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CHARACTERISATION OF OLIGOMERIC AND POLYMERIC PROCYANIDINS FROM GRAPE SEEDS BY LIQUID SECONDARY ION MASS SPECTROMETRY

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Key Word Index—*Vitis vinifera*; Vitaceae; grape seeds; proanthocyanidins; condensed tannins; liquid secondary ionisation mass spectrometry.

Abstract—Several fractions of oligomeric and polymeric procyanidins have been obtained from grape seeds by gel chromatography on Fractogel TSK. Their molecular weights (Mw) were directly determined by Liquid Secondary Ion Mass Spectrometry (LSIMS). We observed that tannins were molecules with a wide range of Mw: from 290 to 3100 (decamers). Catechins (Mw 290), procyanidin dimers (Mw 578), (−)-epicatechin gallate (Mw 442), procyanidin dimer gallates (Mw 730), procyanidin dimer digallates (Mw 882), procyanidin trimers (Mw 866) and procyanidin trimer gallates (Mw 1018), were identified as $(M-H)^-$ quasimolecular ions by negative LSIMS. Some structures were elucidated by partial thiolysis and enzymatic hydrolysis.

INTRODUCTION

The proanthocyanidins, known as condensed tannins, are widely distributed in the plant kingdom and represent a ubiquitous group of plant phenolics [1–3]. In grape seeds, procyanidins represent in general the major part of the total polyphenol extract, and their extreme complexity is the result of the large number of different compounds with very similar structures. They are, in fact, composed of chains of flavan-3-ols units, (+)-catechin (**1**) and (−)-epicatechin (**2**), linked together through C(4)—C(6) and C(4)—C(8) interflavanoid bonds, and various gallate esters [7, 8], Fig. 1. Condensed procyanidins exist as oligomers containing up to five or six catechins units, and as more condensed polymers. The structures of larger oligomeric and polymeric procyanidins are practically unknown.

The procyanidins are partially responsible of the organoleptic characteristics of grapes and wines (e.g. astringency and bitterness) as a result of their tanning properties, which depend on the procyanidin structures and increase with their degree of polymerisation [9–12]. Several techniques including ^{13}C NMR [14], mass spectrometry [15, 16], vapour pressure osmometry (VPO), low-angle laser light scattering (LALLS) [17, 18], gel permeation chromatography (GPC) [19] and thioacidolysis [20, 21], have been used to determine the Mw and the weight distribution of the condensed tannins in plants. However, analysis through most of these techniques requires a previous sample derivatisation (except for FAB mass spectrometry), in order to estimate the weight average of plant polyphenols.

This paper reports a direct and fast measurement of Mw of oligomeric and polymeric procyanidins by Liquid Secondary Ion Mass Spectrometry (LSIMS) in negative mode.

RESULTS AND DISCUSSION

Procyanidins were isolated from grape seeds tissues (Sémillon) as described elsewhere [22]. Column chromatography of procyanidins extract on TSKgel HW-40(s) (350 × 16 mm i.d.) gave several fractions, labelled **A** to **I**, differing in their composition in oligomeric procyanidins, Fig. 2.

For each fraction, the Mw of the eluted compounds was determined directly by LSIMS and required no pre-preparation. After several preliminary assays, glycerol was finally chosen as the matrix, and spectra were acquired in negative mode (because polyphenols are easily deprotonated). For the chosen LSI—mass spectrometry analysis conditions (see Experimental),

