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Identification of anthocyanin derivatives in grape skin extracts and red wines by liquid chromatography with diode array and mass spectrometric detection

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Abstract

The different options MS detection are investigated in order to achieve the best conditions for detection and identification of anthocyanins by LC–MS. A method for separation of these compounds that enables the main molecules in wines to be identified by direct analysis, without any previous preparation, is proposed. The anthocyanin composition of different red grape skin extracts and commercial monovarietal wines were determined by this method. Some recently described anthocyanin derivatives that are supposed to be formed during wine maturation were also investigated. Results showed that some of these derivatives are present in grape and young wine. The combination of diode array detection and MS analysis has been demonstrated to be essential for identification of anthocyanin derivatives.

Keywords: Food analysis; Fruits; Wine; Anthocyanins

1. Introduction

Anthocyanins are responsible for the bright red colour of young red wines and they are some of the main phenolic compounds in red wines. Different methods have been applied for their separation and identification. During the early 1980s, anthocyanins and their acylated and cinnamic derivatives, were mainly identified by taking into account the different absorption rates at some selected wavelengths [1,2]. In the late 1980s the diode array detection (DAD) coupled to high-performance liquid chromatography (HPLC) allowed identification of the monomer anthocyanins [3–6]. In recent years, the coupling of mass spectrometry and DAD to HPLC enabled the unambiguous identification of anthocyanins and related compounds [7,8].

During wine ageing the polyphenolic content of wine becomes progressively more complex directly affecting the wine colour characteristics [9,10]. Evidence of condensation occurrence in red wines is the disappearance of free anthocyanins, which are progressively replaced by polymeric coloured compounds. This phenomenon is thought to be responsible for the coloration change from red–bluish, in young wines, towards the orange–brown colour of matured wines.

Several hypotheses have been postulated: formation of orange xanthylium-based [11], condensation between anthocyanin and vinylphenol [12] or indirect condensation involving acetaldehyde [13–16]. Recently some unidentified peaks eluting soon after the anthocyanins, have been characterised as pyruvic acid derivatives [8]. Indeed, another malvidin-3-

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glucoside derivative has been identified and named vitisin B [7]. A very important property of these anthocyanins, is the stability of the colour when bleaching by SO_2 and pH changes take place [17]. Fulcrand et al. [12] and Francia-Aricha et al., [18] have recently described some other anthocyanin pigments in an LC–DAD system formed by condensation between malvidin-3-glucoside and dimers of flavan-3-ol.

Most of the studies regarding identification of anthocyanins and their derivatives have been done in model solutions, few works having been done in wine. Yet, those studies done over wine have been developed over fortified or aged wines that allow an easy isolation and identification of new pigments. In this work, some of the new pigments have been identified directly over grape skin and over different types of wine made from different grape varieties and with a different ageing process. Also, separation, detection and identification of some monomer anthocyanins and their acetic, coumaric and caffeic derivatives have been carried out by LC–DAD–MS.

2. Experimental

2.1. Chemicals

Milli-Q water was used in all work. HPLC-grade methanol (Lab-Scan, Dublin, Ireland) and formic acid (Merck, Darmstadt, Germany) were used after filtration through a 0.45-μm pore size membrane. Standards, obtained from Extrasynthèse (Lyon, Genay-France), malvidin-3-glucoside (Mv-3-gls), cyanidin-3-glucoside (Cy-3-gls), peonidin-3-glucoside (Pn-3-gls) and malvidin chloride (Mv) were of HPLC grade and they were diluted in methanol–water (40:60).

2.2. Samples

2.2.1. Grape skin extract

Tinto Fino and Cabernet Sauvignon (*Vitis vinifera*) grapes were used for grape skin extract elaboration according to the following procedure: 100 berries were peeled, the skin being separated from the rest of the grape, i.e. seeds and pulp. The skin was macerated with methanol–formic acid (95:5) for 3 days. Every day, the coloured liquid was separated from the solid matrix and replaced with fresh solvent. The last of the extractions was favoured by applying ultrasound for 5 min. The three extracts obtained were mixed and concentrated by vacuum distillation up to 25 ml volume, avoiding temperatures of higher than 35°C. Distilled water was added to the sample up to 50 ml and kept at −18°C until analysis.

2.2.2. Wine

Monovarietal commercial wines, Cabernet Sauvignon, Graciano, Garnacha and Tinto Fino (*Vitis vinifera*) both young (1997 vintage) and 2-year bottle-aged wines (1995 vintage) were analysed.

