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Varietal differences among the anthocyanin profiles of 50 red table grape cultivars studied by high performance liquid chromatography☆

F. Pomar[1], M. Novo[2], A. Masa*

Misión Biológica de Galicia, Consejo Superior de Investigaciones Científicas (CSIC), Viticulture Department, Apdo. 28, 36080 Pontevedra, Spain

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Abstract

In order to develop a method that allows to distinguish between grape cultivars, the anthocyanin profiles of 50 accessions from the "Misión Biológica de Galicia" germplasm collection were studied by high performance liquid chromatography (HPLC). Nineteen anthocyanins were totally or partly identified and significant quantitative differences between the studied anthocyanin markers were found. With this method all 50 cultivars examinated could be easily distinguished from each other. In addition, the HPLC fingerprints and the relative-area anthocyanins plot for every cultivar has been elaborated and stored in a database. To test the validity of this method, several unknown samples have been analysed comparing their anthocyanin profile with the fingerprint database, and we may conclude that this has been proved to be of great value for grape cultivar recognition.

Keywords: Chemotaxonomy; Grape cultivar identification; Anthocyanin analysis

1. Introduction

Traditionally, morphological and agronomical characteristics have been the main criteria for differentiating grapevine cultivars, but it is well known that many of those characters are strongly influenced by environmental conditions. Furthermore, the great intravarietal variability that exists recommends the use of more precise methods, and a wide range of biochemical and molecular markers (DNA, enzymes and diverse metabolic compounds) have been used successfully to characterize and classify grape germplasm collections [1–4].

Among the metabolic compounds, which have frequently been used as chemical markers in chemotaxonomy, the anthocyanins stand out [1,5–7] and it is well known that their distribution in grape is complex and varies according to the cultivar [8]. The anthocyanins are part of the group of compounds collectively known as flavonoids, and they are responsible for the red to black colours of grape cultivars and also contribute to the organoleptic and chemical properties of grape and wines because of their interaction with others phenolic compounds as well as with proteins and polysaccharides [8]. The glycosides are more stable than free aglycones (anthocyanidins), that are highly reactive compounds and do not occur naturally [9]. In *Vitis vinifera* L. red cultivars there are only cyanidin, delphinidin, petunidin, peonidin and malvidin 3-monoglucosides along with the corresponding acetyl, *p*-coumaroyl and caffeoyl derivatives. Cyanidin is the precursor pigment of the other anthocyanidins, and it can be transformed into peonidin by the action of a 3′-*O*-methyltransferase, or into delphinidin by the action of a 3′-hydroxylase. A 3′-5′-*O*-methyltransferase transforms delphinidin into petunidin, and petunidin into malvidin (Fig. 1). The higher or lower activity of the enzyme 3′-hydroxylase would produce different ratios between di- and trihydroxy-substituted anthocyanins [2]. Advances in C18

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* Corresponding author. Tel.: +34 986 854 800; fax: +34 986 841 362.
E-mail address: amasa@mbg.cesga.es (A. Masa).
[1] Present address: Centro de Investigaciones Agrarias de Mabegondo (Xunta de Galicia), P.O. 10, 15080-A Coruña, Spain.
[2] Present address: Departamento de Biología Animal, Biología Vegetal y Ecología, Universidad de A Coruña, 15071-A Coruña, Spain.