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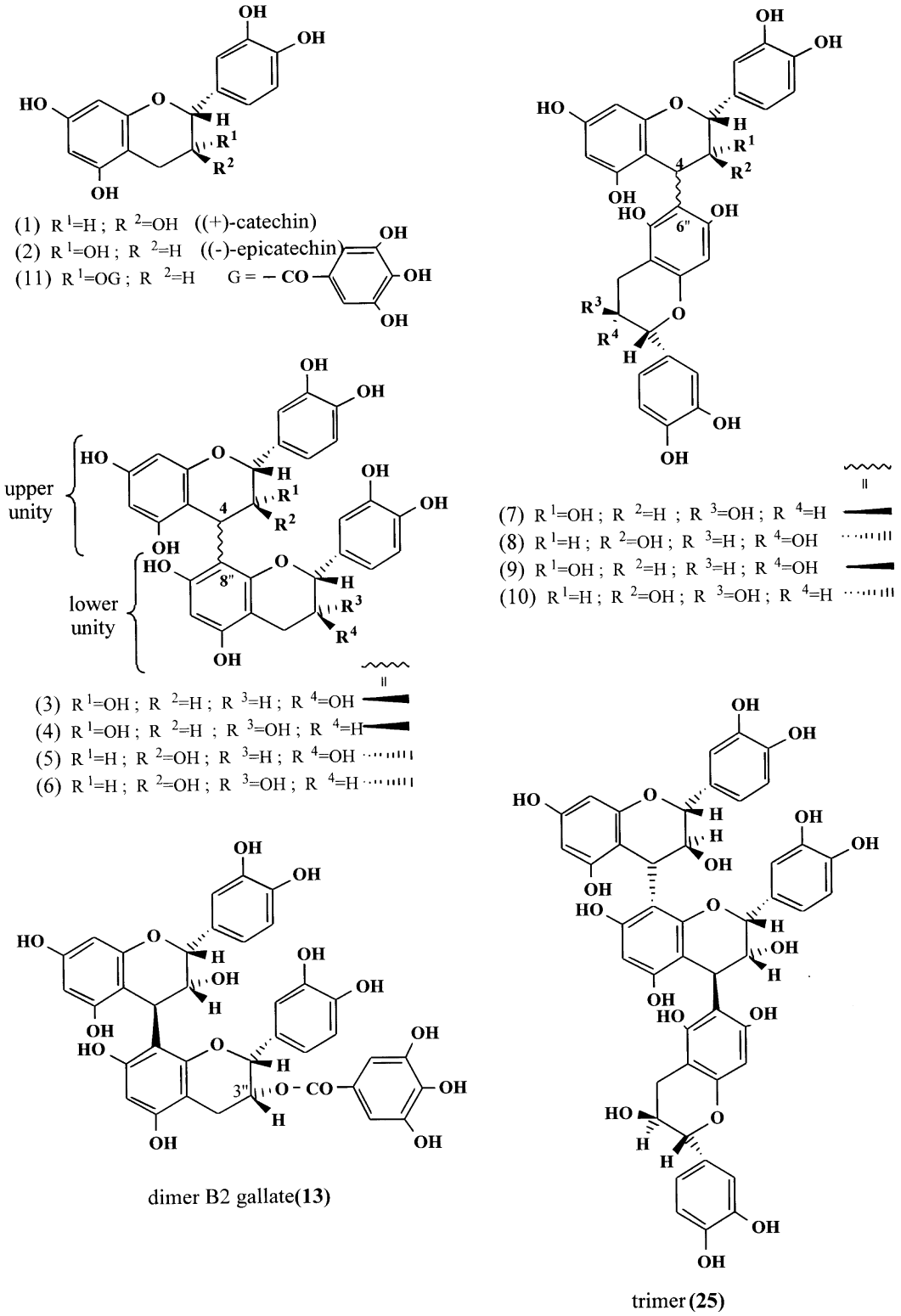

Fig. 1. Structures of catechins, procyanidin dimers and trimers.

each spectrum gave intense $[M-H]^-$ quasimolecular ions of procyanidins, with a faint fragmentation.

Figure 3 shows the LSIMS spectra for the condensed tannins extracted from grape seeds. The spectrum between 200 and 1300 Da, corresponds to the quasimolecular ions of oligomeric procyanidins. **B** to **I**

