Available online at www.sciencedirect.com
SCIENCE DIRECT®
Analytica Chimica Acta 513 (2004) 305–318
ANALYTICA CHIMICA ACTA
www.elsevier.com/locate/aca

Separation of pyranoanthocyanins from red wine by column chromatography

C. Alcalde-Eon, M.T. Escribano-Bailón, C. Santos-Buelga, J.C. Rivas-Gonzalo*

Unidad de Nutrición y Bromatología, Facultad de Farmacia, Universidad de Salamanca, Campus Miguel de Unamuno, E-37007 Salamanca, Spain

Received 10 July 2003; received in revised form 14 October 2003; accepted 20 October 2003

Available online 16 January 2004

Abstract

With the aim of monitoring the formation of anthocyanin-derived pigments and contributing to the study of their chromatic properties, stability and relative contribution to the colour of red wines, a method for fractionation of the colouring material was set up. The method was based on the distinct reactivity of the different pigment families towards bisulfite (hydrogen sulfite). The wine, acidified and bleached with $NaHSO_3$, was placed in a Toyopearl® HW-40(s) gel column and submitted to elution with ethanol. Two fractions with different pigment compositions were collected and analysed by liquid chromatographay diode array detection-mass spectrometry. Compounds present in each fraction were identified according to their UV-visible and MS^n mass spectra, showing that the first one was mostly constituted of pyranoanthocyanins, whereas the second basically contained anthocyanins and anthocyanin-flavanol condensation products. A large variety of new pigments were detected, some of which had not been previously reported in red wines, as far as we know. Characteristic MS^2 and MS^3 fragmentation patterns were observed within each family of compounds, which could be further applied for characterisation of unknown pigments in other wines.

Keywords: Anthocyanin-derived pigments; Wine colour; Size exclusion chromatography; Liquid chromatography-mass spectrometry

1. Introduction

Different types of pigments are involved in the changes of colour that take place in red wine during ageing, namely the pyranoanthocyanins, originated by cycloaddition of diverse compounds at C_4 and hydroxyl group in position 5 of the anthocyanins, and the products resulting from the condensation between anthocyanins and flavanols either direct or mediated by acetaldehyde or other compounds.

In red wines, pyranoanthocyanins have been identified, resulting from the reaction between anthocyanins and 4-vinylphenol [1,2], pyruvic acid (also named A-type vitisins) [3–7], acetaldehyde (B-type vitisins) [4] or vinyl-flavanols [8]. The colours of this type of pigments are more stable against the increase of pH and the bleaching by bisulfite than those of the anthocyanins [4,6,9] and present an orange-red hue, being able to contribute to the explanation of the shade of more aged wines.

The formation of the pigments resulting from the condensation mediated by acetaldehyde between anthocyanins and flavanols requires an acidic pH [10], which has been attributed to the greater facility of acetaldehyde to form a cation, which is necessary to produce the condensation reaction [11]. The formation of these reddish-blue pigments is very rapid, as is their loss, with even their disappearance in model assays taken to more acid pH values (e.g. at pH 3.2).This is basically attributed to their precipitation, as a consequence of the formation of more condensed structures. In fact, as time passes, in the assays carried out at pH 3.2 the formation of new peaks is observed in the chromatograms at later retention times and with similar spectra to those of the reddish-blue pigments. A violet precipitate appears simultaneously in the vials and a decrease is produced in the colour intensity of the solutions. In any case, there are sufficient data to state that they are relatively unstable pigments, although they are less sensitive to bleaching by sulphur dioxide and the increase in pH than the anthocyanins [12].

Recently, using liquid chromatography-mass spectrometry (LC-MS), a red pigment has been found, even in young wines, which could derive from a direct catechin-

* Corresponding author. Tel.: +34-923-294-537; fax: +34-923-294-515.
E-mail address: jcrivas@usal.es (J.C. Rivas-Gonzalo).

0003-2670/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.aca.2003.10.076

anthocyanin condensation reaction [13]. Its molecular weight exactly fits with the weight of a possible catechin-malvidin-3-glucoside dimer in which the anthocyanin is found in the flavylium form. The formation of this type of pigment was postulated by Somers [14], but its presence in wine has never been detected, among other reasons because they were too diluted among the high concentrations of anthocyanins. Most probably the formation can be explained in accordance with the reaction between a carbo-cation, originating from the interflavanic breakdown of condensed tannins, and anthocyanin. This structure, with the anthocyanin as an inferior subunit, has also been proposed by Remy et al. [15] after submitting the phenolic fractions isolated from aged wines to selective hydrolyses and subsequent LC-MS analyses.

Very recently, in Port wines, blue pigments have been isolated and their structures, not previously reported in wine, characterisized by means of electrospray ionisation (ESI)/MS and nuclear magnetic resonance (NMR) spectroscopy. Their mechanism of formation does not directly involve the original anthocyanins, but their derived pyranoanthocyanins, which would react at their C_{10} position with the vinyl group of an 8-vinylflavanol adduct, resulting from the depolymerisation of oligomers from condensation mediated by acetaldehyde [16]. The moment of appearance of these pigments is conditioned by the synthesis of pyranoanthocyanins, by the existence of condensation mediated by acetaldehyde between flavanols, by the depolymerisation of these condensation products and by the later reaction of the vinyl adducts with the pyranoanthocyanins.

Some properties of the stability of these pigments have been established and some approximations have been made regarding the role they play in the colour throughout the life of a wine. The hypotheses about the mechanisms of formation of new pigments, supported by the data on wines in which they are detected, allows speculation about the conditions which can control their appearance during the wine-making and ageing processes of the wine. Nevertheless, these hypotheses need to be scientifically tested and more studies are necessary to ascertain the chromatic properties and the stability of the different pigments to draw conclusions about their true importance regarding the colour of red wines. Nonetheless, one of the main problems is that compounds with great differences of concentrations co-exist which makes their detection and their separation difficult. This is particularly difficult among the anthocyanins and their corresponding type A vitisins, which in the majority of the reversed phase LC methods elute very close together.

Methods have recently been developed for separating wine pigments based on cation exchange chromatography in the absence and presence of excess bisulfite (hydrogen sulfite) [17]. Thus, authors using sulfoxyethyl cellulose and taking advantage of the different bleaching ability for anthocyanins and their derivatives, have separated anthocyanins and anthocyanin-derivatives which form bisulfite addition products from pyranoanthocyanins, 4-substituted anthocyanins, which do not form these adducts.

The aim of this paper is to describe a method for fractionation of the colouring material of red wine using size exclusion chromatography (SEC) in order to facilitate the identification of anthocyanin-derived pigments. Chromatographic and UV-visible and MS characteristics have been used to determine these pigments.

2. Materials and methods

2.1. Sample fractionation

The study was carried out with a 2-year-old red wine from the Alentejo Demarcated Region (Portugal).

For fractionation of the wine, 10 ml of the wine was acidified with 3 M HCl to pH 1, in order to convert all the anthocyanins present in the sample into their respective cationic and coloured forms and to favour the reactions between them and the bisulfite in excess subsequently added to the sample. In some assays performed in our laboratory it was determined that the amount of sodium bisulfite necessary to bleach most of the free anthocyanins is $400\,g\,l^{-1}$ of wine. After complete dissolving the bisulfite in the wine, 15 min passed before placing the sample on the chromatographic column to allow the reaction between bisulfite and anthocyanins and between bisulfite and derived pigments which can form bisulfite addition products.

The treated wine was placed on a 200 mm × 15 mm i.d. Toyopearl® HW-40(s) gel column (Tosoh, Japan). Flow rate was regulated at $0.2\,ml\,min^{-1}$ using a peristaltic pump. The elution solvent was 95% ethanol. With this solvent the majority of the pigments retained in the column were eluted. When practically no more coloured compounds eluted from the column, the solvent was changed to 100% methanol until total elution of the pigments non-eluted with ethanol occurred. The different coloured bands formed during the elution as well as the bleached eluates were collected separately according to visual detection. The eluates were immediately acidified to pH 1 in order to revert the existing bisulfite-anthocyanin adducts and then were concentrated under vacuum and re-dissolved in water.

2.2. LC-DAD analysis

LC-diode array detection (DAD) analysis was performed in a Hewlett-Packard 1100 series liquid chromatograph, and detection was carried out using a photodiode detector. An AQUA C18 reverse phase, 5 μm, 150 mm × 4.6 mm column (Phenomenex®, Torrance, CA) thermostatted at 35 °C, was used.

The solvents used were: (A) an aqueous solution (0.1%) of trifluoroacetic acid (TFA) and (B) 100% HPLC-grade acetonitrile, establishing the following gradient: isocratic 10% B for 5 min, from 10 to 15% B for 15 min, isocratic 15% B

for 5 min, from 15 to 18% B for 5 min, and from 18 to 35% B for 20 min, at a flow rate of 0.5 ml min^{-1}. Detection was carried out at 520 nm as the preferred wavelength.