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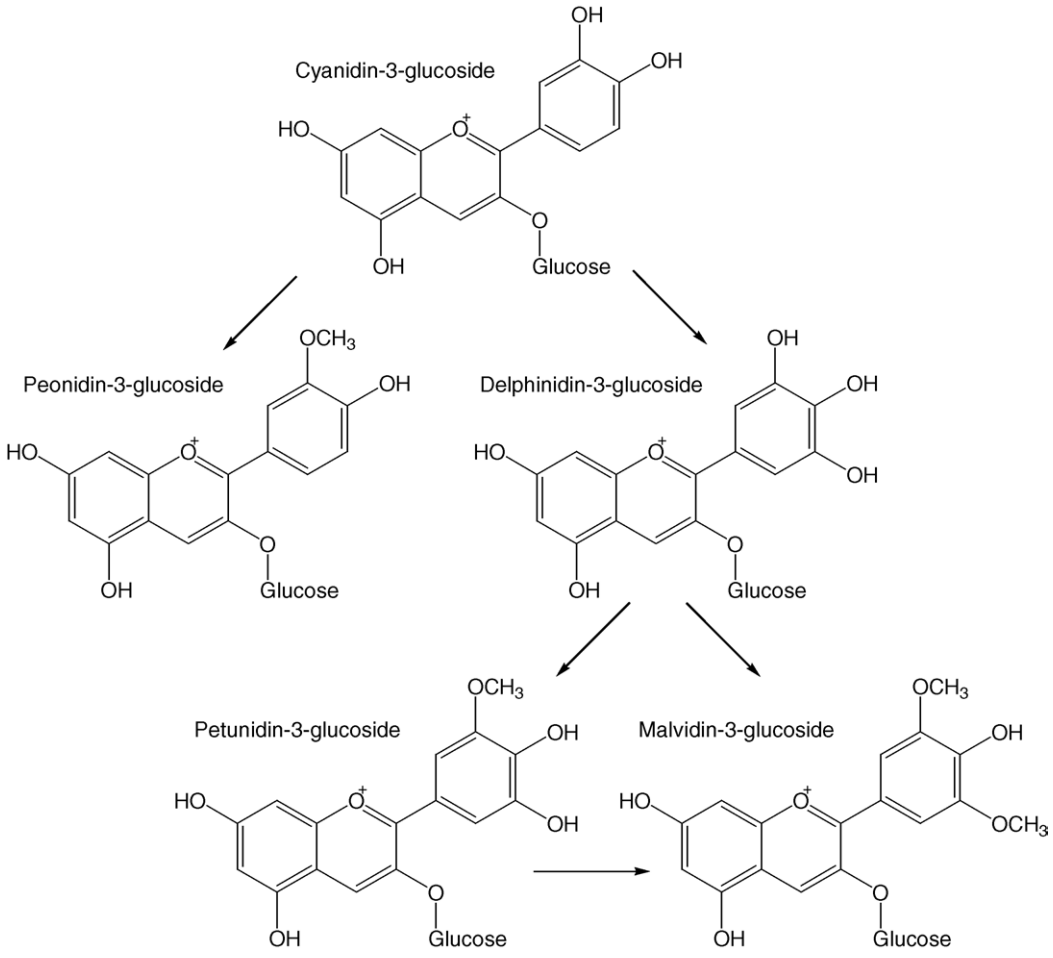

Fig. 1. Scheme illustrating the final reactions involved in the anthocyanin biosynthesis.

reversed-phase HPLC made possible to determinate cultivar anthocyanin profiles and to calculate those ratios, that have been used for the identification of grape cultivars because it seems to be characteristic of cultivar and independent of the production area [1,7,8].

The composition of anthocyanins is primarily determined by genetic factors, however, the content of anthocyanins in grapes changes during their maturation, and seasonal conditions and the physical and chemical characteristics of the soil also influence the distribution of anthocyanins in grapes. Nevertheless, most of references coincide with the fact that the non-genetic factors such as several environmental conditions or viticultural practices have a greater effect on the concentration of anthocyanins rather than on their relative distribution [9].

The main objective of this paper was to develop a chromatographic method that allows to identify and classify the 50 cultivars present at the "Misión Biológica de Galicia" grape germplasm collection. This method was based on the analysis and comparison of the HPLC anthocyanin profiles of cultivars and has been proved to be useful for the identification of grape cultivars.

2. Experimental

2.1. Plant material

Fifty grapevine accessions from the grape germplasm collection of the "Misión Biológica de Galicia" (Pontevedra, Northwest of Spain) were selected. Berries at technological ripening were harvested in late summer and immediately frozen at −23 °C until extraction.