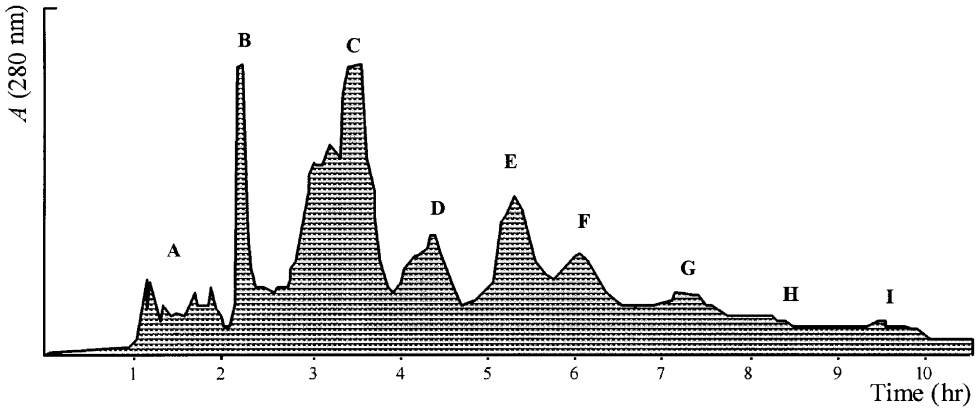

Fig. 2. Fractogel TSL fractionation of procyanidin oligomers from a grape seed phenolic extract (350 × 16 mm i.d.; eluent, MeOH; flow-rate, 0.85 ml min^{-1}).

refer to the molecular ion peaks of eluted procyanidins and their respective fractions. The procyanidin oligomers were isolated by HPLC semi-preparative scale and their structures were elucidated by enzymatic hydrolysis and partial acid-catalysed degradation by thiolysis [8, 23]. The values of Mw measured by LSIMS in the different fractions and the proposed structures are summarised in Table 1. Though enzymatic hydrolysis will not discern the position of the gallate ester linkage in the procyanidins, it is known, from literature, to be usually situated at C3 of the pyran rings [8].

The fraction **A** contains protocatechuic, gallic and caffeic acids, identified by co-elution with authentic standards by analytical HPLC. The evidence of phenolic acids structures is supported by LSI mass spec-

Table 1. Structures of grape seed flavan-3-ol derivatives (and phenol acids) from TSKgel chromatography fractions, values on analytical HPLC retention times and products of partial thiolysis of trimer followed by reduction with Raney nickel. cat = (+)-catechin; epi = (−)-epicatechin

Fraction (m/z $[M-H]^-$)	Compounds identified	R_t (min)	Products of thiolysis
A (153; 169; 179)	protocatechic, gallic and caffeic acids	16.5, 9.7, 38.3	—
B (289)	(+)-catechin (**1**)	29.5	—
	(−)-epicatechin (**2**)	48.6	—
C (441; 577)	(−)-epicatechin gallate (**11**)	74.7	—
	dimers B1 (**3**), B2 (**4**), B3 (**5**), B4 (**6**), B5 (**7**) and B7 (**9**)	*	—
D (577; 865)	dimers B6 (**8**) and B8 (**10**)	39.9; 47.0	—
	epi-(4β-8)-epi-(4β-8)-epi (trimer C1) (**22**)	50.8	(**2**) and (**4**)
E (729; 865)	gallate esters of dimers B1, B2, B3, B4 and B5 (**12**–**16**)	†	—
	trimer epi-(4β-8 or 6)-epi-(4β-6 or 8)-epi (**23**)	28.0	(**2**), (**4**) and (**7**)
	trimer cat-(4α-8)-epi-(4β-8)-epi (**24**)	23.6	(**1**), (**2**), (**4**) and (**6**)
F (729; 865)	procyanidin B6 gallate (**17**)	34.7	—
	trimer cat-(4α-8)-epi-(4β-6)-cat (**25**)	24.8	(**1**), (**6**) and (**9**)
	trimer cat-(4α-8)-cat-(4α-8)-epi (**26**)	21.8	(**1**), (**2**), (**5**) and (**6**)
G (865; 1017)	trimer epi-(4β-8)-cat-(4α-6)-epi gallate (**27**)	55.5	(**2**), (**4**) and (**10**)
	trimer epi-(4β-8)-epi-(4β-6)-cat gallate (**28**)	38.0	(**1**), (**2**), (**4**) and (**9**)
	trimer cat-(4α-6 or 8)-epi-(4β-6)-epi gallate (**29**)	44.1	(**1**), (**2**) and (**7**)
	trimer epi-(4β-6 or 8)-epi-(4β-8 or 6)-epi gallate (**30**)	34.1	(**2**) and (**7**)
	trimer epi-(4β-6)-cat-(4α-6)-epi gallate (**31**)	47.5	(**2**), (**9**) and (**10**)
H (881; 1153)	digallate esters of dimers B1, B2, B3 and B7 (**18**–**21**)	‡	—
I (1017; 1170)	trimer gallates and digallates	—	

* 21.8, 37.7, 23.4, 32.2, 83.7 and 59.3, respectively.
† 37.5, 57.3, 57.9, 29.6 and 77.6, respectively.
‡ 24.4, 47.1, 28.1 and 60.6, respectively.

