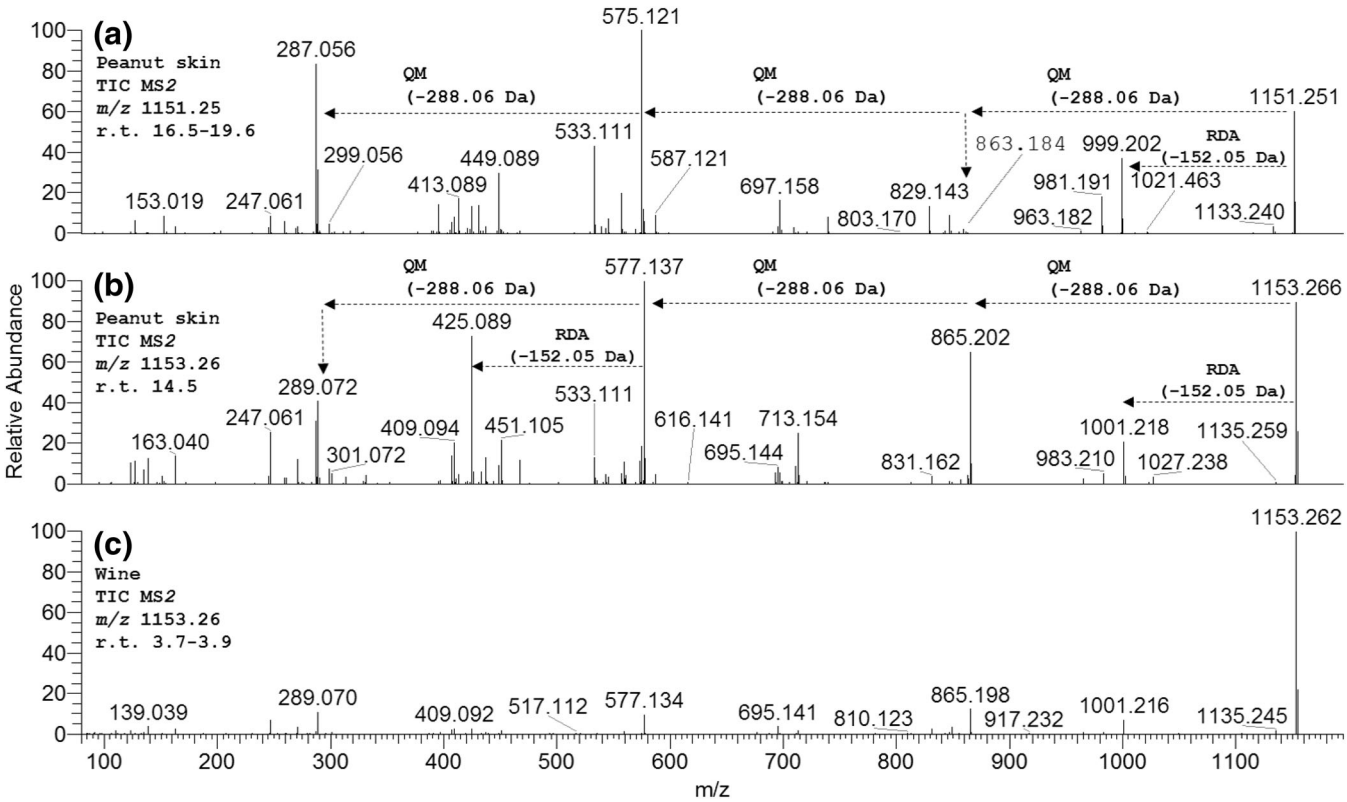

Figure 3. HPLC-MS/MS analysis of peanut skin and wine. (**a**) MS/MS spectrum of precursor *m/z* 1151.245 in peanut skin at 16.5–19.6 min; (**b**) MS/MS spectrum of precursor *m/z* 1153.261 in peanut skin at 14.5 min; (**c**) MS/MS spectrum of precursor *m/z* 1153.261 in wine at 3.7–3.9 min (see Longo et al. (2018) for more details on cyclic tetramer (wine sample) fragmentation)