2.3. Analysis

2.3.1. Apparatus

A Hewlett-Packard 1100 Series LC–MS liquid chromatograph–mass spectrometer fitted with 7125 Rheodyne injector (Fisons Instrument, 20-μl loop) has been used. A photodiode array detector was coupled directly to the sprayer needle where ions were generated by atmospheric pressure chemical ionisation (APCI) or electrospray ionisation (ESI) in both positive and negative ionisation modes. Nitrogen was used as nebulizing and drying gas and different fragmentor voltages were applied.

2.3.2. Method

The HPLC method used was the one proposed by González-San José et al. [2]. Samples were analysed on a reversed-phase column (Spherisorb ODS2, 150×4.6 mm I.D., particle size 5 μm) at constant temperature, 30°C. The mobile phase was: solvent A, water–formic acid (90:10); and solvent B, methanol–water–formic acid (45:45:10). The flow rate was a 0.8-ml/min linear gradient from 35 to 95% solvent B in 20 min, from 95 to 100% (solvent B) in 5 min, and holding 100% (solvent B) for an additional 5 min.

2.3.3. Detection

DAD: simultaneous detection was allowed at 313 and 530 nm. The UV–Vis spectra (scanning from 240 to 600 nm) were recorded for all peaks.

ESI conditions: anthocyanin glucoside standards

were initially studied in flow injection analysis (FIA). ESI conditions were: nebulizer pressure, 380 Pa; drying gas flow and temperature 10 l/min and 350°C, respectively; and V_{cap} 4000 V. Positive- and negative-ion spectra were recorded in the ESI mode with various orifice potentials (from 40 to 210 V). Some ions were selected for detection when samples were analysed.

APCI conditions: carrier gas flow, 3 l/min; corona discharge needle, 5000 V at 90°C.

3. Results

The versatility of APCI and ESI sources in LC–MS was exploited for direct injection of the non-volatile thermally labile products into the mass spectrometer without need of previous sample preparation. Some analyses were done using selected standards in order to determinate the best MS analysis conditions.

Though APCI could be an appropriate technique for MS analysis, some initial analysis of standards carried out in FIA mode showed poor sensitivity. Therefore subsequent work was performed by ESI, both positive- and negative-ion detection mode, and using two types of eluents: methanol–water (50:50) and methanol–water–formic acid (45:45:10).

The former solvent was used due to the recent trend to eliminate acid of the medium when MS analysis was performed. The second eluent was studied because it is the solvent B used in the González-San José et al. [2] HPLC elution method.

3.1. Methanol–water

Different fragmentor voltages in positive mode lead to different results depending on the compound being analysed. For instance, low voltages (40, 60 V) applied to malvidin chloride analysis showed adducts having a higher molecular mass than malvidin itself (m/z 547, 563). Only fragmentor voltages higher than 80 V allowed observation of the molecular malvidin ion peak M^+ (m/z 331), whose relative abundance decreased again when using voltages higher than 120 V.

When fragmentor voltages lower than or equal to 80 V, were applied to anthocyanin glucoside derivative analyses, the molecular ion was the most abundant peak although some adducts were observed. Using voltages higher than 120 V, peaks to the corresponding aglycones (anthocyanidins) begin to appear (m/z: Mv, 331; Cy, 301; and Pn, 286), but the molecular ion disappears.

3.2. Methanol–water–formic acid (45:45:10)

Addition of formic acid in the eluent, largely improved anthocyanin identification in positive mode. Low fragmentor voltages (60 V) did not produce adducts, and the molecular ion and other ions (aglycones) able to identify the compound, were clearly observed. Using high fragmentor voltages ($>$80 V), an important background noise was detected and relative abundance of molecular ion decreased. Therefore, acidified medium and 60 V fragmentor voltage were selected according to the best obtained results.

The negative-ion spectra could be discussed in terms of the corresponding deprotonated species. The behaviour under negative and positive modes is similar, sensitivity and peak abundance being lower for the negative ionisation. Therefore, the positive mode is most satisfactory for detection of anthocyanin compounds, probably because they take the form of the flavylium cation when pH is low. Then, negative mode should only be used if no possibility of using the positive mode exists, for example, in simultaneous analyses of phenolic acids or proanthocyanidins which are better detected in negative ionization mode.

After determining the MS analysis conditions, they were applied coupled to separation by HPLC using the method proposed by González-San José et al. [2] which allows a high peak separation and characterisation due to its mass and UV–Vis spectra.