2.2. Chemicals

The anthocyanin standards were obtained from Extrasynthese (Genay, France) and sugar standards from Fluka (Buchs, Switzerland). Acetonitrile, ethanol and methanol were HPLC grade solvents from Merck (Darmstadt, Germany) and formic acid HPLC grade was from Fluka. Phthalic acid was for chromatography from Merck and all the other chemicals of analytical-reagent grade were from Merck, Scharlau (Barcelona, Spain) or Panreac (Barcelona, Spain). HPLC-grade water was obtained from a Milli-Q System (Millipore, Bedford, MA, USA).

2.3. Extraction

Peels (5 g fresh weight) were manually separated from pulp and extracted with 40 mL of 2% formic acid in methanol for 30 min in the dark and with shaking in a water bath from Grant Instruments (Cambridge, UK). This procedure was repeated four times. The extracts obtained were mixed, filtered (Whatman 4), evaporated to dryness in a vacuum evaporator (Büchi, Flawil, Switzerland) at 35 °C, diluted with 50 mL of distilled water and extracted in separatory funnel three times with *n*-hexane (v/v) to eliminate lipophylic compounds and five times with EtOAc (v/v) to wash polyphenolic compounds such as phenolic acids and flavonols. The aqueous extract obtained was newly evaporated to dryness, resolubilized with 2% formic acid in methanol and applied onto a polyvinylpolypyrrolidone column (300 mm × 20 mm I.D.). The column was washed with Milli-Q water to eliminate free sugars and anthocyanins were eluted with 2% formic acid in methanol. The eluate was concentrated to dryness in a vacuum evaporator and resolubilized with 10% formic acid in methanol. This experiment was performed five times for each sample and the extracts were used for the separation and identification of the anthocyanic pigments.

2.4. Chromatographic analysis

The chromatographic analysis were carried out on a Waters (Milford, MA, USA) high-performance liquid chromatograph equipped with a 2690 Waters Separations Module and a 996 Waters photodiode array detector. The HPLC column was a C18 Kromasil 100 (4 μm particle size, 250 mm × 4 mm I.D.) from Tracer Analitica (Barcelona, Spain) and was protected with a C18 Nova Pack guard precolumn from Waters. The oven temperature was set at 30 °C. Extracts were passed through a 0.45 μm filter (Millex-HV, Millipore) and a volume of 20 μL of solution injected through the RP-C18 column for analytical HPLC. Flow rate was 1.0 mL/min and the mobile phase consisted on an acetronitrile/formic acid/water (45:10:45, v/v/v) as solvent A and 10% formic acid as solvent B. The gradient profile was 25% A at 0 min, 35% A at 15 min, 50% A at 20 min, 55% A at 25 min, 65% A at 40 min. The mobile phase was returned to its initial conditions in 5 min. Data were recorded on a computer with the Millenium^{32} software from Waters, chromatograms were acquired at 546 and 313 nm and photodiode array spectra were recorded between 270 and 600 nm.

Parallelly at HPLC analyses, anthocyanic extracts were separated on succesive preparative descending paper chromatography (PC) (3 MM Whatman paper) with the upper phase of BAW (*n*-butanol/acetic acid/water, 4:1:5, v/v/v) and ascending preparative thin layer chromatography (TLC) (200 mm × 200 mm × 0.1 mm cellulose, Merck) with the same solvent. The different pigments obtained were studied by co-chromatography with anthocyanins standards in analytical HPLC, TLC (200 mm × 200 mm × 0.1 mm cellulose, Merck) and PC (1 M Whatman paper) and according to their spectral properties. The spectral measurements were performed with a DU-640 spectrophotometer from Beckman Coulter (Fullerton, CA, USA). Spectral analysis (between 270 and 600 nm) were achieved in methanol containing 0.01% HCl and shifts were recorded after addition of $AlCl_3$ (three drops of 5%, w/v, in ethanol). Further evidence for the structure of pigments was obtained by chromatographing the products of acid, alkaline and enzymatic hydrolysis, which were carried out following the methods described by Markham [10]. Aglycones were identified by co-chromatography with standards and by UV–vis spectral analysis. Sugars were identified by chromatographic comparison with authentic sugar markers on 3 MM Whatman paper with BBPW (*n*-butanol/benzene/pyridine/water, 5:1:3:3, v/v) as solvent. Detection was achieved by dipping the chromatogram into a solution of aniline hydrogen phthalate (Partridge's reagent) that was prepared dissolving aniline (0.92 mL) and phtalic acid (1.6 g) in *n*-butanol/ether/water (49:49:2, v/v/v) [10].