2.3. LC-MS analysis

LC-MS analyses were performed using a Finnigan™ LCQ MS detector (Thermoquest, San Jose, CA) equipped with an API source, using an electrospray ionisation (ESI) interface. The LC system was connected to the probe of the mass spectrometer via the UV cell outlet. Both the sheath gas and the auxiliary gas were a mixture of nitrogen and helium. The sheath gas flow was 1.2 l min^{-1} and the auxiliary gas flow, 6 l min^{-1}. The capillary voltage was 4 V and the capillary temperature 195 °C. Spectra were recorded in positive ion mode between *m/z* 120 and 1500. The mass spectrometer was programmed to do a series of three consecutive scans: a full mass, an MS^2 scan of the most abundant ion in the full mass, and an MS^3 of the most abundant ion in the MS^2. The normalised energy of collision was 45%.

3. Results and discussion

3.1. LC-DAD analysis of the wine

Five milliliters of the wine was concentrated under vacuum and re-dissolved in acidic water (HCl, pH 0.5) up to a final volume of 2 ml. This solution was filtered through a Millex® HV Syringe Driven Filter Unit (0.45 μm) before the LC analysis. Fig. 1 shows the chromatogram of the wine before fractionation. Due to the complexity of the sample, it was difficult to achieve a total identification of the compounds present in the wine and the "hump" that appeared from 33 min of the chromatogram indicated that there were a lot of different compounds co-eluting within this time range. Only the identity of some of the compounds (see Table 1) could be assigned by means of their retention times and UV-visible spectra. Therefore, a fractionation of this wine was needed to obtain a better analysis of the pigments present.

Table 1
Retention times and UV-visible spectral data from the compounds that were identified in the wine before its fractionation

Peak number	Retention time (min)	Absorption maxima (nm)	Identification
1	21.6	506	Delphinidin-3-glucoside pyruvic derivative
2	21.8	276, 527	Delphinidin-3-glucoside
3	28.4	278, 527	Petunidin-3-glucoside
4	29.4	298, 507	Petunidin-3-glucoside pyruvic derivative
5	33.7	278, 516	Peonidin-3-glucoside
6	35.2	290, 394, 503	Peonidin-3-glucoside pyruvic derivative
7	35.8	280, 527	Malvidin-3-glucoside
8	37.0	272, 367, 507	Malvidin-3-glucoside pyruvic derivative
9	51.4	264, 296, 423, 503	Malvidin-3-glucoside-4-vinylphenol
10	52.2	276, 511	Malvidin-3-glucoside-4-vinylguaiacol?

The numbers of the peaks correspond to Fig. 1.

3.2. Fractionation of the wine

In the elution of the acidified and bleached wine through the column with 95% ethanol, four different coloured bands were formed. As previously stated, not only were the eluates corresponding to the coloured bands collected, but also were all the eluates less coloured or colourless eluting before and after the bands. After acidification of these eluates to pH 1 with 3 M HCl, all became coloured. The collection began when the eluate before the first band became coloured after acidification. The collection of this eluate

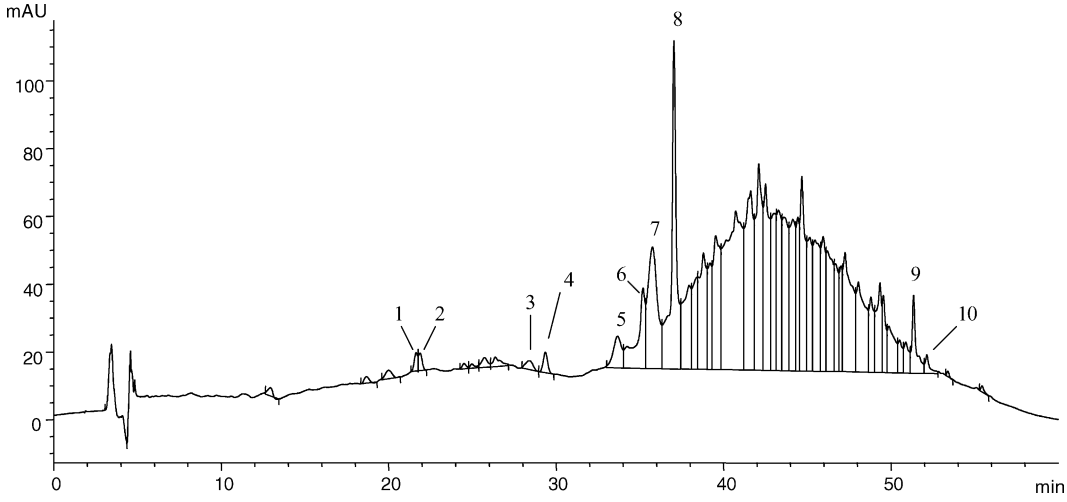


Fig. 1. Chromatogram of the wine sample recorded at 520 nm.

(eluate 1) continued until the elution of the first coloured band started. Eluate 1 was practically colourless before acidification, and, afterwards, it became orange-pink. The first band was orange-pink. Eluate 2, corresponding to that band, acquired a more intense orange hue when acidified. Eluate 3 corresponded to the end of the band and, before acidification, it was less coloured and had a more brownish hue than eluate 2. At pH 1 it also became orange-pink, but much less intense than eluate 2. The second band was orange-brown coloured and its upper limit was not well defined. Only one eluate (eluate 4) was collected from the beginning of this band to the beginning of the next and its colour, once acidified, was similar to those of eluates 1, 2 and 3 but redder. Eluate 5 corresponded to a very narrow purple band (third coloured band) that eluted immediately before a huge purple-violet band (fourth band, eluate 6). After the elution of the fourth band, the eluate was practically colourless, but a coloured zone still remained in the upper part of the column. The elution solvent was changed to 100% methanol in order to elute these retained pigments. A new purple-brown band appeared and moved slowly through the column. Eluate 7 corresponded to this band. The elution with methanol was stopped when the eluate had no more colour, neither before nor after the acidification to pH 1. At this step, the gel almost recovered its original white colour, and it could be assumed that the colouring material of the wine had been collected.

Preliminary LC-DAD analyses of all these eluates were carried out in order to determine the chemical nature of the major components of each. Eluates 1, 2, 3 and 4 showed similar chromatograms, with peaks that had the same retention times and UV-visible spectra in all cases, only differing in the proportions in which they were present in the different eluates. The UV-visible spectra of the majority of the compounds present in these eluates were characteristic of different types of pyranoanthocyanins. The other compounds had retention times and UV-visible spectra characteristic of the monoglucosides of the anthocyanins, but the amount of these compounds was much lower than that of the pyranoanthocyanins. Eluate 5 had a major peak (ca. 60% of the total area) whose retention time and UV-visible spectrum allowed its identification as malvidin-3-glucoside. Along with this compound, and in smaller amounts, the presence of delphinidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside and the acetyl and *p*-coumaroyl derivatives of malvidin-3-glucoside was established taking into account their retention times and spectral data. Eluates 6 and 7 showed similar chromatograms, in which the anthocyanins constituted the majority of the compounds. The UV-visible spectra of the other compounds present in these eluates were characteristic of the anthocyanin-flavanol condensation products.

Taking into account these data, and in order to simplify the analysis of the pigments present in this red wine, the different eluates were gathered in two fractions, according to the chemical nature of the compounds found in them. Thus, "fraction A" was formed by the combination of eluates 1, 2, 3 and 4 and "fraction B" by the combination of eluates 5, 6 and 7.

3.2.1. Fraction A

Fig. 2 shows the chromatogram recorded at 520 nm corresponding to fraction A, in which 30 compounds have been reported. Their retention times, UV-visible absorption

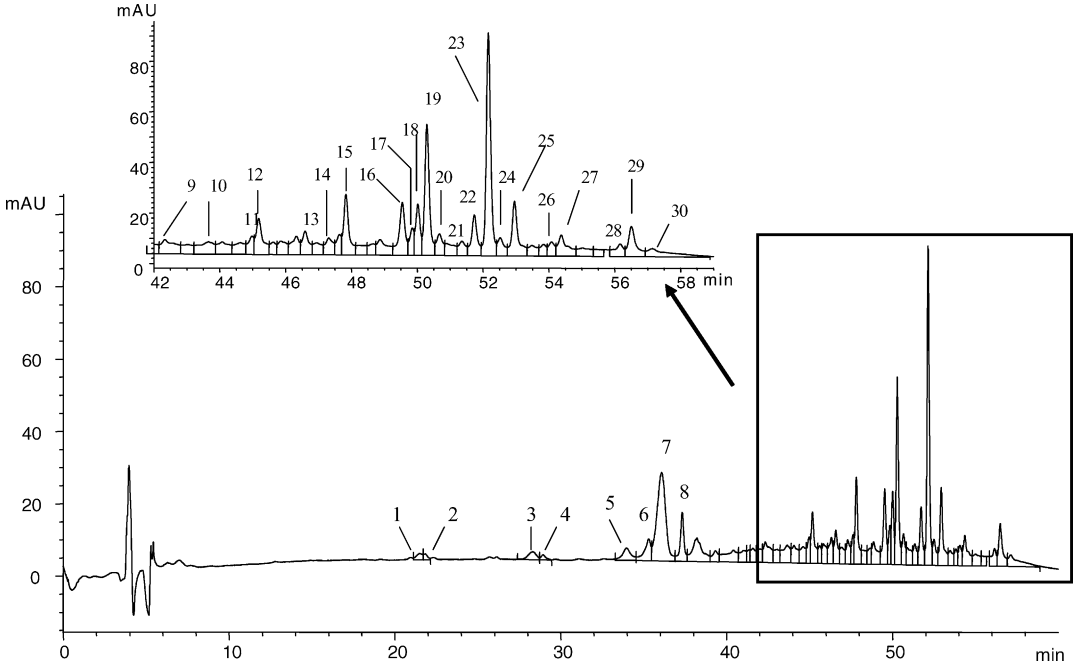


Fig. 2. Chromatogram of fraction A of the wine recorded at 520 nm.

