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Detection of non-coloured anthocyanin–flavanol derivatives in Rioja aged red wines by liquid chromatography–mass spectrometry

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ABSTRACT

Anthocyanins, responsible for wine colour, are involved in many reactions during wine ageing. Anthocyanin–flavanol associations give rise to derivatives in flavylium form that provide blue hues, but also derivatives that do not directly influence wine colour. These colourless derivatives remain mostly unknown but their roles during wine ageing are important for controlling wine quality.

Colourless anthocyanin–flavanol derivatives formed during wine ageing have been studied in three aged red wines from *Rioja* using a combined method with Column Chromatography (CC) and High Performance Liquid Chromatography with Diode Array and Mass Spectrometric detections (HPLC-DADMS).

Twenty-six compounds have been detected: 17 dimers with the anthocyanin in flavene form with possible anthocyanin–flavanol (type 1) and flavanol–anthocyanin (type 2) structures, and 9 with an A-type bicyclic anthocyanin–flavanol structure (type 3). Although some of malvidin derivatives have been previously reported, this is the first time that these derivatives (including different isomers) have also been detected for delphinidin, petunidin and peonidin.

1. Introduction

The colour of red wines is strongly related with their quality because of its relationship with organoleptic properties. Anthocyanins and flavanols play a major role in the quality of wine contributing to sensory characteristics such as colour stability, astringency and bitterness [1–3].

Wine colour changes during wine maturation and ageing, from the initial red of young wines to a more reddish-brown hue of aged wines, are attributed to reactions that take place between anthocyanins, a type of flavonoids responsible for the colour of red grapes and young wines, and other molecules present in the medium, leading to the formation of new more stable pigments. Major anthocyanins present in Vitis vinifera grapes are the 3-O-monoglucosides of delphinidin, cianidin, petunidin, peonidin, and malvidin, the differences among them are related to the number and position of hydroxyl and methoxyl groups in B-ring. Among the most important derivatives are those formed by reactions between anthocyanins and flavanols, another class of flavonoids present in wine. Anthocyanins can react either with flavanol monomers [(+)catechin, (−)epicatechin, (+)gallocatechin and (−)epigallocatechin] or with flavanol oligomers [4]; such reactions can occur directly or through a molecule of acetaldehyde which generates an ethyl bridge between both parts.

At wine pH, both anthocyanins and flavanols can react as nucleophilic as well as electrophilic agents [5,6]. Reactions of anthocyanins with flavanols can form F–A (flavanol–anthocyanin) or A–F (anthocyanin–flavanol) derivatives depending on the relative position of the flavanol and anthocyanin in the structure. Interflavanic bonds can be formed between carbon C4 of the upper unit and carbon at positions 6 or 8 of the lower unit.

In the formation of F–A compounds, the anthocyanin in its hydrated form (AOH) [7] acts as a nucleophilic agent. On the other hand, cleavage of the interflavanic linkage of a flavanol oligomer provides a carbocation F^+, that reacts at C4 position with the C8 or C6 of the anthocyanin, giving rise to a colourless compound F–AOH which dehydrates to form the pigment in flavylium form F–A^+ [8].

If anthocyanin is preserved as the flavilyum form A^+, the derived compounds are coloured forms that provide a bluish hue to aged wines, due to bathochromical shifts in visible absorption maxima. These compounds have been widely reported in wines [9–14] and in model solutions [15,16].

Also, malvidin-3O-glucoside (Mv-3-glc) and its corresponding (+)-catechin-Mv-3-glc derivative, both in the colourless flavene

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form, have been detected in wines and model solutions, indicating that the flavene form of anthocyanin also has a nucleophilic character and the reaction with carbocation of flavanol takes place (Fig. S1A, in Supplementary information), as in the case of the anthocyanin in the AOH hydrated form [7].

In the formation of A–F pigments (Fig. S1B, in Supplementary information), anthocyanin is initially in the flavylium form and acts as an electrophilic agent. A nucleophilic addition of the flavanol through its C6 or C8 takes place to yield an A–F derivative, where the anthocyanin moiety would be in the colourless flavene form [17–19]. This compound could oxidise to the corresponding flavylium form (A^+–F) and subsequently to a yellow xanthilium salt. Also, this adduct can evolve into a colourless bicyclic A–F structure with an A-type interflavanic bond [20], characterised by an extra ether bond between C2 of the anthocyanin and C7 or C5 of the flavanol [16,21], that has been characterised by NMR [22]. The formation of the A-type A-F compounds from the flavene form may occur by an intramolecular addition of the hydroxyl group of flavanol to the double bond C2–C3 [21]. The occurrence in red wine of A–F colourless derivatives was also demonstrated [23].