main peak associated to the *m/z* 1153.2608 tetramer, as earlier reported for wine and a cranberry extract [16]. However, the increased concentration and ionization potential employed allowed us to also investigate species eluting at higher retention times (> 7 min), where the minor components appeared more intense than in earlier tests [16]. In analogy with previous measurements on the cranberry extract [16], these peaks should be assigned to A-type analogues. In fact, as shown with cranberries and wine, non-cyclic procyanidins (A- and B-type alike) displayed higher affinities for the RP stationary phase than the cyclic ones. In the peanut skin extract, *m/z* 1153.2608 extracted ion chromatogram (Fig. 2b) showed multiple peaks at retention times > 7 min. However, a contribution from *m/z* 1151.2452 species overlapped that of *m/z* 1153.2608 below 16 min to such an extent that mixed MS/MS spectra could not be prevented at 14.6–15.3 min and 16.4–16.8 min. Hence, the *m/z* 1153.261 MS/MS spectrum (Fig. 3b) for the peanut skin extract was shown only for retention times where no *m/z* 1151.2452 peak interfered. Besides, *m/z* 1151.2452 (Fig. 2a) extracted ion chromatogram showed its main isobaric peaks at 15.2 min and 16.6 min. The *m/z* 1151.251 spectrum (Fig. 3a) was accordingly displayed at r.t. > 16.5 min to avoid mixed spectra with the *m/z* 1153.261 precursor; *m/z* 1153.261 MS/MS spectrum (Fig. 3b) for the peanut skins extract is shown at 14.5 min.

MS/MS fragmentations for B-type cyclic and non-cyclic (A- and B-types) analogues were discussed earlier [16, 20, 21]. Also, the A-type species followed similar considerations regarding the main fragmentation mechanisms observed for non-cyclic and cyclic B-type procyanidins [16]. Quinone methide (QM, − 288.06 Da) mechanism produced losses of entire monomer units, breaking the precursor ion structure one monomer (epi)catechin at a time. Besides, both the Retro Diels-Alder (RDA) mechanism (− 152.05 Da; for *m/z* 1151.245: *m/z* 999.202, 423.111; for *m/z* 1153.261 in peanut skin: *m/z* 1001.118, 713.154, 425.089) with possible further losses of water molecules (e.g., for precursor *m/z* 1151.245: *m/z* 981.192; for *m/z* 1153.261 in peanut skin: *m/z* 983.210) and the heterocyclic ring fission (HRF) mechanism (− 126.03 Da; for *m/z* 1151.245: *m/z* 449.089; for *m/z* 1153.261 in peanut skin: *m/z* 451.105) contributed. These mechanisms have been investigated and reported in detail for procyanidins [20]. They are summarized in Figure SI 1 (in the Supporting information).

The most evident difference among the A-type procyanidin with two A-linkages (Fig. 3a), the A-type procyanidin with one A-linkage (Fig. 3b), and the cyclic B-type procyanidin (Fig. 3c) was the fragmentation degree displayed by non-cyclic and cyclic species, which has already been described for wine and cranberries [16]. The cyclic B-type underwent fragmentation to a much smaller extent than any non-cyclic analogue at this collision energy

(in this study, normalized collision energy was 15%). Another evident discrepancy was the different distribution in retention times observed for *m/z* 1153.2608 in peanut skin (Fig. 2b) and in wine (Fig. 2c) (as in cranberries). This observation was analogous to that observed with non-cyclic B-types [16]. Non-cyclic analogues also showed a wider distribution of isomers in peanut skin. Notably, the same effect was observed for the tetramer *m/z* 1151.2452 (Fig. 2a) with two A-linkages.