Fig. 1a,b show the chromatograms obtained for young and aged wines, whose peak number information are given in Table 1, which compiles the molecular masses of the molecular ions and the most important ion fragments that help to identify each peak, together with the retention time and UV–Vis spectra.

The most abundant compounds found in grape and wine are the glucoside derivatives, particularly delphinidin-3-glucoside, petunidin-3-glucoside and

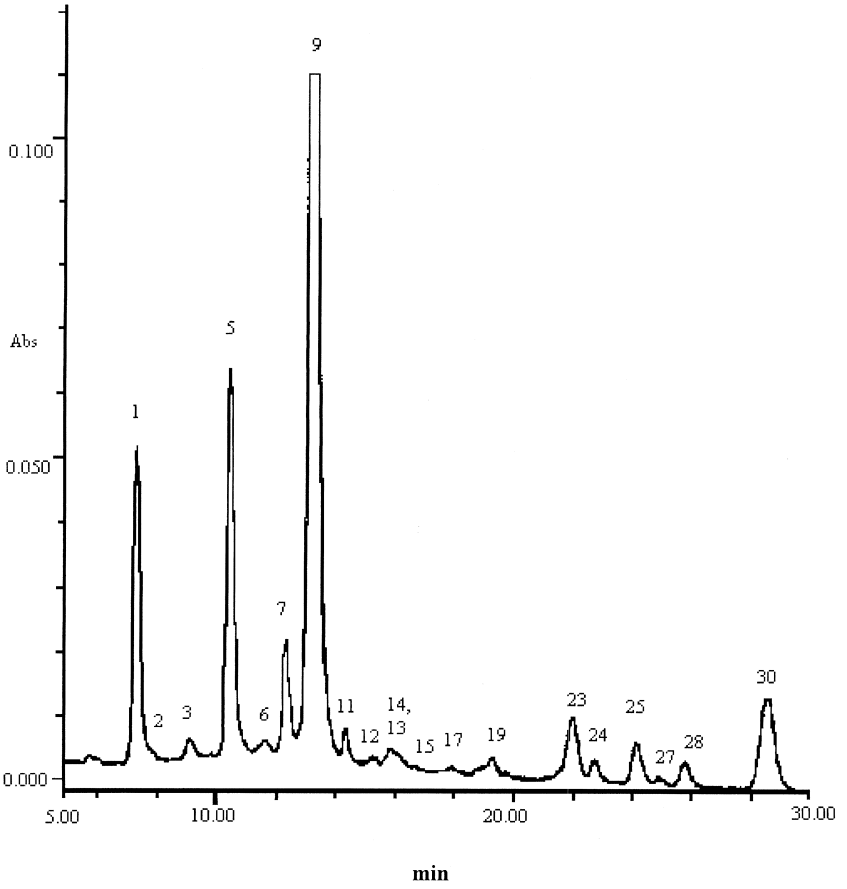

Fig. 1. (a) Chromatogram of young Tinto Fino wine. (b) Chromatogram of aged Tinto Fino wine. Peak numbers correspond to Table 1.

malvidin-3-glucoside (peaks 1, 5 and 9), while very small amounts of cyanidin 3-glucoside and peonidin-3-glucoside were found (peaks 3 and 7) even being negligible in some cases. Low amounts of some coumaric and acetic esters of anthocyanins may also be identified, and even some caffeic derivatives such as malvidin-(6 caffeoil)-3-glucoside (peak 25) have been detected. All the mentioned compounds are present in grapes. Therefore, large amounts of them may be found in skin extracts and young wines. As wine ages, some new anthocyanin derivatives begin to appear (see Fig. 1a,b).

One of the large chromatographic peaks to be pointed up, is peak 11 (m/z 561, λ_{max}=512), its spectral features are the ones found for the pigment isolated by Bakker et al. [7] in Oporto aged wines. Such pigment differs from Mv-3-gls by 68 mass units and it was named vitisin A. Recently, Fulcrand et al. [8] have isolated and characterised, in a model solution, a similar compound the structure of which suggested condensation between Mv-3-gls and pyruvic acid, they named it a pyruvic derivative of Mv-3-gls (Mv-py-gls).

Peak 15 presents a mass for the molecular ion (m/z 517) and a maximum absorbance at λ_{max}=496 nm. These data correspond to a compound, identified by Bakker and Timberlake [17] in Oporto aged wine, and named vitisin B. The structure of this compound is derived from Mv-3-gls adding a –C=C– group in site 4 and an oxygen atom in site 5.