As mentioned, anthocyanin–flavanol (A–F) associations may give rise to coloured derivatives in the flavylium form, which provide blue hues to wine, or to derivatives in flavene or in bicyclic forms that do not directly influence the wine colour, as they are the respective non-coloured forms, but obviously reduce the formation of the coloured ones. Many studies have been carried out about coloured anthocyanin derived pigments [24]. However non coloured derived compounds remain notably less known and this type of derivatives (with different structures) derived from the anthocyanins delphinidin-, petunidin-, peonidin- and malvidin-3O-glucoside have not been previously reported to our knowledge, neither in flavene form nor like A-type bicyclic derivatives. The aim of this work is to provide a new insight in these non-coloured anthocyanin derivatives, their identification and the study of the Mass Spectrometry (MS) fragmentation patterns of these compounds present in aged red wines of the Protected Denomination of Origin (PDO) Rioja.

2. Materials and methods

Reagents, samples and the experimental procedure have been previously described [25]. Briefly, two different fractionation methodologies (one based in column chromatography (CC) and a more complete procedure including solid phase extraction and column chromatography (SPE+CC)) were followed for the identification of non-coloured anthocyanin–flavanol derivatives in three different Rioja aged red wines. For the CC procedure, 10 mL of wine was filtered and was injected in a 250×15 mm^2 i.d. glass column packed with Toyopearl HW-40S (Tosoh Bioscience, Stuttgart, Germany). Methanol was used as an elution solvent at a flow rate of 0.5 mL/min using a HPLC pump and 30 fractions of 6 mL for each wine were collected. These fractions were concentrated to dryness under a stream of nitrogen, redissolved in 400 μL of the initial HPLC mobile phase (88% of a TFA:H_2O 0.5:99.5 v/v solution and 12% acetonitrile, in volume) solution and filtered with 0.45 μm PTFE filters before being analysed. For the SPE+CC procedure, two empty commercial cartridges (IST, Hengoed Mid, Glam, UK, 150 mL capacity) were filled with 40 g of C18 modified silica (IST, particle size 60 μm). Both top and bottom of the solid phase were covered with 20 μm polyethylene frits (IST). The cartridges were activated with 120 mL of methanol, washed with 2×120 mL of ultrapurified water and preaconditionated with 120 mL of phosphate buffer (1 M, pH=7.0). 200 mL of red wine was desalcoholized in a Büchi (Büchi Labortechnik, AG, Suiza) R200 rotary evaporator at 25 °C for 30 min. Two aliquots of 60 mL each with desalcoholized wine were loaded in two preaconditioned cartridges. Elution began with 200 mL of 0.125 M phosphate buffer (pH=7.0) to elute phenolic acids, followed by 200 mL of ethyl acetate for the elution of monomer and oligomer flavanols and, finally, 250 mL of methanol for the elution of anthocyanins and anthocyanin derived pigments. The two methanolic extracts from both cartridges were mixed and evaporated to dryness in the rotary evaporator and redissolved in 15 mL of methanol. The procedure continued as the previous one (CC procedure), loading 10 mL of the redissolved extract in the glass column packed with Toyopearl HW-40S. Derivatives identification was carried out by High Performance Liquid Chromatography with Diode Array and Mass Spectrometric detections (HPLC-DAD–MS) in an Alliance 2695 instrument coupled to a diode-array detector (DAD) model 2996 and a Micromass Quattro micro triple quadrupole mass spectrometer equipped with a Z-spray electrospray ionisation (ESI) source (Waters Corporation, Milford, MA, USA). The identification of the molecular and fragment ions was made by the MS full scan data. The correct alignment of the different fragment ions was ascertained by observing the extracted ion chromatogram (EIC) at the *m/z* of each fragment ion to check if they eluted at the same retention time. The flow rate and column temperature were set to 0.8 mL/min and 30 °C, respectively. Vial samples were kept in the injector at 4 °C and 50 μL of each fraction was injected. Nitrogen was used as the desolvation gas, at 300 °C and a flow rate of 450 L/h; no cone gas was used. A potential of 3.2 kV was used on the capillary for positive ion mode. The source block temperature was held at 120 °C. Mass spectra, within the *m/z* range 50–1500, were recorded in positive mode at four cone voltages (15, 30, 45 and 60 V).

3. Results and discussion

Twenty-six non-coloured derivatives formed by direct condensation between anthocyanins and flavanols have been identified in this work. Among them, differences in retention times and in relative intensities of fragment ions in mass spectra were observed.

Although partition coefficient calculations have limitations to estimate the relative elution order of different compounds, with the aim of assigning one of the two isomeric structures, flavene or bicyclic, to the identified derivatives, these calculations were carried out with the software ChemBio 3D Ultra (V 11.0.1, CambridgeSoft). Therefore, we used the values obtained to assign a most probable structure between the two candidates. The results suggested that A-type bicyclic structures are less polar than the flavene ones. As an example, the calculated partition coefficient was 0.49 for A-type bicyclic Mv-3-glc-(epi)catechin and −0.78 for Mv-3-glc-(epi)catechin in flavene form. This elution order is in concordance with that reported for A-type and B-type procyanidins [26].

3.1. General fragmentation pattern

The mass spectra of the 26 derivatives show the protonated molecule $[M+H]^+$ and adducts with Na^+ and K^+, that facilitates their identification. Protonated molecules $[M+H]^+$ were detected at *m/z* 755, 769, 753 and 783 for delphinidin-3-glucoside-(epi)catechin, petunidin-3-glucoside-(epi)catechin, peonidin-3-glucoside-(epi)catechin and malvidin-3-glucoside-(epi)catechin derivatives, respectively, (compounds **1-24**) and at *m/z* 799 in the case of malvidin-3-glucoside-(epi)gallocatechin derivatives (compounds **25** and **26**).