Then, HDX was applied to the peanut skin extract and to the SPE wine fraction F3, and the results are displayed in Fig. 4. The wine F3 sample showed one main peak in the extracted ion chromatogram of *m/z* 1174.3926 (Fig. 4f), corresponding to the cyclic B-type tetramer (spectrum in Fig. 4c). With the new applied analysis conditions (ionizing potential + 4 kV, the wine sample purified by SPE), new minor features appeared at higher retention times (r.t. > 7 min in Fig. 4f) compatible with tetrameric A-type procyanidin isomers (spectrum in Fig. 4h), in analogy with what had been seen in cranberries [16]. Instead, the main peaks in the peanut extract appeared either for *m/z* 1173.3864 A-type tetramer (EIC in Fig. 4d; MS^1 in Fig. 4e, and Fig. 4g), and for *m/z* 1170.3644 A-type tetramer (EIC in Fig. 4a; MS^1 in Fig. 4b), which is likely assignable to an A-type tetrameric procyanidin with two A-linkages (by forming a new A-linkage, this one has a [D+H] less than the *m/z* 1173.3864 A-type).

An important consideration must be made at this point. The spectra of all species under investigation presented an over-deuteration effect, which causes the isotopic pattern to be distorted. At any tested ionization potential (+ 2.6, + 2.8, + 3.0, + 3.2, + 3.5, + 3.8 and + 4 kV), this phenomenon took place with non-predictable effects on the isotopic profile shape. Surprisingly, the highest potential tested did not show the rightmost shift as one would expect. The mechanism involving non-labile C–H hydrogens in flavan-3-ols undergoing HDX was discussed by Niemeyer et al. [22]. However, in that case, the study was performed in negative ion mode; thus, those considerations may not apply here. Accordingly, the relative peak intensities in the pattern could also depend on the specific conformation of the oligomer, as reported in that report for (epi)catechin gallate. This effect was observed here by comparison between the spectra of *m/z* 1173.3864 (in the peanut skin extract) at ~ 10.0 and ~ 11.4 min (Fig. 4e, g respectively).

The lowest peak in the "isotopic" pattern (at least for the potential applied here) should be reasonably produced by HDX of all and only the labile phenolic protons since no energetic barrier impedes the exchange. Notably, peaks preceding the leftmost peak with a considerably tiny contribution were also consistently observed (− 1 Da and − 2 Da) for all the species, but these are very probably caused by a minimal percentage of residual H_2O (which is guaranteed < 1% in the used deuterium oxide).

Another issue may arise if more procyanidins with close molecular weights and with comparable relative abundances overlap in time (as for retention times higher than 7 min). This occurred for non-cyclic A- and B-type species, which share similar ranges of retention time with this chromatographic method. However, comparison of the extracted ion chromatogram profiles and of spectral patterns can aid the analysis, particularly if the overlapping species possess different relative abundances.

Beside tetramers, pentamers, and hexamers were also investigated in water and deuterium oxide. Only the cyclic B-type tetramer was earlier addressed by HDX [16]. The results of the analysis in deuterium oxide are reported in Figs. 5 and 6 respectively for pentamers and hexamers. Table SI 1 summarizes the results for dimeric and trimeric, tetrameric, pentameric, and hexameric procyanidins in both water and deuterium oxide. For the discussion of these results, it must be noted that the resolution of the configuration of the stereogenic centers, the preferences of inter-monomers linkages (C4-C6 or C4-C8) and even the position of the A-linkages in the A-type procyanidins are beyond the purpose of the applied method and must be considered unresolved. The resolution of these structural features may be achieved after isolation and purification of each single compound, after selecting a suitable natural source, and further structural characterization by either NMR or other techniques (e.g., X-ray crystallography). With HDX, only the assessment of the macro-cyclic B-type structure is achieved.

Regarding the heavier oligomers investigated herein (the pentameric and hexameric cyclic procyanidins), the presence in wine of two probable cyclic pentamers (in water, r.t. 4.0 and 4.4 min; in deuterium oxide, 4.9 and 5.4 min) and two cyclic hexamers (in water, r.t. 5.9 and 10.2 min; in deuterium oxide, 6.8 and 11.1 min) was observed (see Table SI 1 in the Supporting Information). In the previous HDX study on wine and cranberry extract [16], only the tetramer could be confirmed, and only one pentameric isomer was detected. In the current study, the confirmation of pentamers and hexamers with HDX was surely due to the higher ionization potentials, to the SPE purification applied, and to the higher sample concentrations. The results confirmed instead previous observations of the presence of one cyclic B-type tetramer and two cyclic B-type pentamers in wine [14, 15]. The assignment of the theoretical masses corresponded to the experimental observation also for the smaller A-type oligomers (dimers and trimers, see Table SI 1), although no equivalent cyclic structures were envisioned for them. In peanut skin, A-type procyanidin pentamers with one A-linkage (chromatogram in Fig. 5d and spectrum in Fig. 5g) and two A-linkages (chromatogram in Fig. 5a and spectrum in Fig. 5b, e) were identified. Moreover, A-type hexamers were observed in peanuts (Fig. 6, chromatograms in Fig. 6a, d).