Table 2
Chromatographic, UV-visible and mass spectral data of the compounds identified in fraction A of the wine

Peak number	Retention time (min)	Molecular ion (M^+) (m/z)	Absorption maxima (nm)	Identification
1	21.0	533	298, **506**	Delphinidin-3-glucoside pyruvic derivative
2	21.5	465	276, 345, 372, **526**	Delphinidin-3-glucoside
3	28.3	479	278, 344, 373, **527**	Petunidin-3-glucoside
4	29.3	547	298, **507**	Petunidin-3-glucoside pyruvic derivative
5	34.0	463	276, 315, 362, **516**	Peonidin-3-glucoside
6	35.3	531	233, 296, 355, **503**	Peonidin-3-glucoside pyruvic derivative
7	36.0	493	277, 348, **527**	Malvidin-3-glucoside
8	37.3	561	236, 268, 299, 372, **507**	Malvidin-3-glucoside pyruvic derivative
9	42.3	531		Unknown
10	43.6	1093	**510**	Malvidin-3-glucoside-4-vinyl-procyanidin dimer
11	45.0	677	277, 310, **513**	Peonidin-3-(*p*-coumaroyl)glucoside pyruvic derivative
12	45.2	707	279, 313, 359, 425, **516**	Malvidin-3-(*p*-coumaroyl)glucoside pyruvic derivative
13	46.6	533		Unknown
14	47.3	775	271, 439, **506**	Peonidin-3-glucoside-4-vinylcatechin
15	47.8	805	268, 319, 412, 446, **507**	Malvidin-3-glucoside-4-vinylcatechin
16	49.5	595	263, 362, 410, **503**	Petunidin-3-glucoside-4-vinylphenol
17	49.8	595	280, 402, 440, **507**	Peonidin-3-glucoside-4-vinylcatechol
18	50.0	805	266, 320, 380, 453, 484, **512**	Malvidin-3-glucoside-4-vinylepicatechin
19	50.3	625	264, 352, 380, **512**	Malvidin-3-glucoside-4-vinylcatechol
20	50.7	951	286, 314, 446, **510**	Malvidin-3-(*p*-coumaroyl)glucoside-4-vinylcatechin
21	51.4	951		Malvidin-3-(*p*-coumaroyl)glucoside-4-vinylepicatechin
22	51.7	579	259, 294, 348, 410, **499**	Peonidin-3-glucoside-4-vinylphenol
23	52.2	609	264, 297, 329, 423, **503**	Malvidin-3-glucoside-4-vinylphenol
24	52.5	609	273, 376, 426, 483, **510**	Peonidin-3-glucoside-4-vinylguaiacol
25	53.0	639	263, 295, 335, 426, **512**	Malvidin-3-glucoside-4-vinylguaiacol
26	54.1	741	279, 312, 377, 408, 438, 474, **508**	Peonidin-3-(*p*-coumaroyl)glucoside-4-vinylcatechol
27	54.4	771	278, 309, 398, 420, 458, **512**	Malvidin-3-(*p*-coumaroyl)glucoside-4-vinylcatechol
28	56.2	725	279, 307, 399, 442, **498**	Peonidin-3-(*p*-coumaroyl)glucoside-4-vinylphenol
29	56.5	755	293, 313, 364, 412, 449, **506**	Malvidin-3-(*p*-coumaroyl)glucoside-4-vinylphenol
30	57.1	785	276, 312, 446, **512**	Malvidin-3-(*p*-coumaroyl)glucoside-4-vinylguaiacol

The number of the peaks correspond to Fig. 2.

maxima and molecular ions in the MS analyses are shown in Table 2.

The chromatographic and UV-visible spectral features of peaks 2, 3, 5 and 7 corresponded to those of the standards of delphinidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside and malvidin-3-glucoside, respectively, when analysed under the same chromatographic conditions. The MS^n data of these peaks were helpful to confirm their identity.

Peaks 1, 4, 6, and 8 showed similar UV-visible spectra (see Fig. 3b), with absorption maxima hypsochromically shifted when compared with those of the monoglucosides.

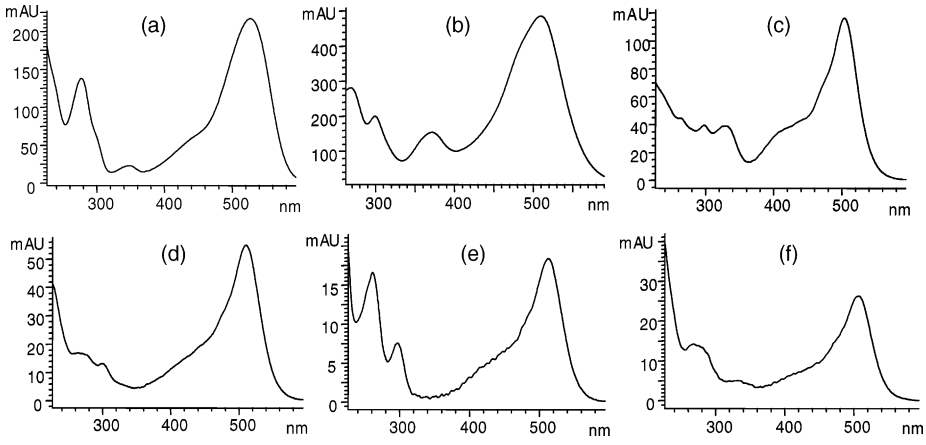


Fig. 3. Characteristic UV-visible spectra of some of the compounds of fraction A: (a) malvidin-3-glucoside, peak 7; (b) malvidin-3-glucoside pyruvic derivative (vitisin A), peak 8; (c) malvidin-3-glucoside-4-vinylphenol, peak 23; (d) malvidin-3-glucoside-4-vinylcatechol, peak 19; (e) malvidin-3-glucoside-4-vinylguaiacol, peak 25; (f) malvidin-3-glucoside-4-vinylcatechin, peak 15.

In the mass spectrum of peak 8, there was an intense signal at *m*/*z* 561, corresponding to the molecular ion. The fragmentation of this ion in the MS^2 analysis gave rise to a major ion at *m*/*z* 399 and a loss in the *m*/*z* 162 signal was observed. This loss can be attributed to the loss of one glucose moiety. All these data allowed us to identify this compound as the pyranoanthocyanin derived from the reaction between malvidin-3-glucoside and pyruvic acid, as described by Fulcrand et al. [18] and also named vitisin A by Bakker and Timberlake [4]. Peak 6 showed a molecular ion at *m*/*z* 531 in the mass spectrum and, as in compound 8, the fragment ion present in the MS^2 spectrum (*m*/*z* 369) originated from the loss of one glucose moiety. This datum and the fact that compound 6 eluted near peonidin-3-glucoside allowed us to propose that this compound should be the pyranoanthocyanin derived from the reaction between peonidin-3-glucoside and pyruvic acid, since this elution feature has been previously established in our laboratory for other anthocyanins and their corresponding pyruvic derivatives [13]. The same reasoning was employed in the identification of peaks 1 and 4. The molecular ion of each peak in the MS analysis (*m*/*z* 533 and 547) was fragmented into a major ion in the MS^2 analysis (*m*/*z* 371 and 385, respectively), corresponding to their aglycone moieties. Thus, compound 1 was identified as the delphinidin-3-glucoside pyruvic acid derivative and compound 4 as the petunidin-3-glucoside pyruvic acid derivative. Compounds 1, 4, 6 and 8 were previously reported in 1- and 2-year-old monovarietal red wines [19,20] and compounds 1 and 8 in port wine [7].

Peaks 11 and 12 had similar UV-visible spectra to those of peaks 1, 4, 6 and 8, but possessed an additional absorption maximum at 313 nm. They also had higher retention times than those of peaks 1, 4, 6 and 8. These data indicated a possible acylation of the sugar moiety with *p*-coumaric acid. The MS analysis confirmed the identity of the compounds. Their molecular ions yielded a signal at *m*/*z* 677 and 707, respectively. In both cases the molecular ion had 146 amu more than those of peaks 6 and 8, which can be attributed to the presence of the *p*-coumaric acid residue in the molecule. Thus, peak 11 originated from the reaction between peonidin-3-(*p*-coumaroyl)glucoside and pyruvic acid, and peak 12, between malvidin-3-(*p*-coumaroyl)glucoside and pyruvic acid. Fig. 4b shows the structure of compounds 1, 4, 6, 8, 11 and 12.

Peaks 9 and 13 showed a molecular ion and a fragmentation pattern identical to those of peaks 6 and 1, respectively, but exact identities for these two compounds has still to be determined.