The description of the fragmentation pattern will be explained using Mv-3-glc-(epi)catechin derivatives as an example, since they

are more abundant in aged wines. Proposed structures for the observed fragment ions are shown in Fig. S2 and S3, in Supplementary information, for the flavene forms in the case of A–F and F–A compounds, respectively, and in Fig. S4 for the bicyclic A-type structure.

Some major fragmentations observed for some or all of these compounds are described in the next lines. The fragmentation pattern is characterised by the loss of glucose (-162 u) from the protonated molecule $[M+H]^+$ at *m/z* 783, giving rise to the aglycone ion $[M+H-glc]^+$, observed at *m/z* 621. Retro Diels-Alder fragmentations

Table 1
Chromatographic, UV–vis and MS spectrum data of the type 1 non-coloured anthocyanin derived compounds as well as their proposed identities. In bold, the most intense fragment ions that allow their classification.

No.	RT (min)	Compound (flavene form)	$[M]^+$ (*m/z*)	Fragment ion (*m/z*)	λ_{max} (nm)	Fractions				
						Wine 1 CC	Wine 2 CC	Wine 3 CC	Wine 2 SPE+CC	Wine 3 SPE+CC
1	11.73	Dp-3-glc-catechin	755	**603**, 593, 585, 467, **441**, 423, **315**	278	n.d.	14–15	n.d.	14	15
2	12.88	Dp-3-glc-epicatechin	755	**603**, 593, 575, 467, **441**, 423, **315**	278	14	15–16	n.d.	14–15	n.d.
3	20.13	Pt-3-glc-catechin	769	751, **617**, 607, 481, **455**, 437, **329**	278	n.d.	12–13	n.d.	11–13	n.d.
4	21.5	Pt-3-glc-epicatechin	769	**617**, 607, 481, **455**, 437, **329**	–	n.d.	13–14	n.d.	12–13	13–14
5	30.86	Pn-3-glc-catechin	753	**601**, 591, 465, **439**, 421, **313**, 301	–	n.d.	12	n.d.	11–12	n.d.
6	32.68	Pn-3-glc-epicatechin	753	**601**, 591, **439**, 421, **313**, 301	277	n.d.	12	n.d.	11–12	n.d.
7	15.20	Mv-3-glc-(C4–C6)-catechin	783	**631**, 621, 495, **469**, 451, **343**, 331	–	n.d.	n.d.	n.d.	10–11	n.d.
8	17.28	Mv-3-glc-(C4–C6)-epicatechin	783	765, **631**, 621, 613, 603, 505, 493, **469**, 451, **343**, 331	278	n.d.	11	11–12	10–11	11–12
9	34.23	Mv-3-glc-catechin	783	765, **631**, 621, 603, 505, 495, **469**, 451, **343**, 331	272	9–10	11–12	11–12	10–15	11–14
10	36.20	Mv-3-glc-epicatechin	783	**631**, 621, 603, 495, **469**, 451, **343**	–	n.d.	n.d.	n.d.	10–11	n.d.

n.d.: not detected; –: not determined.

Table 2
Chromatographic, UV–vis and MS spectrum data of the type 2 non-coloured anthocyanin derived compounds as well as their proposed identities. In bold, the most intense fragment ions that allow their classification.

No.	RT (min)	Compound (flavene form)	$[M]^+$ (*m/z*)	Fragment ion (*m/z*)	λ_{max} (nm)	Fractions				
						Wine 1 CC	Wine 2 CC	Wine 3 CC	Wine 2 SPE+CC	Wine 3 SPE+CC
11	16.10	Catechin-Pt-3-glc	769	751, 617, **607**, 599, 481, **455**, 437, 329, **317**	–	n.d.	13	n.d.	12–13	n.d.
12	16.85	Epicatechin-Pt-3-glc	769	**607**, 481, **455**, 437, 329, **317**	279	n.d.	n.d.	n.d.	12	n.d.
13	26.03	(Epi)catechin-Pn-3-glc	753	601, **591**, 465, **439**, 421, 313, **301**	278	n.d.	12	n.d.	11–12	n.d.
14	25.27	(Epi)catechin-Mv-3-glc	783	765, 631, **621**, 613, 603, 495, **469**, 451, 343, **331**	271	n.d.	11–12	12	10–12	11–12
15	27.55	Catechin-Mv-3-glc	783	631, **621**, 495, 493, **469**, 451, 343, **331**	278	9–10	11–12	n.d.	10–11	11–12
16	28.50	Epicatechin-Mv-3-glc	783	631, **621**, 495, **469**, 451, 343, **331**	–	n.d.	n.d.	n.d.	10–11	n.d.
17	51.32	(Epi)catechin-Mv-3-glc	783	631, **621**, 603, 495, **469**, 451, 343, **331**	277	n.d.	n.d.	n.d.	12–13	n.d.

n.d.: not detected; – not determined.