For the first time, HDX allowed for confirmation of the cyclic hexamer in wine (Fig. 6g), which was previously only proposed [16]. Besides, a minor isomer still compatible with a cyclic B-type hexameric structure was observed in wine at r.t. 6.8 min (chromatogram in Fig. 6e and spectrum in Fig. 6c) in analogy with the presence of two cyclic pentamer isomers.

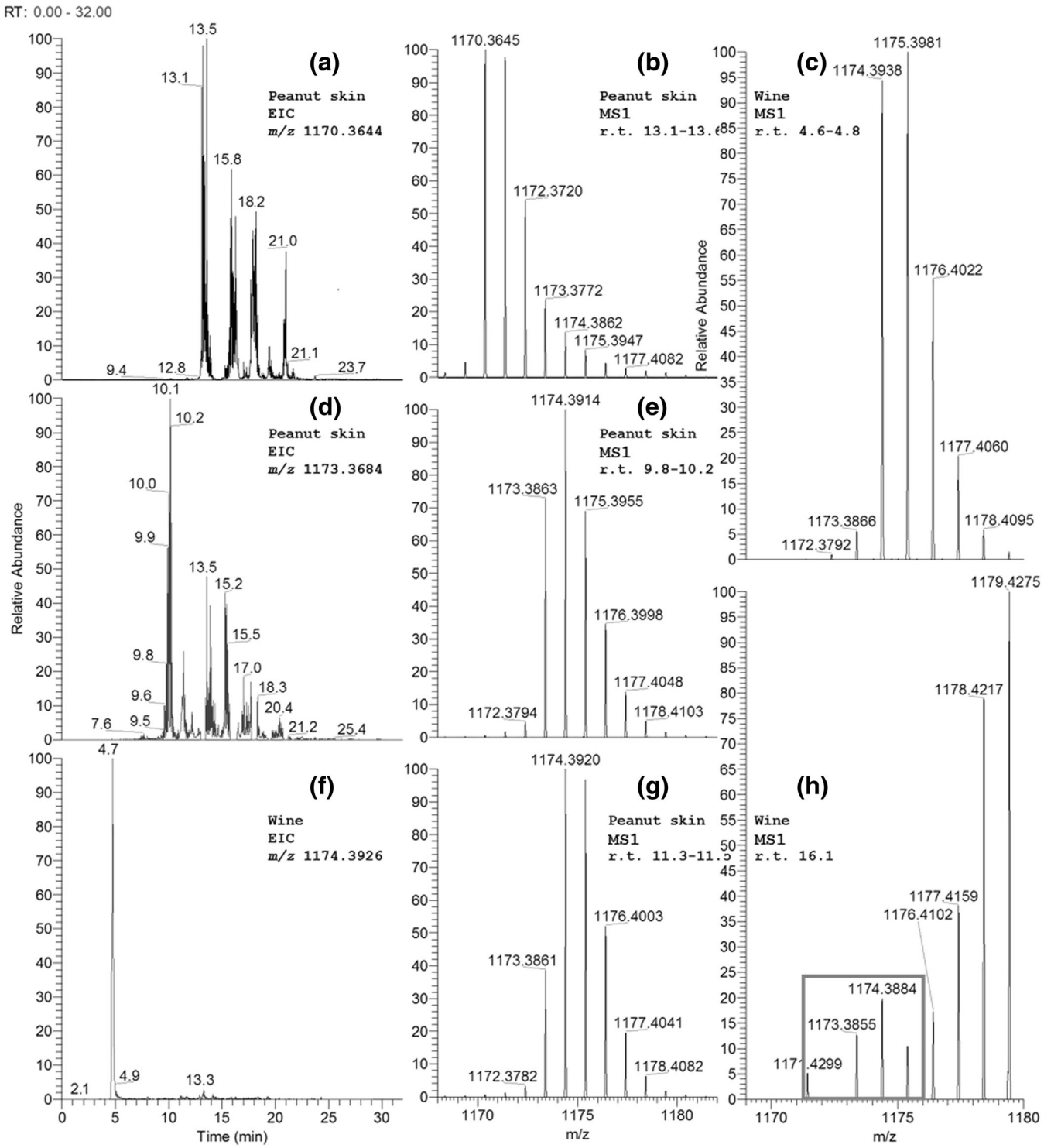

Figure 4. HDX applied to the HPLC-HRMS analysis of peanut skin and wine: tetramers. (**a**) Extracted ion chromatogram of *m/z* 1170.3644 (4 ppm filter applied) in peanut skin extract; (**b**) MS^1 {D} spectrum of peanut skin (*m/z* 1168–1182 shown range) at 13.1–13.6 min; (**c**) MS^1 {D} spectrum of wine (*m/z* 1169–1180 shown range) at 4.6–4.8 min; (**d**) extracted ion chromatogram of *m/z* 1173.3864 (4 ppm filter applied) in peanut skin extract; (**e**) MS^1 {D} spectrum of peanut skin (*m/z* 1168–1182 shown range) at 9.8–10.2 min; (**f**) extracted ion chromatogram of *m/z* 1174.3926 (4 ppm filter applied) in wine extract; (**g**) MS^1 {D} spectrum of peanut skin (*m/z* 1168–1182 shown range) at 11.3–11.5 min; (**h**) MS^1 {D} spectrum of wine (*m/z* 1169–1180 shown range) at 16.1 min (the spectrum of A-type tetramer *m/z* 1173.3864 is indicated in the box)