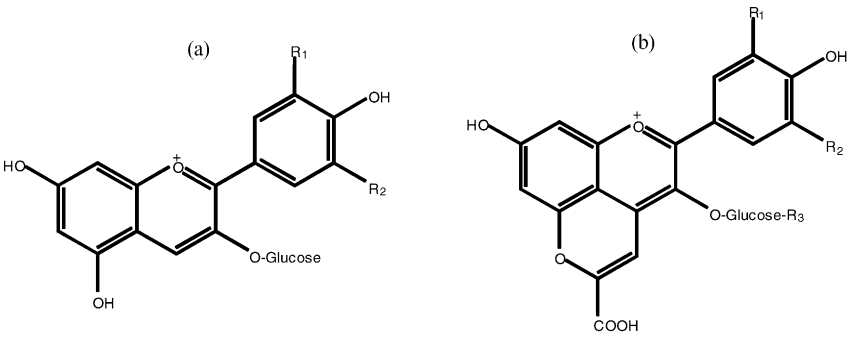
The UV-visible spectrum of peak 23 showed an absorption maximum in the visible region at 503 nm. This maximum is at much shorter wavelength than the maxima of anthocyanins and lower than those of the pyranoanthocyanins originated by reaction with pyruvic acid (see Fig. 3c). The molecular ion corresponding to this peak gave rise to an intense signal in the mass spectrum at *m*/*z* 609, and its fragmentation in the MS^2 analysis produced a fragment ion at *m*/*z* 447 by loss of 162 amu. With all these data we could identify peak 23 as the pyranoanthocyanin that originated from the cycloaddition between malvidin-3-glucoside and vinylphenol. This compound has been studied widely [9] and has previously been reported in red wines [2,17,19,21,22] and Port wines [7].

Peaks 16 and 22 had UV-visible spectra with very similar features to those of peak 23. In their mass spectra an intense signal appeared at *m*/*z* 595 and 579, respectively, corresponding to their molecular ions. The fragmentation pattern of these molecular ions, the loss of one glucose moiety originating in the aglycone moiety (*m*/*z* 433 and 417, respectively), was the same as in peak 23, so it can be concluded that peak 16 was the petunidin-3-glucoside vinylphenol adduct and peak 22, which eluted immediately before the vinylphenol derivative of malvidin-3-glucoside, was the peonidin-3-glucoside vinylphenol adduct.

Peaks 28 and 29 had similar UV-visible spectra to those of peaks 22 and 23, but they possessed an additional shoulder around 310 nm, typical of acylation with *p*-coumaric acid. Their molecular ions (*m*/*z* 725 and 755, respectively) were fragmented in the MS^2 analyses, in both cases producing a major fragment ion by loss of 308 amu (*p*-coumaroylglucose residue) which corresponded to the aglycone moiety of the peonidin-vinylphenol adduct (*m*/*z* 417) and to the aglycone moiety of the malvidin-vinylphenol adduct (*m*/*z* 447), respectively. Compound 29 was initially described by Fulcrand et al. [2] and both compounds have already been reported in Port wines [7].

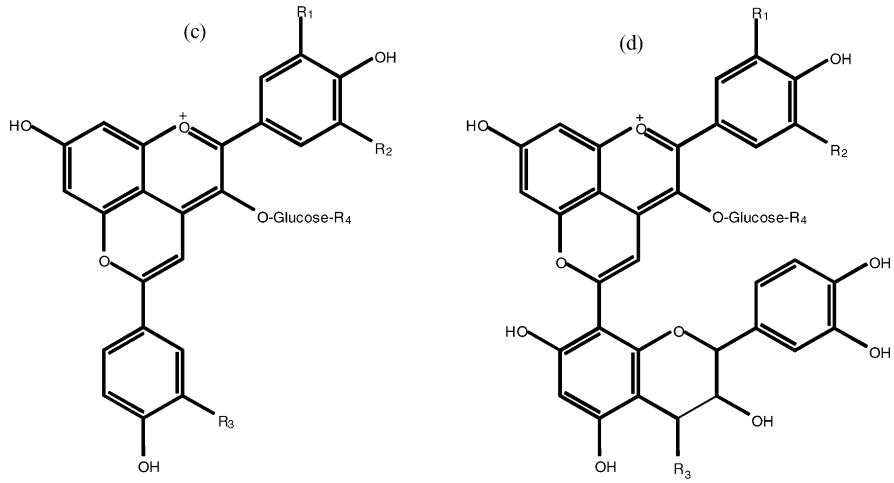
Peak 19 had a similar UV-visible spectrum to that of peak 23 (malvidin-3-glucoside-4-vinylphenol), with a narrow and pointed absorption band in the visible region, but with the absorption maximum 4 nm higher than the latter (see Fig. 3d). The molecular ion of compound 19 gave a signal in the mass spectrum at *m*/*z* 625, 16 amu higher than the molecular ion of peak 23. The aglycone (*m*/*z* 463), which originated from the loss of one sugar moiety in the MS^2 analysis, also possessed 16 additional amu when compared to the aglycone of compound 23. In accordance with this, and taking into account that compound 19 eluted earlier than compound 23, we proposed that compound 19 should have the same structure as compound 23 with an additional hydroxyl group in its structure. We assigned it the following identity: malvidin-3-glucoside-4-vinylcatechol. This compound has recently been synthesized [23] and its presence in red wine has been confirmed [21,23,24].

The molecular ion of peak 17 yielded a signal at *m*/*z* 595 in the conventional mass spectrum, and was fragmented in the MS^2 analysis into a major ion (*m*/*z* 433), originated by loss of one glucose moiety and corresponding to the aglycone. This compound was 30 amu less than compound 19 and eluted earlier than it, so it could be identified as the pyranoanthocyanin formed by reaction between vinylcatechol and peonidin-3-glucoside. To our knowledge this compound has not previously been described in red wines.



Peak 2: R_1=R_2=OH
Peak 3: R_1=OH; R_2=OCH_3
Peak 5: R_1= OCH_3; R_2=H
Peak 7: R_1=R_2=OCH_3

Peak 1: R_1=R_2=OH; R_3=H
Peak 4: R_1=OH; R_2=OCH_3; R_3=H
Peak 6: R_1= OCH_3; R_2=H ; R_3=H
Peak 8: R_1=R_2=OCH_3; R_3=H
Peak 11: R_1= OCH_3; R_2=H ; R_3= *p*-coumaric acid
Peak 12: R_1=R_2=OCH_3; R_3= *p*-coumaric acid



Vinyphenol derivatives (R_3=H)
Peak 16: R_1=OH; R_2=OCH_3; R_4=H
Peak 22: R_1= OCH_3; R_2=H ; R_4=H
Peak 23: R_1=R_2=OCH_3; R_4=H
Peak 28: R_1= OCH_3; R_2=H ; R_4= *p*-coumaric acid
Peak 29: R_1=R_2=OCH_3; R_4= *p*-coumaric acid
Vinylcatechol derivatives (R_3=OH)
Peak 17: R_1=OCH_3; R_2=H ; R_4=H
Peak 19: R_1=R_2=OCH_3; R_4=H
Peak 26: R_1= OCH_3; R_2=H ; R_4= *p*-coumaric acid
Peak 27: R_1=R_2=OCH_3; R_4= *p*-coumaric acid
Vinylguaiacol derivatives (R_3=OCH_3)
Peak 24: R_1=OCH_3; R_2=H ; R_4=H
Peak 25: R_1=R_2=OCH_3; R_4=H
Peak 30: R_1=R_2=OCH_3; R_4= *p*-coumaric acid

Peak 10: R_1=R_2= OCH_3; R_3=(epi)catechin; R_4=H
Peak 14: R_1= OCH_3; R_2=R_3=R_4=H
Peak 15: R_1=R_2=OCH_3; R_3=R_4=H (catechin)
Peak 18: R_1=R_2=OCH_3; R_3=R_4=H (epicatechin)
Peak 20: R_1=R_2=OCH_3; R_3=H; R_4= *p*-coumaric acid (catechin)
Peak 21: R_1=R_2=OCH_3; R_3=H; R_4= *p*-coumaric acid (epicatechin)

Fig. 4. Structures of all the compounds found in fraction A: (a) anthocyanins; (b) A-type vitisins; (c) pyranoanthocyanins originated by reaction between anthocyanins and vinylphenol, vinylcatechol or vinylguaiacol; (d) pyranoanthocyanins originated by reaction between anthocyanins and vinyl(epi)catechin.

Peaks 26 and 27 possessed an additional shoulder around 310 nm in their UV-visible spectra when compared to peaks 17 and 19. Their molecular ions (*m*/*z* 741 and 771) had 146 amu more than peaks 17 and 19, whereas their aglycones were the same in each case (peaks 17 and 26 at *m*/*z* 433 and peaks 19 and 27 at *m*/*z* 463). This means that compounds 26 and 27 are the peonidin and malvidin 3-(*p*-coumaroyl)glucoside vinylcatechol adducts, respectively. The presence of compound 27 in red wines has been reported previously [21,24], but not the presence of compound 26.

The molecular and fragment ions of peaks 24 and 25 were 30 amu greater than those of peaks 22 and 23 (peonidin and malvidin-3-glucoside-vinylphenol adducts, respectively),

and 14 amu greater than those of peaks 17 and 19 (peonidin and malvidin-3-glucoside-vinylcatechol adducts, respectively). These values corresponded, in the first case, to an additional $–OCH_3$ group in the vinylphenol structure and in the second case, to a methylation of a hydroxyl group in the vinylcatechol structure. Their UV-visible spectra were similar to those of pyranoanthocyanins formed between peonidin and malvidin-3-glucosides and vinylcatechol (Fig. 3e). Thus, these two compounds were identified as peonidin and malvidin-3-glucoside-4-vinylguaiacol adducts. Compound 25 has been reported previously in red wines [17,21].