Table 3
Chromatographic, UV–vis and MS spectrum data of the type 3 non-coloured anthocyanin derived compounds as well as their proposed identities. In bold, the most intense fragment ions that allow their classification.

No.	RT (min)	Compound	$[M]^+$ (*m/z*)	Fragment ion (*m/z*)	λ_{max} (nm)	Fractions				
						Wine 1 CC	Wine 2 CC	Wine 3 CC	Wine 2 SPE+CC	Wine 3 SPE+CC
18	29.07	Dp-3-glc-A-(epi)catechin	755	**603**, **593**, 585, 467, 441, 315	278	n.d.	17–18	n.d.	16–17	19
19	37.20	Pt-3-glc-A-catechin	769	**617**, **607**, 455, 329	279	n.d.	n.d.	n.d.	14	n.d.
20	42.55	Pt-3-glc-A-epicatechin	769	**617**, **607**, 589, 481, 455, 437, 329, 317	277	n.d.	14–15	n.d.	13–14	n.d.
21	55.42	Pn-3-glc-A-(epi)catechin	753	**601**, **591**, 573, 465, 439, 421, 313, 301	279	n.d.	13	n.d.	12–13	n.d.
22	33.43	Mv-3-glc-A-(epi)catechin	783	765, **631**, **621**, 603, 505, 495, 469, 451, 343, 331	276	9–10	10–12	11–12	9–11	11–12
23	57.38	Mv-3-glc-A-catechin	783	765, **631**, **621**, 603, 505, 495, 469, 451, 343, 331	–	11	12–13	13	11–17	12–14
24	60.90	Mv-3-glc-A-epicatechin	783	**631**, **621**, 495, 469, 451, 343, 331	277	n.d.	n.d.	n.d.	11–12	n.d.
25	23.77	Mv-3-glc-A-(epi) gallocatechin	799	**637**, **631**, 619, 511, 469, 451, 343, 331	270	11	12–13	13	11–13	12–14
26	39.85	Mv-3-glc-A-(epi) gallocatechin	799	**637**, **631**, 619, 511, 469, 451, 343, 331	271	n.d.	13–14	n.d.	13–14	14

n.d.: not detected; –: not determined.

of flavanol C-ring, involving a loss of −152 u, both from the protonated molecule and the aglycone ion are also observed at *m/z* 631 and 469, respectively. Dehydrations from the ions at *m/z* 783, 621, 631 and 469 (protonated molecule, aglycone ion and retro Diels-Alder product reaction ions) can be observed at *m/z* 765, 603, 613 and 451, respectively, although structure of fragment ion at *m/z* 613 could not be deduced.

It is widely reported that for coloured F–A$^+$ pigments the fragmentation of the 1 and 4 bonds of the flavanol C-ring (loss of −126 u) involves that flavanol is the upper unit [20,27]. For non-coloured A–F compounds with an A-type bicyclic bond, Remy-Tanneau et al. [22] explain the loss of −126 u as the loss of the anthocyanin A-ring, which leads to the presence of fragment ions at *m/z* 495 for Mv-3-glc and (epi)catechin derivatives and at *m/z* 511 in the case of (epi)gallocatechin ones; both ions are observed for the proposed pigments in our mass spectra. We have observed this loss of 126 u also from the retro Diels-Alder reaction product (*m/z* 631) giving rise to a fragment ion at *m/z* 505, supporting that statement. Another possible explanation for the fragment ion at *m/z* 495 could be the loss of flavanol from the protonated molecule, but it is not consistent with the presence of the fragment ion at *m/z* 511 in the case of the Mv-3-glc and (epi)gallocatechin derivatives for A-type compounds. Further retro Diels-Alder fragmentation of this ion at *m/z* 495 is also observed at *m/z* 343.

In the case of flavene derivatives, the loss of −126 u could also generate a fragment ion at *m/z* 495, but its structure as well the corresponding fragment ions at *m/z* 505 and 343 remain unknown. This fragment ion at *m/z* 495 could also come from