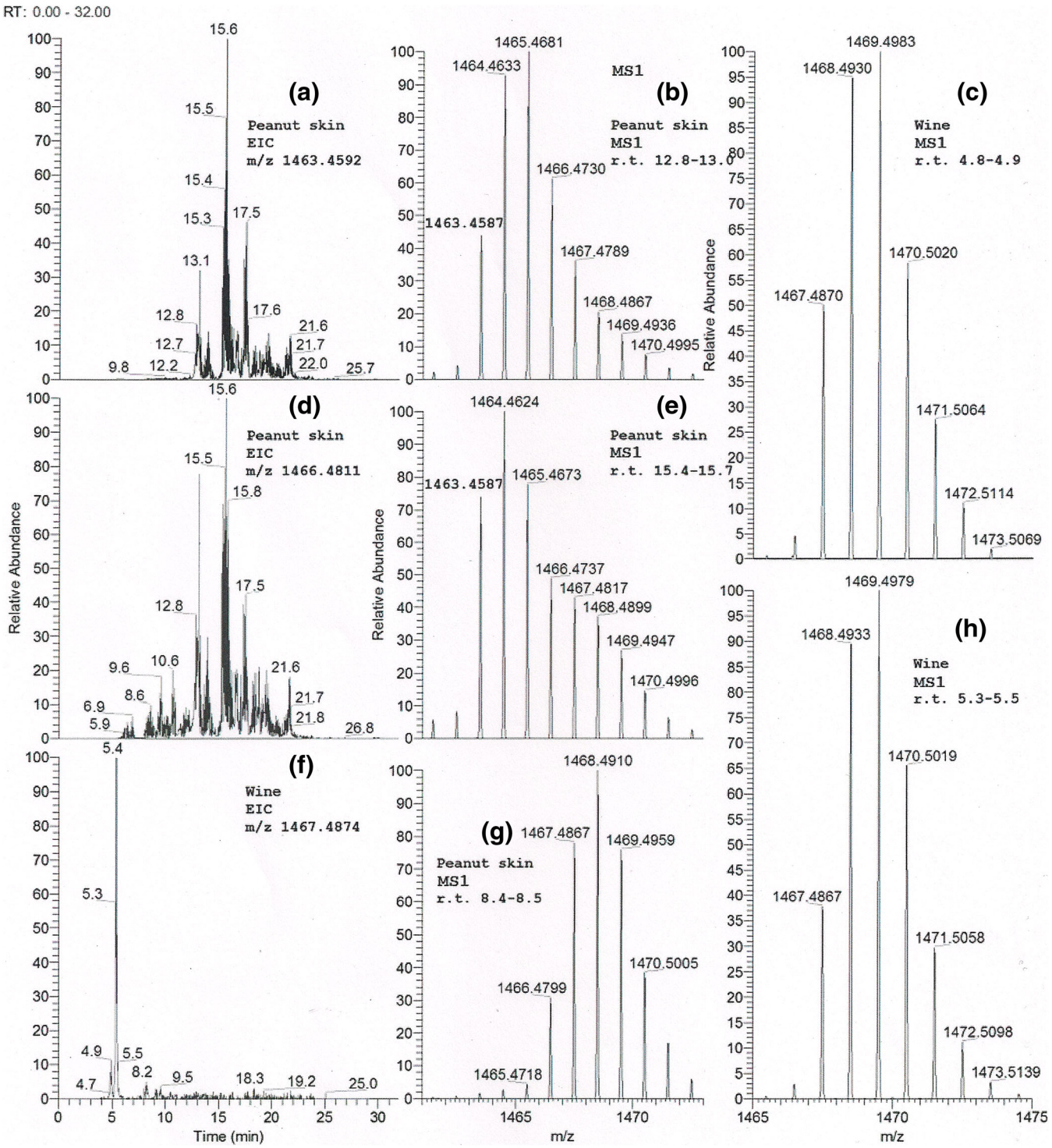

Figure 5. HDX applied to the HPLC-HRMS analysis of peanut skin and wine: pentamers. (**a**) Extracted ion chromatogram of *m/z* 1463.4592 (4 ppm filter applied) in peanut skin extract; (**b**) MS^1 {D} spectrum of peanut skin (*m/z* 1461–1473 shown range) at 12.8–13.0 min; (**c**) MS^1 {D} spectrum of wine (*m/z* 1465–1475 shown range) at 4.8–4.9 min; (**d**) extracted ion chromatogram of *m/z* 1466.4811 (4 ppm filter applied) in peanut skin extract; (**e**) MS^1 {D} spectrum of peanut skin (*m/z* 1461–1473 shown range) at 15.4–15.7 min; (**f**) extracted ion chromatogram of *m/z* 1467.4874 (4 ppm filter applied) in wine extract; (**g**) MS^1 {D} spectrum of peanut skin (*m/z* 1461–1473 shown range) at 8.4–8.5 min; (**h**) MS^1 {D} spectrum of wine (*m/z* 1465–1475 shown range) at 5.3–5.5 min