Peak 30 showed a molecular ion in the conventional mass spectrum at *m*/*z* 785, and its fragmentation in the MS^2 analysis yielded a major fragment at *m*/*z* 477, originated by the loss of 308 amu, and corresponding to the aglycone of peak 25. This compound was identified as malvidin-3-(*p*-coumaroyl)glucoside-4-vinylguaiacol adduct.

Fig. 4c shows the structure of the compounds corresponding to peaks 16, 17, 19, 22, 23, 24, 25, 26, 27, 28, 29 and 30.

The UV-visible spectra of compounds 15 and 18 were similar to those of pyranoanthocyanins, with a hypsochromic shift in the visible absorption maximum when compared to the monoglucosides of the anthocyanins, and with a narrower shape of the absorption band in the visible region (Fig. 3f). The molecular (*m*/*z* 805) and fragment ions originated in the MS^2 and MS^3 analyses were the same for both compounds. In their MS^2 spectra, there were two major signals at *m*/*z* 643 and 491. The first originated from the loss of one glucose moiety and the second by the loss of 314 amu. In the MS^3 spectra, there was only a major signal at *m*/*z* 491, and it originated from the loss of 152 amu from the aglycone moiety. The formation of compounds with these features was first described in model solutions containing malvidin-3-glucoside, catechin and acetaldehyde [11]. Taking into account the structure of the compounds first described by Fulcrand et al. [2], these compounds were finally identified as pyranoanthocyanins originating from the reaction between malvidin-3-glucoside and vinylcatechin or vinylepicatechin, and, more recently, their presence in red and Port wines [7,17,21,25] have been reported. The identities of compounds 15 and 18 were also confirmed by the loss of 152 amu observed in the MS^3 analyses. This loss indicated that catechin or epicatechin was present in the compound, because these flavanols lose this fragment when they undergo a retro Diels–Alder cleavage, a common pathway of their fragmentation. According to the results obtained in the isolation and characterization studies carried out by Mateus et al. [25] in compounds of this kind, and taking into account their retention times and elution orders in the reverse phase, peak 15 should be malvidin-3-glucoside-4-vinylcatechin and peak 18 should be the epicatechin adduct.

Peak 14 had a molecular ion (*m*/*z* 775) and fragment ions (*m*/*z* 613 and 461) 30 amu lower than those of peaks 15 and 18. Since it eluted just before peak 15, and this is the adduct containing catechin, the compound proposed for peak 14 was: peonidin-3-glucoside-4-vinylcatechin. To our knowledge this is the first time that the presence of this compound has been reported in red wines.

The molecular ions of compounds corresponding to peaks 20 and 21 (*m*/*z* 951) were 146 amu greater than those of compounds 15 and 18 and the fragment ions of the former in the MS^2 and MS^3 were the same as those of the latter. This was indicative of the acylation of the sugar moiety with *p*-coumaric acid. Thus, compound 20 was malvidin-3-(*p*-coumaroyl)glucoside-4-vinylcatechin and compound 21 was the corresponding vinylepicatechin adduct. These two compounds have already been isolated and structurally identified [26].

The compound of peak 10 also belongs to the family of pyranoanthocyanins originated from the reactions between anthocyanins and flavanols in the presence of acetaldehyde. Its molecular ion (*m*/*z* 1093) and the fragmentation patterns in the MS^2 and MS^3 analyses are consistent with the structure proposed for this compound: malvidin-3-glucoside-4-vinylprocyanidin dimer. The formation of this kind of compound was first observed in model solutions [8], but their presence in red wines [17,21,24] and port wines [7,25] has also been reported.

Fig. 4d shows the structure of compounds 10, 14, 15, 18, 20 and 21.

3.2.2. Fraction B

Fig. 5 shows the chromatogram of fraction B recorded at 520 nm.

Thirty five different compounds were detected, and we could identify 29 of them. Their retention times, UV-visible absorption maximums and molecular ions in the MS analyses are shown in Table 3. Some of the peaks contained more than one compound. This is the case of peaks 18, 19 and 21, which contained, in the first two cases, two different compounds and, in the third case, three different compounds.

The monoglucosides of the anthocyanins and their respective acylated derivatives accounted for almost 70% of the total area in the chromatogram shown in Fig. 5.

Peaks 4, 6, 8, 9 and 10 were identified, by comparison of their chromatographic and spectroscopic features with those of standards, as delphinidin-3-glucoside, cyanidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside and malvidin-3-glucoside, respectively.

Peaks 23, 25 and 31 corresponded to different malvidin-3-acylglucosides. The molecular ion of peak 23 was 42 amu greater than that of malvidin-3-glucoside, which can be attributed to the acylation of the sugar with acetic acid. This hypothesis was confirmed by its UV-visible spectrum and by its fragmentation pattern. The molecular ion (*m*/*z* 535) originated only one fragment ion in the MS^2 analysis, corresponding to the aglycone of malvidin (*m*/*z* 331). This fragment ion originated by loss of 204 amu, corresponding to the loss of one glucose moiety linked to an acetic acid residue. The UV-visible spectra of peaks 25 and 31 had an additional shoulder in the region of 330 nm in the first case and

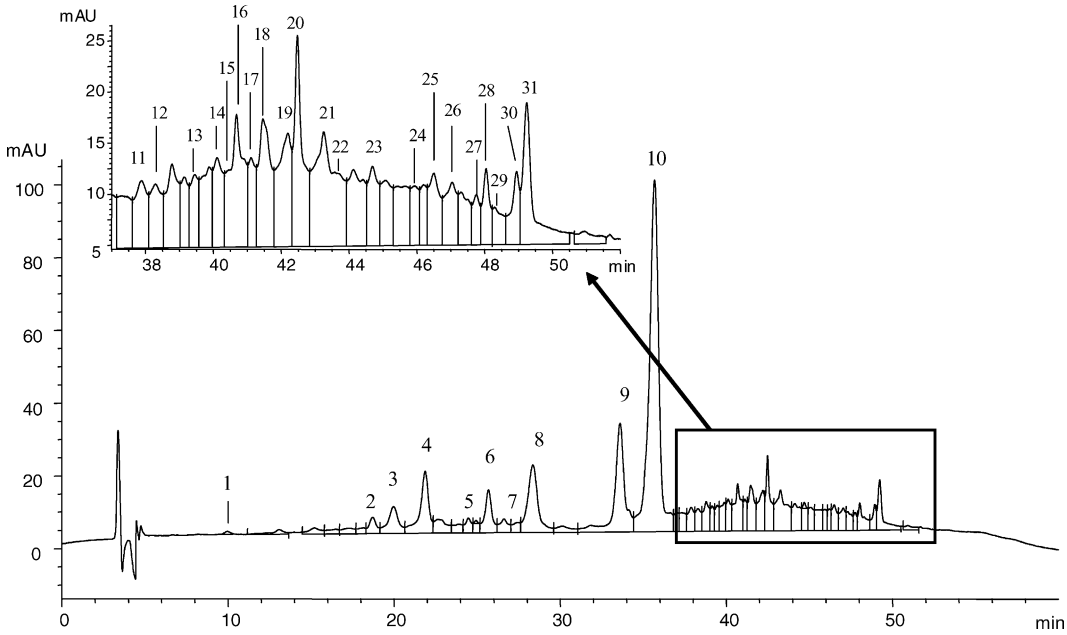


Fig. 5. Chromatogram of fraction B of the wine recorded at 520 nm.

in the region of 310 nm in the second case (see Fig. 6a). The molecular ion of compound 25 (*m*/*z* 655) was 162 amu greater than that of malvidin-3-glucoside and the molecular ion of compound 31 (*m*/*z* 639) only 146 amu greater. The fragmentation of these molecular ions gave rise, in both cases, to the fragment ion corresponding to the aglycone of malvidin. All these data allowed us to identify these peaks as follows: peak 25, malvidin-3-caffeoylglucoside and peak 31, malvidin-3-(*p*-coumaroyl)glucoside.

The conventional mass spectrum of peak 26 showed a signal at *m*/*z* 625, corresponding to the molecular ion. In the MS^2 analysis, this molecular ion was fragmented, giving rise to a major ion at *m*/*z* 317 by loss of 308 amu. This ion corresponded to the mass of the aglycone of petunidin and originated by loss of one glucose moiety linked to a *p*-coumaric acid residue. Thus, compound 26 was petunidin-3-(*p*-coumaroyl)glucoside.

Peak 30 was identified as peonidin-3-(*p*-coumaroyl)glucoside, taking into account the data obtained from its UV-visible spectrum (there was an absorption maximum in the region of 310 nm) and from its mass spectrum (molecular ion at *m*/*z* 609) and MS^2 and MS^3 spectra (loss of 308 amu from the molecular ion to produce the aglycone, *m*/*z* 301).

Fig. 7a shows the structure of compounds 4, 6, 8, 9, 10, 23, 25, 26, 30 and 31.