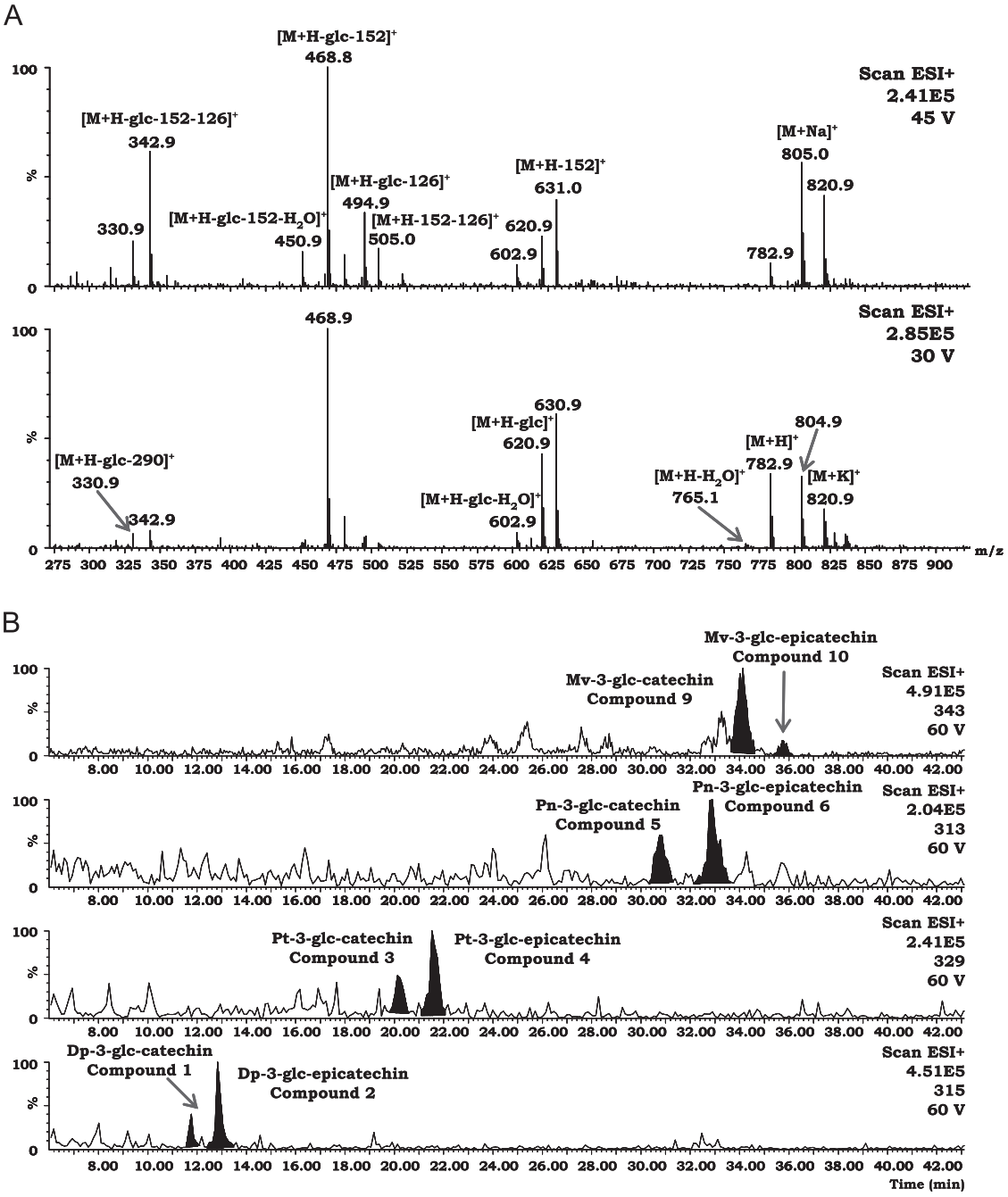

Fig. 1. ESI(+)-MS spectrum at 30 and 45 V voltage cone values of compound 9 (Mv-3-glc-catechin, in flavene form), belonging to anthocyanin–flavanol type 1 derivatives (A). Extracted ion current chromatograms (EIC) at *m/z* 343 (malvidin derivatives), 313 (peonidin), 329 (petunidin) and 315 (delphinidin) for type 1 derivatives, extracted from the total ion current (TIC) chromatogram of one of the analysed fractions (B).

the loss of flavanol from the protonated molecule, in which the anthocyanin retains the flavene form.

Moreover, fragment ions corresponding to malvidin-3-glucoside and the aglycone malvidin are often observed at *m*/*z* 493 and 331, respectively, after the loss of the flavanol unit, which involves a loss of 290 u for (epi)catechin and 306 u for (epi)gallocatechin derivatives. These fragments can be explained as a recovery of the cationic flavylium form due to the loss of the flavanol unit.

3.2. Types of compounds identified

As mentioned before, some differences were observed in retention times and in relative ion intensities in the mass spectra of the detected compounds; thus, a classification of these derivatives into three different groups was done. These group data are shown in Tables 1–3; retention times, wavelengths of absorption maxima and *m*/*z* values for the protonated molecule and major fragments are included in these tables.

Type 1 and 2 compounds, with shorter and closed retention times, could be considered as flavene derivatives (probably more polar than A-type bicyclic derivatives, as suggested by the partition coefficient calculations). A previous paper from our lab showed that F–A coloured pigments elute before than the corresponding coloured A–F derivatives [25]. Therefore type 1 compounds, that have longer retention times, could be assigned as A–F flavene structures while type 2 would be identified as F–A flavene