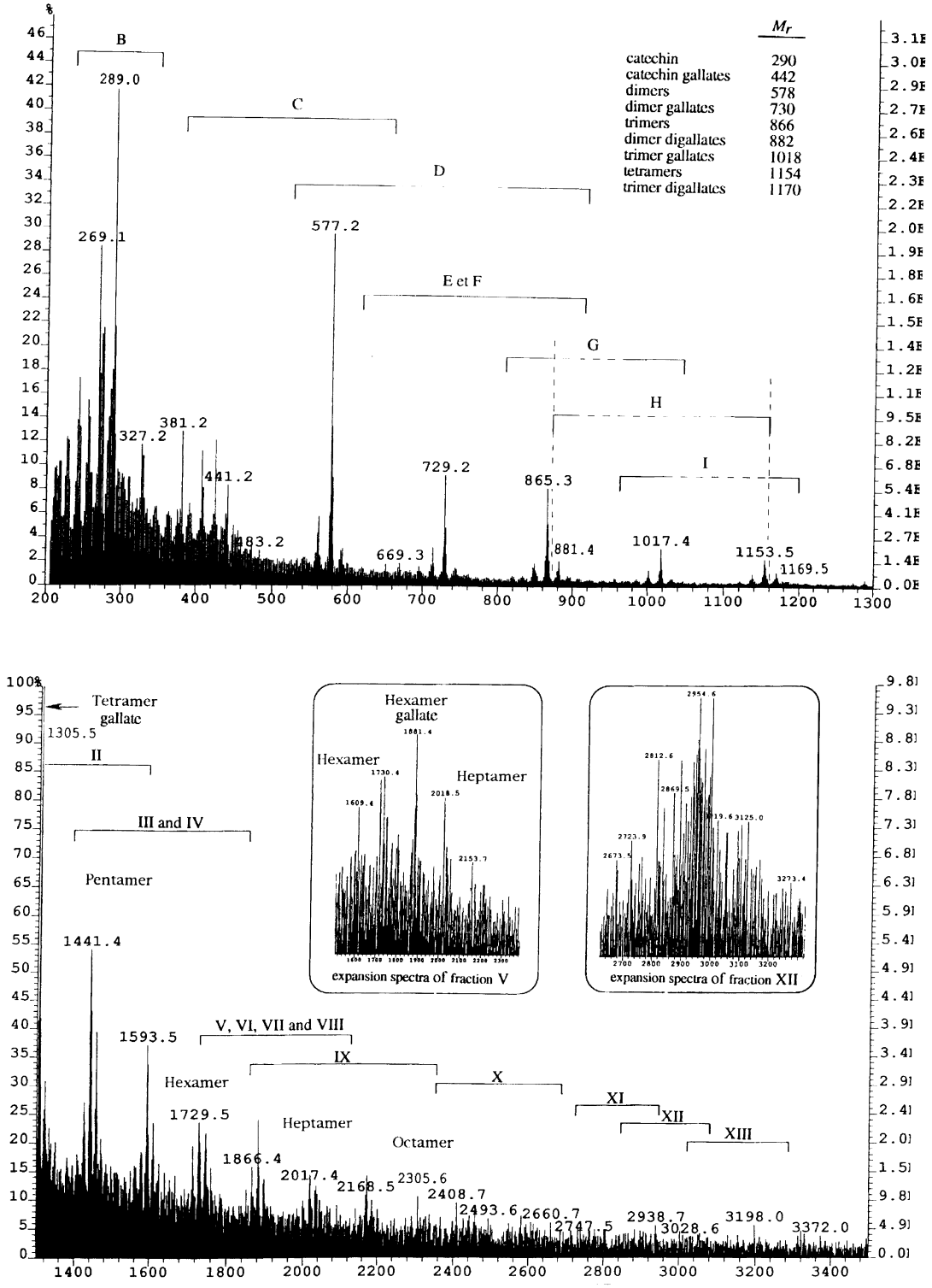

Fig. 3. LSIMS spectrum of oligomeric (200–1300) and polymeric (1300–3500) procyanidins from grape seeds polyphenolic extract.