Peak 3 showed a UV-visible spectrum (Fig. 6b) similar to those of anthocyanins but with an absorption maximum at higher values. Moreover, the shoulder in the region

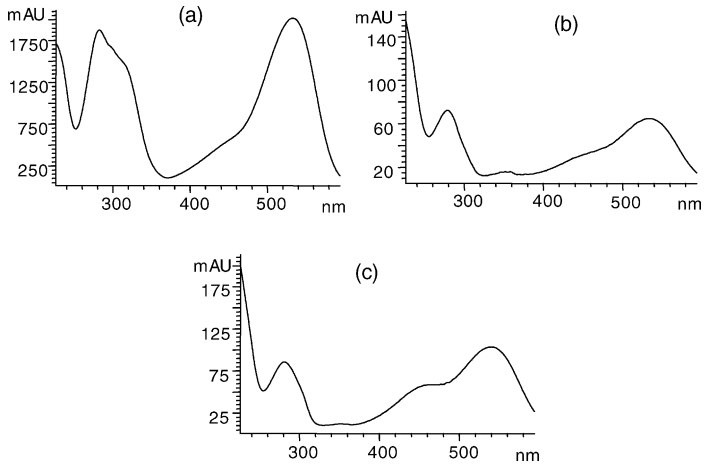


Fig. 6. Characteristic UV-visible spectra of some of the compounds of fraction B: (a) malvidin-3-(*p*-coumaroyl)glucoside, peak 31; (b) direct condensation product between catechin and malvidin-3-glucoside, peak 3; (c) one of the two possible dimers resulting from the condensation mediated by acetaldehyde between malvidin-3-glucoside and catechin, peak 20.

Table 3
Chromatographic, UV-visible and MS spectral data of the compounds identified in fraction B of the wine

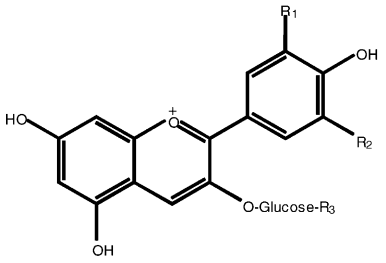
Peak number	Retention time (min)	Molecular ion (M^+) (m/z)	Absorption maxima (nm)	Identification
1	9.9	753		Direct condensation product between catechin and delphinidin-3-glucoside
2	18.7	751	279, 321, 349, 404, 459, **530**	Direct condensation product between catechin and peonidin-3-glucoside
3	20.0	781	288, 309, 455, **532**	Direct condensation product between catechin and malvidin-3-glucoside
4	21.8	465	276, 345, 372, **526**	Delphinidin-3-glucoside
5	24.5	867	282, 333, 383, 459, **538**	Unknown
6	25.7	449	280, 326, 374, **518**	Cyanidin-3-glucoside
7	26.6	867	283, 332, 361, 399, 474, **535**	Unknown
8	28.4	479	278, 344, 373, **527**	Petunidin-3-glucoside
9	33.6	463	276, 315, 362, **516**	Peonidin-3-glucoside
10	35.6	493	277, 348, **527**	Malvidin-3-glucoside
11	37.8	947		Unknown
12	38.3	947		Unknown
13	39.4	1097		Malvidin-3-glucoside-8-ethyl-procyanidin dimer
14	40.1	795	283, 336, 466, **537**	Petunidin-3-glucoside-8-ethyl-catechin
15	40.4	795		Petunidin-3-glucoside-8-ethyl-catechin
16	40.7	889	281, 348, 463, **539**	Unknown
17	41.1	825	278, 342, 454, **538**	Malvidin-3-glucoside-8-ethyl-gallocatechin
18a	41.5	809	282, 336, 424, 454, **538**	Malvidin-3-glucoside-8-ethyl-catechin
18b	41.6	825		Malvidin-3-glucoside-8-ethyl-gallocatechin
19a	41.9	897	278, 361, 390, 474, **533**	Direct condensation product between catechin and peonidin-3-(*p*-coumaroyl)glucoside
19b	42.2	779	276, 355, 438, 466, **530**	Peonidin-3-glucoside-8-ethyl-catechin
20	42.5	809	282, 345, 466, **540**	Malvidin-3-glucoside-8-ethyl-catechin
21a	43.1	927		Direct condensation product between catechin and malvidin-3-(*p*-coumaroyl)glucoside
21b	43.3	809	284, 325, 454, **540**	Malvidin-3-glucoside-8-ethyl-epicatechin
21c	43.4	927		Direct condensation product between catechin and malvidin-3-(*p*-coumaroyl)glucoside
22	43.5	895		Unknown
23	44.7	535	278, 449, **530**	Malvidin-3-acetylglucoside
24	45.8	941		Petunidin-3-(*p*-coumaroyl)glucoside-8-ethylcatechin
25	46.5	655	280, 327, **530**	Malvidin-3-caffeoylglucoside
26	47.0	625		Petunidin-3-(*p*-coumaroyl)glucoside
27	47.7	925		Peonidin-3-(*p*-coumaroyl)glucoside-8-ethyl-catechin
28	48.0	955	280, 484, **538**	Malvidin-3-(*p*-coumaroyl)glucoside-8-ethyl-catechin
29	48.3	955		Malvidin-3-(*p*-coumaroyl)glucoside-8-ethyl-catechin
30	48.9	609	281, 312, **518**	Peonidin-3-(*p*-coumaroyl)glucoside
31	49.2	639	284, 309, **533**	Malvidin-3-(*p*-coumaroyl)glucoside

The number of the peaks correspond to Fig. 5.

of 440 nm was more pronounced than in the case of the monoglucosides of the anthocyanins. This compound showed a molecular ion at m/z 781 in the conventional mass spectrum and in the MS^2 analysis it was fragmented, producing several ions at m/z 619, 601, 373, 467, 493 and 331, in order of relative abundance. The first fragment ion was formed by the loss of 162 amu (a glucose moiety) and was fragmented in the MS^3 analysis giving rise to ions at m/z 467, 373, 601, 479, 493, 343 and 331 by loss of 152, 246, 18, 140, 126, 276, and 288 amu, respectively. The presence of the ion at m/z 331, in both MS^2 and MS^3 spectra, indicated that the compound was a malvidin derivative. The loss of 152 amu was indicative of a retro Diels–Alder cleavage. Moreover, the loss of 288 amu indicates the loss of one catechin unit, according to the fragmentation pattern of oligomeric cocoa procyanidins when analysed by LC-MS and MS^2 techniques [27]. The main pathways to fragmentation of oligomeric procyanidins are the retro Diels–Alder reactions and the cleavage of the interflavanoid linkages, releasing flavanol units. When a T-unit (top unit), linked to the rest of the procyanidin by one interflavanoid linkage at C_4, is released from the oligomer, owing to the cleavage of the interflavonoid bond, a loss of 288 amu is observed. Taking into account these data we propose that the compound of peak 3 originated by direct condensation between catechin and malvidin-3-glucoside. This compound has already been detected in red wines [13,15,22] and the data obtained in the analysis of peak 3 are consistent with those reported in these works.

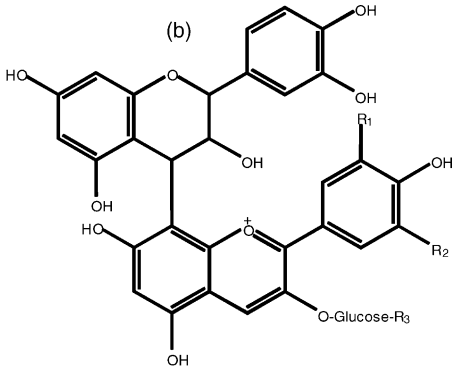
Peaks 1 and 2 had molecular ions at m/z 753 and 751, respectively. Their fragmentation pattern was identical to that of peak 3, which indicated that compounds 1 and 2 belonged to the same family of compounds as those in peak 3. Peak 1 was the direct condensation product between

(a)



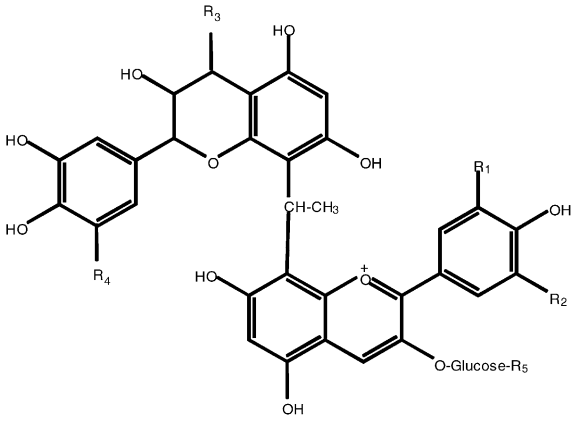
Peak 4: R_1=R_2=OH; R_3=H
Peak 6: R_1=OH; R_2=R_3=H
Peak 8: R_1=OH; R_2=OCH_3;R_3=H
Peak 9: R_1= OCH_3; R_2=R_3=H
Peak 10: R_1=R_2=OCH_3; R_3=H
Peak 23: R_1=R_2=OCH_3; R_3= acetic acid
Peak 25: R_1=R_2=OCH_3; R_3= caffeic acid
Peak 26: R_1=OH; R_2=OCH_3; R_3= *p*-coumaric acid
Peak 30: R_1= OCH_3; R_2=H; R_3= *p*-coumaric acid
Peak 31: R_1=R_2=OCH_3; R_3= *p*-coumaric acid

(b)