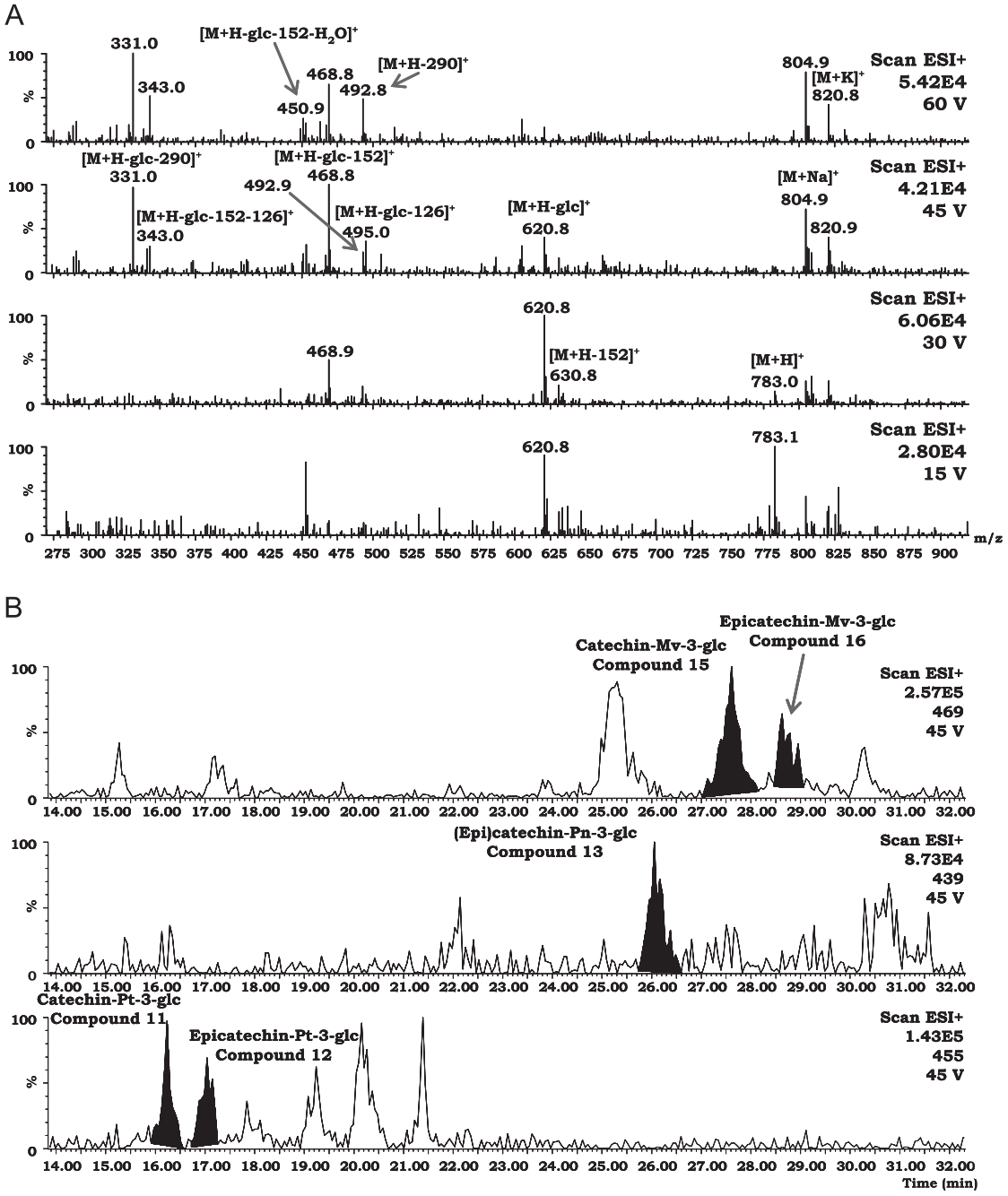

Fig. 2. ESI(+)-MS spectrum example of compound 15 (catechin-Mv-3-glc in flavene form), belonging to flavanol–anthocyanin type 2 derivatives (A). Extracted ion current chromatograms (EIC) at *m*/*z* 469 (malvidin derivatives), 439 (peonidin) and 455 (petunidin) for type 2 derivatives, extracted from the total ion current (TIC) chromatogram of one of the analysed fractions (B).

structures. Taking this assignation into account, type 3 derivatives, those showing longer retention times, could be interpreted as A-type bicyclic derivatives.

Mass spectra of compounds **1–10** (Table 1) show high intensity fragment ions corresponding to the retro Diels-Alder fragmentation of the protonated molecule, of the aglycone ion and the product ion resulting from the cleavage of anthocyanin C-ring at 1 and 4 bonds after the retro Diels-Alder fragmentation. These compounds, for which the MS spectra are dominated by the retro Diels-Alder fragmentation, are identified as type 1 compounds. An example of this kind of fragmentation can be observed in Fig. 1A, which shows the ESI(+)-MS spectrum of the compound **9**, Mv-3-glc-catechin.