Peaks 2, 6 and 10 differ from their respective glucosides: Dp-3-gls, m/z 533; Pt-3-gls, m/z 457; and Pn-3-gls, m/z 531, by 68 mass units, as peak 11 from Mv-3-gls. These derivatives elute right after

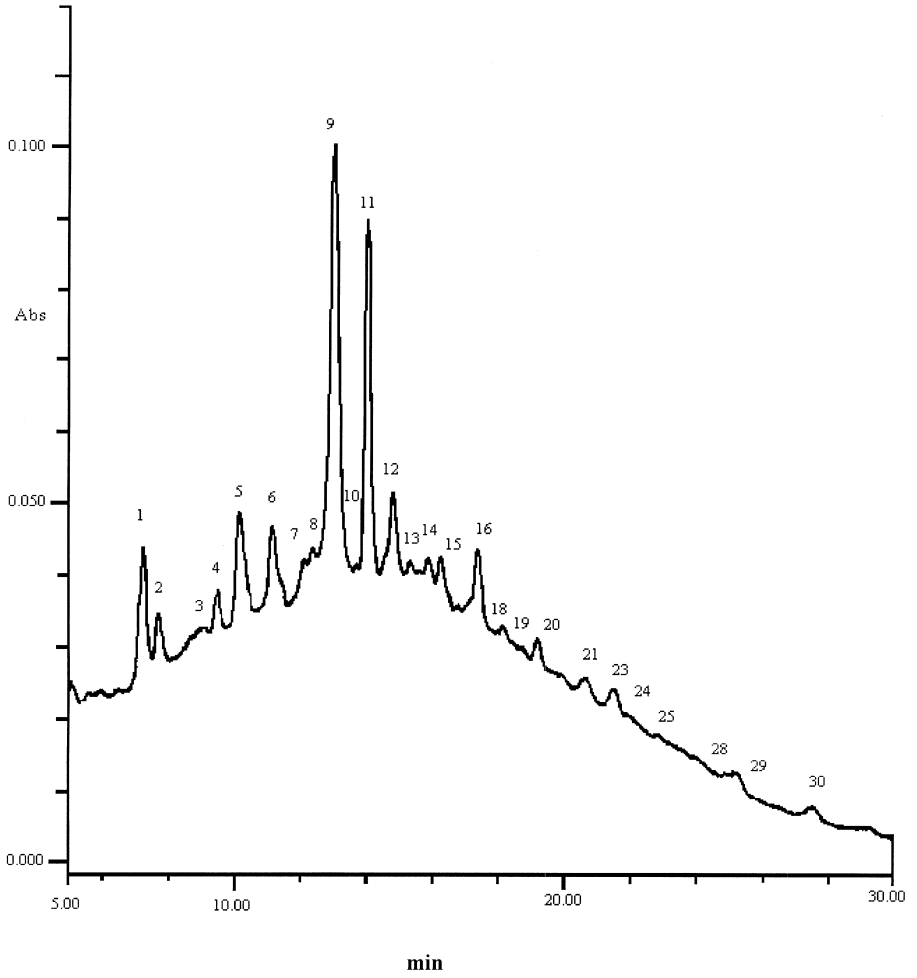

Fig. 1. (*continued*)

their respective glucosides. Their absorption spectra show a shift of λ_{max} towards lower values (515–517 nm) than their glucosides, as the Mv-3-gls derivative did compared to Mv-3-gls. Such spectral features indicate that peaks 2, 6 and 10 are derivatives that have a structure similar to peak 11.

The malvidin derivatives peaks 11 and 15, are distinguished by a high resistance to colour losses when adding SO_2, greater colour expression at higher pH values, and increased stability than anthocyanin glucosides [17]. Because of the greater stability, this group of anthocyanin derivatives appears not to be affected by the ageing reactions undergone by monomer anthocyanins after several months of wine maturation. Therefore peak 11 may be, in some cases, the most abundant anthocyanin. In addition, it may also be found in young wines (Fig. 1a) and in skin extracts (Table 2) recently prepared though in a very small amount in relation with other anthocyanins.