Peak 1: R_1=R_2=OH; R_3=H
Peak 2: R_1=OCH_3; R_2=R_3=H
Peak 3: R_1= OCH_3; R_2=R_3=H
Peak 19a: R_1= OCH_3; R_2=H; R_3= *p*-coumaric acid
Peak 21a: R_1=R_2=OCH_3; R_3= *p*-coumaric acid
Peak 21c: R_1=R_2=OCH_3; R_3= *p*-coumaric acid

(c)



Peak 13: R_1=R_2=OCH_3; R_3= (epi)catechin; R_4=H; R_5=H
Peak 14: R_1=OH; R_2=OCH_3; R_3=H; R_4=H; R_5=H
Peak 15: R_1=OH; R_2=OCH_3; R_3=H; R_4=H; R_5=H
Peak 17: R_1=R_2=OCH_3; R_3=H; R_4=OH; R_5=H
Peak 18a: R_1=R_2=OCH_3; R_3=R_4=R_5=H
Peak 18b: R_1=R_2=OCH_3; R_3=H; R_4=OH; R_5=H
Peak 19b: R_1= OCH_3; R_2=R_3=R_4=R_5=H
Peak 20: R_1=R_2=OCH_3; R_3=R_4=R_5=H
Peak 21b: R_1=R_2=OCH_3; R_3=R_4=R_5=H (epicatechin)
Peak 24: R_1=OH; R_2=OCH_3; R_3=H; R_4=H; R_5= *p*-coumaric acid
Peak 27: R_1= OCH_3; R_2=R_3=R_4=H; R_5= *p*-coumaric acid
Peak 28: R_1=R_2=OCH_3; R_3=R_4=H; R_5= *p*-coumaric acid
Peak 29: R_1=R_2=OCH_3; R_3=R_4=H; R_5= *p*-coumaric acid

Fig. 7. Structures of the compounds found in fraction B: (a) anthocyanins and acylated anthocyanins; (b) direct condensation products between flavanols and anthocyanins; (c) dimers resulting from the condensation mediated by acetaldehyde between anthocyanins and flavanols.

delphinidin-3-glucoside and catechin and peak 2 the direct condensation product between peonidin-3-glucoside and catechin. The identification was established in accordance with their retention times, *m*/*z* of the molecular ions and the presence in the MS^3 spectra of a fragment ion at *m*/*z* 303, in the first case and a fragment ion at *m*/*z* 301 in the second case, corresponding to the aglycones of delphinidin and peonidin, respectively. To our knowledge, this is the first time that the presence of the direct condensation reaction product between peonidin-3-glucoside and catechin has been reported in red wines.

Compounds 21a and 21c showed a major signal in their conventional mass spectra at *m*/*z* 927, corresponding to the molecular ion. The fragmentation pattern was identical in

both cases. The fragment ions originated in the MS^2 analysis and MS^3 were the same as those of compound 3. The molecular ion of compounds 21a and 21c was 146 amu greater than that of compound 3 and their retention times were higher than that of compound 3. These data allowed the identification of these compound as direct condensation reaction products between malvidin-3-(*p*-coumaroyl)glucoside and catechin. These two compounds are isomers but we still do not know if they are *cis–trans* isomers (owing to the presence of *p*-coumaric acid) or if there are differences in the nature of the flavanol involved in the reaction (epicatechin instead of catechin in compound 21a).

Peak 19a possessed a molecular ion 30 amu lower than those of compounds 21a and 21c. The fragmentation pattern was similar to that of those compounds. Moreover, in its MS^3 spectrum there was a signal at *m/z* 301, indicative of the presence of peonidin. This compound was identified as the direct condensation product between peonidin-3-(*p*-coumaroyl)glucoside and catechin.

Fig. 7b shows the structure of compounds 1, 2, 3, 19a, 21a and 21c.

Peaks 18a, 20 and 21b showed the same molecular ion (*m/z* 809) in their conventional mass spectra. Their UV-visible spectra were very similar, with absorption maxima in the visible region at longer wavelength than those of the anthocyanins and also longer than those of the direct condensation products between flavanols and anthocyanins. Fig. 6c shows the UV-visible spectrum of compound 20. These compounds were identified as malvidin-3-glucoside-ethyl-(epi)catechin adducts, formed by the acetaldehyde-mediated condensation reaction between malvidin-3-glucoside and flavanols. This kind of compounds has been synthesized and studied in model solutions [8,11,28] and its presence in red wines has been demonstrated [19,20,22]. In the condensation reaction through an ethyl bridge between malvidin-3-glucoside and (epi)catechin, reproduced in model solutions, two diasteroisomeric dimers appeared owing to the presence of an asymmetric carbon in the ethyl bridge, one of them always being produced in larger amounts [11]. In accordance with this fact and the results previously reported, compounds 18a and 20 corresponded to the two diasteroisomers formed in the acetaldehyde-mediated reaction between malvidin-3-glucoside and catechin, and compound 21b should correspond to one of the possible dimers originated in the same type of reaction between epicatechin and malvidin-3-glucoside. The fragmentation patterns of these three compounds in the MS^2 and MS^3 analyses were identical. The fragmentation of the molecular ion produced several signals in the MS^2 spectra at *m/z* 357, 519, 647, 331, 495 and 657 (order of relative abundance), by loss of 452, 290, 162, 478, 314 and 152 amu, respectively. The proposed structure for these three compounds allowed the explanation of all these losses. Thus, the losses of 162 and 152 amu, corresponded, as previously stated, to the loss of one glucose moiety and the loss of the fragment released by the retro Diels–Alder reaction. The loss of 290 amu was attributed to the loss of catechin. The loss of 452 amu was assigned to the simultaneous loss of catechin and the glucose moiety (290 + 162 amu). The loss of 478 amu originated the aglycone of malvidin and corresponded to the simultaneous loss of catechin with the ethyl bridge and the glucose moiety. The loss of 314 was due to the loss of the glucose moiety and the fragment released by the retro Diels–Alder reaction. It is worth indicating that the loss of the lower catechin unit (also named B-unit or base unit) from a procyanidin oligomer, after cleavage of the interflavonoid linkage at C_8 or C_6 in the MS^2 analysis, was observed in the spectrum as a loss of 290 amu [27]. This is the loss observed in the fragmentation of molecular ions of compounds 18a, 20 and 21b, and this loss is different from that observed in the direct condensation products. This would indicate that the positions of the flavanols involved in the condensation reactions are different in the case of direct condensation (C_4) from those involved in the case of acetaldehyde-mediated condensation (C_6 or C_8).

Compounds 14, 15 and 19b had similar UV-visible spectra to those of compounds 18a, 20 and 21b (malvidin-3-glucoside ethyl-catechin adducts). Peaks 14 and 15 showed a molecular ion (*m/z* 795) 14 amu lower than those of the malvidin-3-glucoside ethyl-catechin adducts and compound 19b had a molecular ion (*m/z* 779) 30 amu lower than them. The fragmentation patterns, and, therefore, the losses observed in the MS^2 analyses of these three compounds, were identical to those of the malvidin-3-glucoside ethyl-catechin adducts. All this indicated that compounds 14, 15 and 19b belonged to the family of compounds originating from condensation between ethylcatechin and anthocyanins. Compounds 14 and 15 were the two possible diasteroisomers of petunidin-3-glucoside-8-ethyl-catechin and compound 19b was the second and more abundant isomer of the peonidin-3-glucoside-8-ethyl-catechin.

Peaks 24, 27, 28 and 29 corresponded to the acylated derivatives of compounds 15, 19b, 18a and 20, respectively. All possessed 146 additional amu in their molecular ion when compared to the molecular ions of their respective non-acylated compounds. These molecular ions were fragmented, following the same patterns as those of the anthocyanin-ethyl-flavanol derivatives. The fragment ions that originated in the MS^2 and MS^3 analyses were identical to those formed in the fragmentation of their respective non-acylated compounds. Thus, compound 24 was identified as petunidin-3-(*p*-coumaroyl)glucoside-8-ethylcatechin, compound 27 as peonidin-3-(*p*-coumaroyl)glucoside-8-ethylcatechin and compounds 28 and 29 as the two possible isomers of the malvidin-3-(*p*-coumaroyl)glucoside-8-ethylcatechin.

Peaks 17 and 18b had similar UV-visible spectra to those of peaks 18a, 20 and 21b, with the absorption maxima bathochromically shifted in the visible region with respect to those of the anthocyanins. Both peaks had the same molecular ion (*m/z* 825), which was 16 amu greater than

that of peaks 18a, 20 and 21b and was indicative of the presence of an additional hydroxyl group in the molecule with respect to the latter ones. The fragment ions originated in the MS^2 analysis were exactly the same as those that originated in the fragmentation of the molecular ions of *m/z* 809, but the losses observed were different in both cases. The MS^2 spectra of peaks 17 and 18b showed several fragment ions originated by losses of 468, 306, 162, 150 and 484 amu. The loss of 306 amu was attributed to the loss of a gallocatechin unit and the loss of 468 to the simultaneous loss of a glucose moiety and the gallocatechin unit. Thus, peaks 17 and 18b could be the two possible isomers of malvidin-3-glucoside-8-ethyl-gallocatechin. The loss of a gallocatechin unit, when it is linked to an anthocyanin through an ethyl bridge, gives rise to a neutral loss of 306 amu in the *m/z* signal. In our laboratory, we have found direct condensation products between anthocyanins and gallocatechin in red wines (non-published data) and we could observe that, when the adducts were fragmented in the MS^2 and MS^3 analyses, the loss of the gallocatechin unit produced a neutral loss of 304 amu instead of the loss of 306 amu. As has been mentioned previously, different positions of the flavanols are involved in each type of condensation reaction.