Several relationships can be found among these compounds taking into account the elution order in reverse phase chromatography of different anthocyanins commonly detected in red wines (delphinidin, cyanidin, petunidin, peonidin and malvidin) [28] and the fact that derivatives with (+)-catechin elute before than their corresponding with (−)-epicatechin ones [29]. The pairs of compounds **1** and **2** (delphinidin derivatives), **3** and **4** (petunidin), **5** and **6** (peonidin), and **9** and **10** (malvidin) should have the same structure but with different anthocyanidins and the first one of

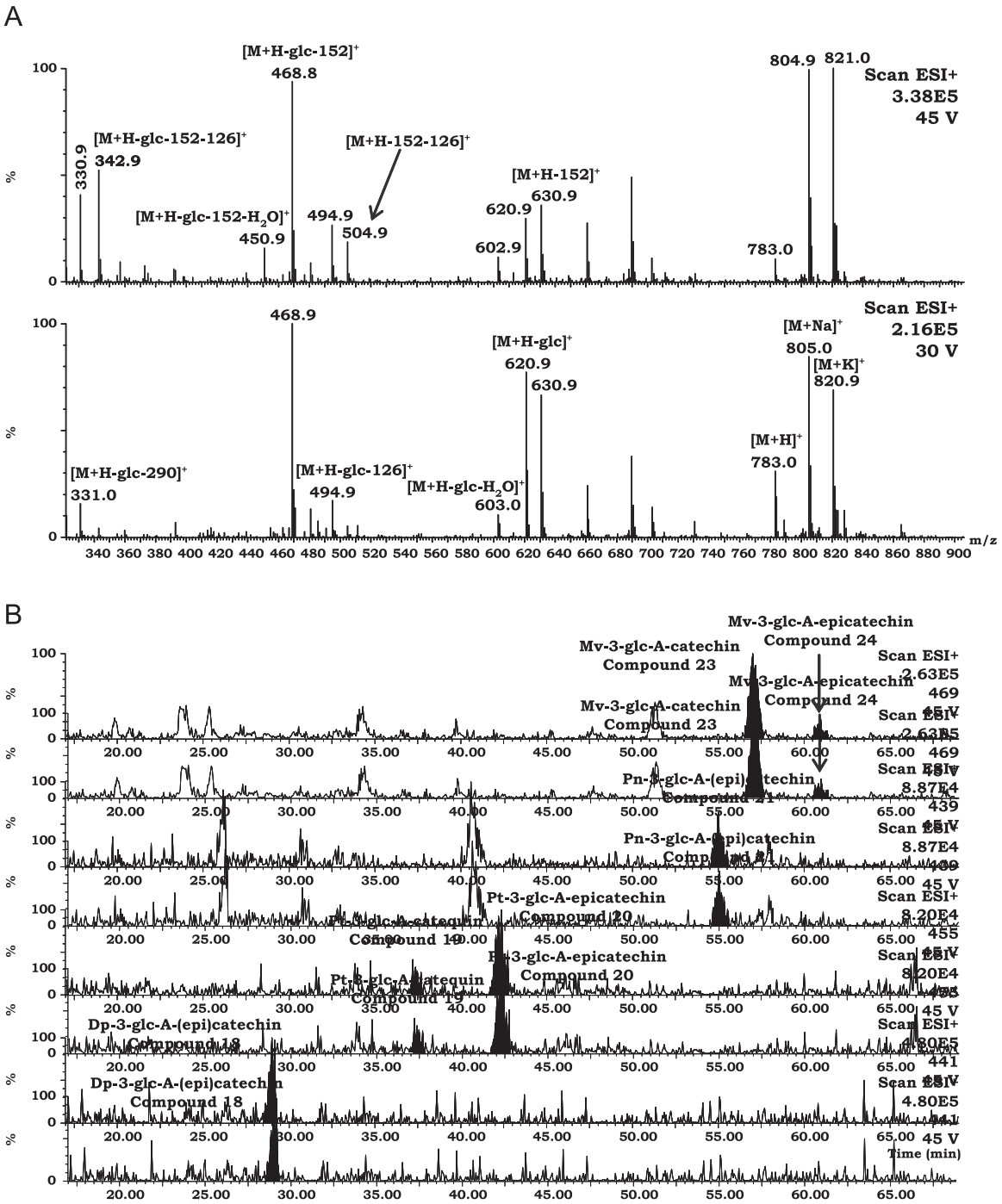

Fig. 3. ESI(+)-MS spectrum example of compound 23 (Mv-3-glc-A-catechin), belonging to anthocyanin–flavanol type 3 derivatives (A). Extracted ion current chromatograms (EIC) at *m/z* 469 (malvidin derivatives), 439 (peonidin), 455 (petunidin) and 441 (delphinidin) for type 3 derivatives, extracted from the total ion current (TIC) chromatogram of one of the analysed fractions (B).

each pair may be the derivative corresponding to (+)-catechin and the second one corresponding to (−)-epicatechin (the elution sequence can also be observed in Fig. 1B). These compounds, that are observed for several anthocyanins, could have the more probable [30] interflavanic linkage C4–C8.

On the other hand, malvidin derivatives **7** and **8**, with very early retention times, could be considered as C4–C6 derivatives, somewhat less probable than C4–C8 ones, which explains why compounds with this structure have not been detected for other anthocyanins. Also, the calculated partition coefficients for A–F derivatives were lower in the case of C4–C6 linkages, supporting this statement. The obtained values were, for example, −0.78 for C4–C8 A–F Mv-3-glc-(epi)catechin and −0.98 for C4–C6 A–F Mv-3-glc-(epi)catechin.