tra, since the respective $[M-H]^-$ ion peaks at *m/z* 153, 169 and 179 were obtained. Fraction **B** exhibit in the LSI mass spectra the $[M-H]^-$ ion peak at *m/z* 289 corresponding to the catechins (**1**) and (**2**). The spectrum of the fraction **C** gave $[M-H]^-$ ion peaks at *m/z* 441 and 577 consistent with a (−)-epicatechin monogallate (**11**) and procyanidin dimers structures, respectively. The dimers B1 to B4 and B7 were ident-

ified by co-chromatography with authentic standards using analytical HPLC and also from partial degradation by thiolysis. The additional dimers B6 and B8 and trimer C1 were eluted later in the next fraction. Effectively, fraction **D** exhibits in the LSI mass spectra the characteristic $[M-H]^-$ ions at m/z 577 and 865, corresponding to the additional dimers and trimer, respectively.

Both **E** and **F** fractions LSI mass spectra gave the $[M-H]^-$ ion peaks at m/z 729 and 865 consistent with a dimer gallates and trimers structures, respectively. These procyanidins were isolated by HPLC from semi-preparative scale and the structures of dimer monogallates (**12**–**17**) and trimers (**23**–**26**) were elucidated by enzymatic hydrolysis and by partial thiolysis with toluene-α-thiol, respectively (see Table 1); the fragments released were identified by comparison of their chromatographic data with those of authentic standards. The spectrum of the fraction **G** exhibited $[M-H]^-$ ion peaks at m/z 865 and 1017 corresponding to trimers (not identified) and trimer gallates, respectively. Procyanidin trimer monogallates (**27**–**31**) purified by HPLC were first submitted to enzymatic hydrolysis and yielded all gallic acid. The trimer moiety structure released was then identified by partial thiolysis as is described above (Table 1). Fraction **H** gave $[M-H]^-$ ion peaks at m/z 881 and 1153 consistent with dimer digallates and tetramers, respectively. Only dimer digallate were suggested by enzymatic hydrolysis to be compounds **18**–**21**. Fraction **I** exhibits in its LSIMS the $[M-H]^-$ ion peaks at m/z 1017 and 1169, consistent with trimer gallates and digallates constitution, respectively (structures not found).

Polymeric procyanidins

Larger molecules of procyanidins are exceedingly polar to be eluted only with methanol. Consequently, the experimental conditions for chromatography on TSKgel HW-40(s) were changed (100×16 mm i.d.) and methanol was slightly acidified with HOAc in order to separate the polymeric procyanidins. The new conditions yielded thirteen fractions (I–XIII) which were directly analysed by LSIMS. Figure 3 shows part of the LSIMS mass spectrum of the polymeric procyanidins in grape seeds extract (1300–3500). The fraction numbers (II–XIII) indicate the range of molecular ions peaks exhibited in the respective spectrum. The molecular weights and compositions of the procyanidins eluted from grape seeds are showed in Table 2.

LSIMS analysis was limited by the mass ranges calibration, which was performed using caesium iodide salt between 200–3500. On the other hand, as the degree of polymerisation of eluted procyanidins increased, the molecular ion peak signals becomes smaller and approaches the background noise, which made difficult their identification. Nevertheless, the quasimolecular peaks exhibited in the last four fractions spectra (X, XI, XII and XIII) gave important knowledge about the size of higher polymers up to 3100.

LSI mass spectrometry is a very attractive technique, because it is fast and requires only little amounts of procyanidin extract to be directly analysed without derivatisation. To our knowledge, the exact molecular weight of larger procyanidins up to seven and more units of catechins, were determined here for the first time. On the basis of our spectrometer data, it is evident that procyanidins exist in grape seeds with an extremely wide Mw range from 578 up to 3100, and are predominantly esterified by one or more molecules of gallic acid.

EXPERIMENTAL

(+)-Catechin (**1**) and (−)-epicatechin (**2**) were purchased from Extrasynthèse and Fractogel TSK HW-40(s) from Merck. Procyanidin dimers B1 to B8 (**3**–**9**) and C1 (**10**) were synthesised following the methods in Refs [24, 25].