Another group of compounds that appears during wine ageing processes, is formed by Mv-3-gls and catechin binding through an ethyl bridge to give two different dimers corresponding to peaks 20 and 21, m/z 809 and $\lambda_{max}=530$ nm (Table 1). Those peaks have been previously described in model solutions [18,19] and in some isolated wine fractions [20], here being found for the first time when analysing

Table 1
Anthocyanin compounds detected in wine samples

Peak no.	t_R (min)	Relative t_R	m/z (M^+)	m/z	Identity
1	6.8	0.56	465	303	Delphinidin-3-glucoside
2	7.3	0.59	533	465,371	Dp-gls-py derivative[a]
3	8.6	0.69	449	287	Cyanidin-3 glucoside
4	9.0	0.72	–		unk.
5	9.6	0.77	479	317	Petunidin-3-glucoside
6	10.6	0.85	547	479,385	Pt-gls-py derivative[a]
7	11.6	0.93	463	301	Peonidin-3-glucoside
8	11.8	0.95	–		unk.
9	12.5	1.00	493	331	Malvidin-3-glucoside
10	13.1	1.05	531	463,301	Pn-gls-py derivative[a]
11	13.5	1.08	561	493,331	Mv-gls-py derivative
12	14.1	1.13	–		unk.
13	14.6	1.17	–		unk.
14	15.2	1.22	507	465, 303	Dp-(6 acetyl)-3 glucoside
15	15.5	1.24	517		Vitisin B
16	16.6	1.33	–		unk.
17	17.2	1.38	491	449, 287	Cy-(6 acetyl)-3 glucoside
18	17.4	1.39	1029	809	Polymer Mv-cat[a]
19	17.8	1.43	521	479, 317	Pt-(6 acetyl)-3 glucoside
20	18.3	1.47	809		Dimer Mv-cat[a]
21	19.7	1.58	809		Dimer Mv-cat[a]
22	20.2	1.62	505	463, 301	Pn-(6 acetyl)-3 glucoside
23	20.7	1.66	535	331	Mv-(6 acetyl)-3 glucoside
24	21.6	1.73	611	303	Dp-(6 coumaroil)-3 glucoside
25	22.8	1.83	655	493,331	Mv-(6 caffeoil)-3 glucoside
26	22.9	1.85	595	287	Cy-(6 coumaroil)-3 glucoside
27	23.6	1.89	625	317	Pt-(6 coumaroil)-3 glucoside
28	24.5	1.96	609	301	Pn-(6 coumaroil)-3 glucoside
29	26.0	2.08	609		Mv condensed[a]
30	26.8	2.15	639	331	Mv-(6 coumaroil)-3 glucoside

[a] Proposed structure.

whole wine. One of the features of these compounds is their λ_{max} shift towards the blue colour. These compounds seem to take part of subsequent polymerisation reactions to form larger compounds, such as the one that gives chromatographic peak 18 (m/z 1029, λ_{max}=530 nm), whose mass spectrum presents a fragment, m/z 809, corresponding to the dimer. This polymer has been previously found by Santos-Buelga et al. [20], after red wine fractionation.

Peak 29 (m/z 609) appears only when analysing aged wines (Table 2) and its λ_{max} presents a 15-unit hypsochromic shift compared to peonidin-3 (6 coumaroil)-glucoside (m/z 609). Peak 29 could correspond to the pigment isolated from the retentate in the membranes used for wine filtration by Fulcrand et al. [15].

Some other compounds have been detected whose structure and features are still unknown and are the subject of further studies. Chromatographic peaks 4 and 8 eluted right after the two minor anthocyanin glucosides. Their absorption spectra indicate their relation to anthocyanins since, though they absorb at 525 nm, they also present a large maximum at 350 nm (Fig. 2). Timberlake and Bridle [21] pointed out that absorption peaks in the near-UV (350–370 nm) indicate that there are substituents in site 4. Similarly, the chromatographic peak 13 (Fig. 2) belongs to a compound that presents an absorption maximum in the near-UV (369). However, the maximum in the visible region (λ_{max}=518 nm) is shifted towards lower wavelengths than anthocyanins. The chromatographic peaks 12 and 16 also show this type of shift in the visible spectrum (λ_{max}=517 and 504 nm, respectively).