Type 2 compounds could be associated with an F–A flavene structure with a C4–C8 linkage between the flavonoids. In the case of these compounds (**11–17**, Table 2), the most intense ions observed are those corresponding to the loss of glucose from the protonated molecule (aglycone) and the retro Diels-Alder fragmentation from the aglycone. Also, the anthocyanidin ion, after losing the glucose and flavanol unit. This fragmentation pathway can be seen in the ESI(+)-MS spectrum of compound **15**, catechin-Mv-3-glc, shown in Fig. 2A.

As in the previous case, the compounds **11** and **12** (petunidin derivatives), **13** (peonidin), and **15** and **16** (malvidin) could also be related (Fig. 2B), having the same structure. However, structures of compounds **14** and **17** could not be hypothesised.

As mentioned before, type 3 compounds could have a bicyclic A-type structure and would exhibit an interflavanic linkage C4–C8 and a linkage C2–O–C7. For these compounds (Table 3), the retro Diels-Alder fragmentation and the loss of glucose from the protonated molecule occur with similar intensities. Fig. 3A shows the ESI(+)-MS spectrum of compound **23**, M-3-glc-A-catechin. The same is observed for compounds **18–24** and (epi)gallocatechin derivatives **25** and **26**.

In this case, a relationship between the compounds **18** (delphinidin), **19** and **20** (petunidin), **21** (peonidin), and **23** and **24** (malvidin) could also be observed, as shown in Fig. 3B, that shows the elution sequence of such derivatives. Compound **22**, in this case, could also have this type of structure but with a less frequent linkage C4–C6 instead of C4–C8 [30] or C2–O–C5 instead of C2–O–C7 [22], however available experimental evidence cannot provide definitive conclusions. Only Mv-(epi)gallocatechin derivatives were detected for the latter type of compounds (compounds **25** and **26**).

All these compounds are mainly detected in wine 2 (the sample analysed by SPE+CC methodology shows all the derivatives). However, wine 1 present only two type 1 derivatives (compounds **2** and **9**), only compound **15** of type 2 and three type 3 derivatives (compounds **22**, **23** and **25**). In the analysis of wine 3, with both CC and SPE+CC methodologies, a higher number of compounds have been detected in comparison with wine 1 (compounds **1**, **4**, **8**, **9**, **14**, **15**, **18**, **22**, **23**, **25** and **26** of the three types). These variations in non-coloured derivatives composition may be due to differences in wine elaboration.

As mentioned previously, all of these three types of anthocyanin–flavanol derivatives do not contribute directly to wine colour. As an example, the absorption spectrum of compound **8**, Mv-3-glc-(C4–C6)-epicatechin, is compared with that of the coloured pigment (+)-catechin-Mv-3-glc, both acquired on-line with DAD detection, (Fig. 4); it can be observed that compound **8** does not exhibit absorption in visible region.

Wine colour is a very important aspect in wine quality evaluation. Anthocyanin reactions that take place during wine ageing result in changes in wine colour towards new hues that have influence on wine sensorial evaluation.

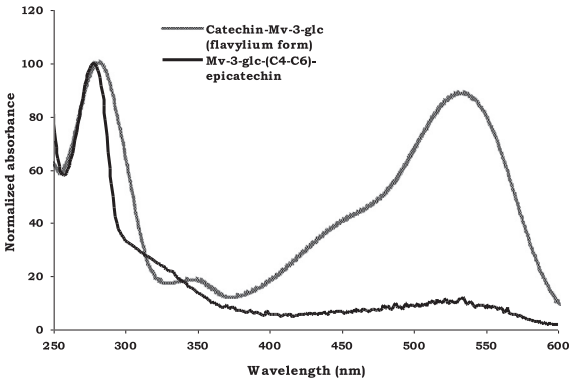

Fig. 4. Comparison of UV–vis normalised absorption spectra of the compound 8, Mv-3-glc-(C4–C6)-epicatechin in flavene form and of the coloured pigment catechin-Mv-3-glc.

This work provides a new insight into the colourless anthocyanin–flavanol derivatives formed during wine ageing, with the detection of 26 different compounds. Improving the knowledge about this kind of colourless compounds is important since they could reduce the formation of coloured derivatives. The presence of different derivatives, both in flavene form and as A-type compounds for delphinidin, petunidin, peonidin and malvidin have not been previously reported to our knowledge. However, further research is needed to assign a concrete structure to each of the fragmentation patterns described here. Techniques such as Nuclear Magnetic Resonance (NMR) would enable the complete structural elucidation but, even in this case, the purification of these derivatives from wine is almost impossible due to their very small concentration.

4. Conclusions

In this work 26 non-coloured anthocyanin–flavanol derivatives have been identified. These derivatives vary in their structures and in their behaviour in chromatographic and mass spectrometric analyses. They have been classified as A–F or F–A flavene compounds, or as A-type bicyclic derivatives using spectral information. However, these identifications are tentative since some of the fragment ions could not be elucidated for flavene compounds and experimental data obtained are not sufficient to get conclusive identifications. Further research would be needed to assign a concrete structure to each of the fragmentation patterns.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.12.066.

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