Table 2. Composition and molecular weights of procyanidin from grape seeds fractions, determined by LSIMS. From each fraction, the underlined compound corresponds to the one which has the more important $[M-H]^-$ ion peak

Fraction	Procyanidins (Mw)
I	catechins (290); catechin gallate (442); dimers (578); dimer gallates (730); trimers (866); dimer digallates (882); trimer gallates (1018); tetramers (1154); trimer digallate (1170)
II	tetramer gallates (1306); pentamers (1422); tetramer digallates (1458); pentamer gallates (1594)
III	tetramer gallates (1306); pentamers (1442); tetramer digallates (1458); pentamer gallates (1594); tetramer tetragallates (1762); hexamers (1730)
IV	pentamers (1442); tetramer digallates (1458); pentamer gallates (1594); tetramer tetragallate (1762); hexamers (1730); pentamer digallates (1746) hexamer gallates (1882)
V and VI	pentamer digallates (1746); hexamer gallates (1882) heptamers (2018)
VII and VIII	hexamer gallates (1882); heptamers (2018); hexamer digallates (2034)
IX	pentamer trigallates (1898); heptamer galates (2170); octamers (2306)
X to XIII	$2900 \leqslant Mw \leqslant 3100$

Extraction

Grape seeds (*Vitis vinifera*) were extracted with 50% aq. ethanol according to the procedure described in Ref. [22].

Isolation of oligomeric procyanidins

The extract was subjected to chromatography over Fractogel TSKL HW-40(s) (350 × 25 mm i.d.) using MeOH as the eluant at 0.85 ml min^{-1} and gave nine frs, noted **A** to **I**, containing various oligomeric procyanidins. Each fr. was freeze-dried after eliminating the solvent with a rotatory evaporator under reduced pressure at 30°. The resulting solids were analysed by HPLC and LSIMS.

Isolation of polymeric procyanidins

The extract was chromatographed on TSKgel HW-40(s) (100 × 16 mm i.d., 0.85 ml min^{-1}) and procyanidins elution was performed with a mixture of MeOH in 5% HOAc during 22 h, giving frs I to IX, and then with MeOH in 10% of HOAc over another 8 h, to obtain frs X to XIII. The frs were first mixed with H_2O and then MeOH was removed. The aq. solns of condensed tannins acidified by HOAc were freeze-dried and the white amorphous resultant solids were analysed by LSIMS.

LSIMS analysis

A few mg of each sample was dissolved in the minimum volume of anhydrous MeOH and then dissolved in a matrix of glycerol. The LSIMS spectra were recorded using a VG Autospec EQ mass spectrometer, equipped with a Cs^+ gun in negative mode (beam energy 35 keV). Calibration was performed with caesium iodide (200–3500 Da).

HPLC analysis

Two Beckman Ultrasphère (C18) ODS (250 × 4.6 mm i.d.) columns disposed in line and protected with a guard column with the same packing, were used for all analysis. The chromatograms were monitored at 280 nm using a UV detector. The elution system consisted of two solvents, A: 2.5% HOAc in H_2O, B: 80% CH_3CN in A and the following gradients; elution starting with 7% B in A isocratic for 5 min; 7–20% B in A, 5–90 min; 20–100% B in A, 90–95 min; 100% B, 95–100 min (isocratic); followed by washing and reconditioning of the column. The analysis was carried out at room temp. at 1 ml min^{-1}.

Degradation with toluene-α-thiol

The flavan-3-ol derivatives eluted from the HPLC columns were collected in a vial and the solvent was evaporated to dryness under vacuum at 30°C. After addition of 45 μl of toluene-α-thiol 5% (w/v) in EtOH, the sol. was sealed in a vial and heated at 100° for 1 h. The solvent was then evaporated and 30 μl of Raney nickel was added under H_2. The vial was sealed and heated at 50° for 1 h and then injected onto the HPLC system to analyse the reaction products.

Enzymatic hydrolysis

The flavan-3-ol derivatives, eluted from the HPLC columns were collected and the solvent was evaporated to dryness under vacuum at 30°. The residue was diluted with 1 ml of acetate buffer 0.2 M (pH 4.5), incubated with an enzyme (1 mg ml^{-1} of phenol heterosidase proceeding from *Aureobasidium pullulans*) at 30° for 2 h. After extraction with ethyl acetate (1 ml) the extract was injected onto the HPLC system.

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