Table 2
Anthocyanin compounds detected in various skin extracts and wines[a]

Peak no.	Skin extract		Wines				
	Ext CS	Ext TF	TF (young)	TF[b] (aged)	CS[b]	Garnacha[b]	Graciano[b]
1	*[c]	*	*	*	*	*	*
2	nd[d]	*	*	*	*	nd	*
3	*	*	*	*	*	*	*
4	nd	nd	nd	*	*	nd	*
5	*	*	*	*	*	*	*
6	*	nd	*	*	*	*	*
7	*	*	*	*	*	*	*
8	nd	nd	nd	*	*	nd	nd
9	*	*	*	*	*	*	*
10	nd	nd	nd	*	*	*	nd
11	*	*	*	*	*	*	*
12	nd	nd	*	*	*	*	*
13	nd	nd	*	*	*	*	*
14	*	*	*	*	*	*	*
15	nd	nd	*	*	*	*	*
16	nd	nd	nd	*	*	*	nd
17	*	*	*	nd	nd	nd	*
18	nd	nd	nd	*	*	nd	
19	*	*	*	*	*	nd	*
20	nd	nd	nd	*	*	*	*
21	nd	nd	nd	*	*	nd	*
22	*	*	nd	nd	*	nd	nd
23	*	*	*	*	*	*	*
24	*	*	*	*	*	nd	*
25	*	*	*	*	nd	nd	*
26	*	*	nd	nd	nd	nd	nd
27	*	*	*	nd	*	*	*
28	*	*	*	*	*	nd	*
29	nd	nd	nd	*	*	*	*
30	*	*	*	*	*	*	*

[a] CS, Cabernet Sauvignon; TF, Tinto Fino.
[b] Two-year aged wines.
[c] Detected.
[d] Not detected.

The identified chromatographic peaks, were analysed in skin extracts from Tinto Fino and Cabernet Sauvignon grapes and wines elaborated from different varieties (Table 2). It can be observed that skin extracts contain glucoside derivatives, acetic and coumaric esters, and a small amount of malvidin derivative peak 11. However they do not contain vitisin B or pigments with higher molecular weights than monomer anthocyanins. Skin extracts from Tinto Fino contain the anthocyanins derivative, peak 2, while they do not contain the derivative peak 6, in contrast to skin extracts from Cabernet Sauvignon that present peak 6 but do not present peak 2.

Most of the pigments found in the skin extracts were also found when analysing young wine made from Tino Fino grapes. The fact that cyanidin-3 glucoside, peonidin-3 (6 acetyl)-glucoside and cyanidin-3 (6 coumaroil)-glucoside were not found in this young wine may be due to the very low concentrations of these compounds detected in grape skin. It may be observed that this wine, though young, contains peaks 11 and 15 and peaks 2 and 6. However, dimers formed by condensation of malvidin and catechin cannot be observed.

Aged wines (Cabernet Sauvignon, Tinto Fino, Graciano and Garnacha) contain pigments that

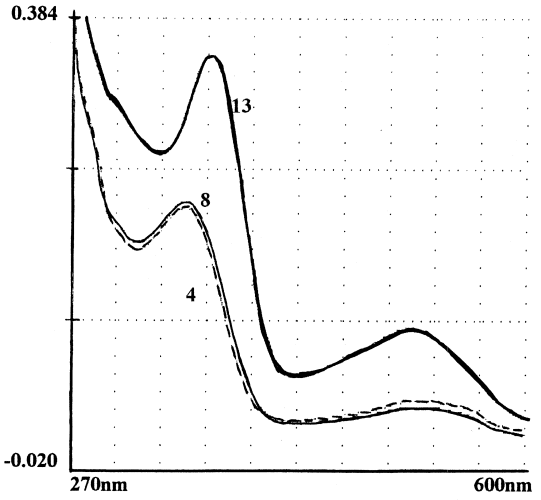

Fig. 2. UV–Vis spectra of peaks 4 (- - -), 8 (—) and 13 (———).

belong to the group with similar characteristics to that described for peak 11 (peaks 2, 6 and 10) and to the group characterised by malvidin-catechin condensation (peaks 18, 20 and 21). In addition, peak 29 is observed, while it was not when analysing skin extracts or young wines.

The new pigments were detected in higher amounts in wines made from Tinto Fino and Cabernet Sauvignon varieties since they have the highest anthocyanin content.

4. Conclusions

The MS data in this work show that mass analyses are best carried out under the following conditions: acid medium, positive ionisation, ESI and low fragment voltages (60 V).

Combining the information given by both DAD and MS is very useful for identification of anthocyanin derivatives formed during wine elaboration and ageing. Generally MS detection allows identification of the group to which the compound belongs, and the anthocyanin source and the hypsochromic shifts observed in the UV–Vis spectrum help to elucidate the final structure.

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