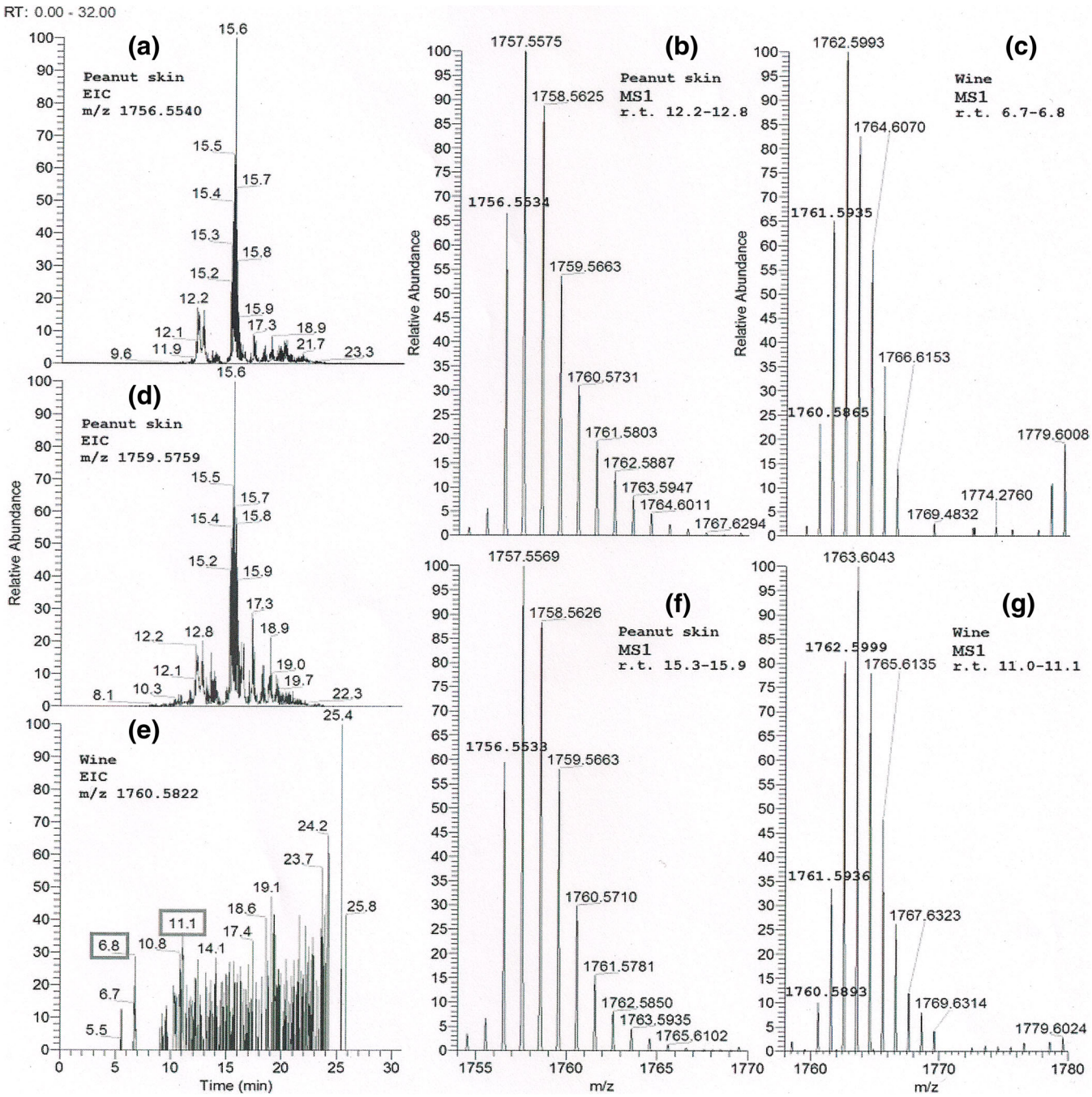

Figure 6. HDX applied to the HPLC-HRMS analysis of peanut skin and wine: hexamers. (**a**) Extracted ion chromatogram of *m/z* 1756.5540 (4 ppm filter applied) in peanut skin extract; (**b**) MS^1 {D} spectrum of peanut skin (*m/z* 1754–1770 shown range) at 12.2–12.8 min; (**c**) MS^1 {D} spectrum of wine (*m/z* 1759–1780 shown range) at 6.7–6.8 min; (**d**) extracted ion chromatogram of *m/z* 1759.5759 (4 ppm filter applied) in peanut skin extract; (**e**) extracted ion chromatogram of *m/z* 1760.5822 (4 ppm filter applied) in wine (peaks associated to cyclic B-type hexamer are highlighted); (**f**) MS^1 {D} spectrum of peanut skin (*m/z* 1754–1770 shown range) at 15.3–15.9 min; (**g**) MS^1 {D} spectrum of wine (*m/z* 1759–1780 shown range) at 11.0–11.1 min

Conclusions

HDX applied to HPLC-HRMS/MS analysis allowed the unambiguous identification of A-type procyanidins (with up to three A-linkages) in peanut skin and B-type (cyclic and non-cyclic) procyanidins in wine for the first time. The previous report [16] could not provide HDX studies on A-type procyanidins and also failed to provide the deuterated counterpart for the B-type cyclic pentamer and hexamer. This new study filled this gap, confirming the usefulness of HDX for structural investigations on cyclic procyanidins complementary to NMR and other structural analytical techniques.

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