Peak 13 showed a molecular ion at *m/z* 1097. Neither its UV-visible spectrum nor its MS^2 and MS^3 spectra could be obtained. Taking into account the nature of the compounds found in this wine fraction and the results concerning this kind of compounds obtained in our laboratory, not only in model solutions [8] but also in red wines [13], the compound of peak 13 was identified as malvidin-3-glucoside-8-ethyl-procyanidin dimer.

Fig. 7c shows the structure of compounds 13, 14, 15, 17, 18a, 18b, 19b, 20, 21b, 24, 27, 28 and 29.

Peak 16 shows a UV-visible spectrum similar to those of anthocyanin-ethyl-flavanol dimers. Its molecular ion showed a signal in the MS spectrum at *m/z* 889 and its fragmentation produced an ion at *m/z* 807 by loss of 82 amu. This ion was fragmented in the MS^3 analysis and gave signals at *m/z* 357 and 645, formed by loss of 450 and 162 amu. This compound has not yet been identified, but it should contain malvidin-3-glucoside, because of the presence in the MS^3 spectrum of the ion at *m/z* 357 (see fragmentation pattern of malvidin-3-glucoside ethyl-catechin adducts, peaks 18a, 20 and 21b). It should also contain catechin with an 82 amu substituent linked to its C_4 position. That linkage should be easier to cleave than the glycosidic one established between the glucose and the anthocyanin. These features would explain why, after the loss of the substituent at the C_4 position of the catechin, the simultaneous loss of the catechin and the glucose moiety gives rise to a loss of 450 amu (288 + 162 amu) instead of 452 amu.

Peaks 5 and 7 had very similar UV-visible spectra and they were also similar to those of anthocyanin-ethyl-flavanol dimers. Their molecular and fragment ions in the MS^2 and MS^3 spectra were identical in both cases and the losses observed in the MS^2 analysis were identical to those observed in the fragmentation of those dimers. The molecular ion (*m/z* 867) and the major fragment ion (*m/z* 415) originated in the MS^2 analysis were 58 amu greater than those of the malvidin-3-glucoside-8-ethyl-catechin. The MS^3 spectrum showed two ions (*m/z* 369 and 397) that originated by loss of 46 and 18 amu, respectively. These two compounds have not yet been identified, but they should have a similar structure to those of the anthocyanin-ethyl-flavanol dimers with a substituent that provides them more polarity, which would explain their earlier elution.

Compound 22 possessed a molecular ion (*m/z* 895) 28 amu greater than those of peaks 5 and 7. Again, the losses observed in its fragmentation were the same as those observed in the fragmentation of the anthocyanin-ethyl-flavanol dimers, which indicated that this compound belongs to the same family of anthocyanin-derived compounds. The major fragment ion in the MS^2 spectrum (*m/z* 443) was fragmented in the MS^3 analysis originating signals at *m/z* 397, 369 and 415. This indicated that compound 22 had the same structure as compounds 5 and 7, but with an additional substituent. Its identity has still to be established.

4. Conclusions

Pigments that are responsible for the colour of red wine have distinct reactivity towards bisulfite and we have checked that sulphited adducts are more retained in Toyopearl® HW-40(s) gel column than those non-sensitive to bisulfite bleaching. Thus, after applying the method to a red wine, acidified and bleached with $NaHSO_3$, two fractions with different pigment composition were collected. Compounds present in each fraction were identified in accordance with their UV-visible and MS^n spectra, showing that the first one was mostly constituted by pyranoanthocyanins, whereas the second one basically contained anthocyanins and anthocyanin-flavanol condensation products.

The fractionation also allows the detection of some minor pigments, among them peonidin derivatives which are analysed with difficulty in the presence of malvidin derivatives. A large variety of new pigments was detected, some of which have not been reported previously in red wines. Characteristic MS^2 and MS^3 fragmentation patterns, which could be further applied for characterisation of unknown pigments in other wines, were observed within each family of compounds. Thus, in pigments resulting from anthocyanin-flavanol direct condensation, irrespective of the flavanol involved, fragmentation always yields a fragment ion 42 amu bigger than that of the anthocyanidin. In the case of anthocyanin-ethyl-flavanol pigments, losses of sugar and flavanol moieties always yield a fragment ion corresponding to the anthocyanidin linked to the ethyl group.

Acknowledgements

This work was supported by grant AGL-2002-00167 from MCYT (Ministerio de Ciencia y Tecnología, Spain) and FEDER (UE Program). We would like to thank Mr. G.H. Jenkins for revising the English version of the manuscript.

References

[1] P.J. Cameira dos Santos, J.Y. Brillouet, V. Cheynier, M. Moutonet, J. Sci. Food Agric. 70 (1996) 204.
[2] H. Fulcrand, P.J. Cameira dos Santos, P. Sarni-Manchado, V. Cheynier, J. Favre-Bonvin, J. Chem. Soc., Perkin Trans. 1 (1996) 735.
[3] J. Bakker, P. Bridle, T. Honda, H. Kuwano, N. Saito, N. Terahara, C.F. Timberlake, Phytochemistry 44 (1997) 1375.
[4] J. Bakker, C.F. Timberlake, J. Agric. Food Chem. 45 (1997) 35.
[5] H. Fulcrand, P.J. Cameira dos Santos, P. Sarni-Manchado, V. Cheynier, M. Moutounet, Phytochemistry 47 (1998) 1401.
[6] C. Romero, J. Bakker, J. Agric. Food Chem. 47 (1999) 3130.
[7] N. Mateus, S. de Pascual-Teresa, J.C. Rivas-Gonzalo, C. Santos-Buelga, V. de Freitas, Food Chem. 76 (2002) 335.
[8] E.M. Francia-Aricha, M.T. Guerra, J.C. Rivas-Gonzalo, C. Santos-Buelga, J. Agric. Food Chem. 45 (1997) 2262.
[9] P. Sarni-Manchado, H. Fulcrand, J.M. Souquet, V. Cheynier, M. Moutounet, J. Food Sci. 61 (1996) 938.
[10] C. García-Viguera, P. Bridle, J. Bakker, Vitis 33 (1994) 37.
[11] J.C. Rivas-Gonzalo, S. Bravo-Haro, C. Santos-Buelga, J. Agric. Food Chem. 43 (1995) 1444.
[12] M.T. Escribano-Bailón, M. Álvarez-García, J.C. Rivas-Gonzalo, F.J. Heredia, C. Santos-Buelga, J. Agric. Food Chem. 49 (2001) 1213.
[13] A.M. Vivar-Quintana, C. Santos-Buelga, E.M. Francia-Aricha, J.C. Rivas-Gonzalo, Food Sci. Technol. Int. 5 (1999) 347.
[14] T.C. Somers, Phytochemistry 10 (1971) 2175.
[15] S. Remy, H. Fulcrand, B. Labarbe, V. Chenyer, M. Moutonet, J. Sci. Food Agric. 80 (2000) 745.
[16] N. Mateus, A.M. Silva, J.C. Rivas-Gonzalo, C. Santos-Buelga, V. de Freitas, J. Agric. Food Chem. 51 (2003) 1919.
[17] R.E. Asenstorfer, Y. Hayasaka, G.P. Jones, J. Agric. Food Chem. 49 (2001) 5957.
[18] H. Fulcrand, C. Benabdeljalil, J. Rigaud, V. Cheynier, M. Moutonet, Phytochemistry 47 (1998) 1401.
[19] I. Revilla, S. Pérez-Margariño, M.L. González-SanJosé, S. Beltrán, J. Chromatogr. A 847 (1999) 83.
[20] A. Heier, W. Blaas, A. Droß, R. Wittkowski, Am. J. Enol. Vitic. 53 (2002) 78.
[21] Y. Hayasaka, R.E. Asenstorfer, J. Agric. Food Chem. 50 (2002) 756.
[22] A.M. Vivar-Quintana, C. Santos-Buelga, J.C. Rivas-Gonzalo, Anal. Chim. Acta 458 (2002) 147.
[23] A.E. Håkansson, K. Pardon, Y. Hayasaka, M. de Sa, M. Herderich, Tetrahedron Lett. 44 (2003) 4887.
[24] A.M. Vivar-Quintana, Doctoral thesis, University of Salamanca, 2001.
[25] N. Mateus, A.M. Silva, C. Santos-Buelga, J.C. Rivas-Gonzalo, V. de Freitas, J. Agric. Food Chem. 50 (2002) 2110.
[26] N. Mateus, E. Carvalho, A.R.F. Carvalho, A. Melo, A.M. González-Paramás, C. Santos-Buelga, A.M.S. Silva, V. de Freitas, J. Agric. Food Chem. 51 (2003) 277.
[27] J. Wollgast, L. Pallaroni, M.E. Agazzi, E. Anklam, J. Chromatogr. A 926 (2001) 211.
[28] N.E. Es-Safi, H. Fulcrand, V. Cheynier, M. Moutonet, J. Agric. Food Chem. 47 (1999) 2096.