2.5. Statistical analysis

An analysis of variance (ANOVA) for the area percent of each chromatographic peak and a principal component analysis (PCA) were performed using the Statgraphics Plus software for Windows 4.0 version (Statistical Graphics, USA). The graphical representations were performed using Sigmaplot 2001 for Windows (SPSS, USA).

3. Results and discussion

3.1. Chromatographic anthocyanin identification

It is well known that the elution order of anthocyanins in reversed-phase chromatography is closely related to their polarity, the most polar ones eluting first followed the less polar ones. Thus, delphinidin-3-monoglucoside elutes first, followed in order by the 3-monoglucosides of cyanidin, petunidin, peonidin and malvidin. The same order of elution was followed by the acetic-acid-acylated anthocyanins as by coumarates and caffeoates. Thus, the order of elution as a function of the polarity of the different compounds together with their spectral properties can be used to characterize the different chromatographic peaks.

The typical HPLC chomatogram of anthocyanin extracts captured at 546 nm, shows 19 peaks (Fig. 2). Attending to the retention time in HPLC and their UV–vis spectral properties, it was possible to identify 16 anthocyanic compounds (Table 1). Their chemical structure was confirm by the result of hydrolysis and co-chromatographic analysis (PC and TLC). In any case glucose was the sugar liberated by

Table 1
Retention times and spectral characteristics of the chromatographic peaks identified

Peak number	t_R (min)	λ_{max} (nm)		Identification
		MeOH/HCl (from literature)[a]	Photodiode array	
1	3.1	278, 542	277, 346, 524	Delphinidin-3-monoglucoside
2	4.2	282, 530	279, 330, 515	Cyanidin-3-monoglucoside
3	5.1	278, 540	277, 347, 526	Petunidin-3-monoglucoside
4	7.6	280, 528	279, 515	Peonidin-3-monoglucoside
5	9.2	278, 538	277, 348, 526	Malvidin-3-monoglucoside
6	11.1	280, 542	280, 523	Delphinidin-3-monoglucoside-acetate
7	15.5	280, 500, 530	281, 514	Cyanidin-3-monoglucoside-acetate
8	17.8	280, 540	278, 528	Petunidin-3-monoglucoside-acetate
9	21.6	–	282, 529	Petunidin-3-monoglucoside-caffeoate
10	21.8	281, 527	280, 518	Peonidin-3-monoglucoside-acetate
11	22.8	280, 538	278, 348, 529	Malvidin-3-monoglucoside-acetate
12	23.3	–	283, 326, 523	?
13	23.4	–	283, 314sh, 520	?
14	23.8	–	281, 329, 534	?
15	24.0	282, 308sh, 538	282, 530	Delphinidin-3-monoglucoside-*p*-coumarate
16	24.1	282, 310sh, 528	282, 529	Cyanidin-3-monoglucoside-*p*-coumarate
17	24.8	283, 312sh, 541	281, 535	Petunidin-3-monoglucoside-*p*-coumarate
18	26.6	283, 312sh, 528	283, 314sh, 519	Peonidin-3-monoglucoside-*p*-coumarate
19	27.3	284, 313sh, 538	282, 534	Malvidin-3-monoglucoside-*p*-coumarate

The symbol '?' in the table is used for 'not identified' anthocyanins.
[a] [14,15].

acid and enzymic hydrolysis and acetic, *p*-coumaric and caffeic were the only organic acids removed by alkali treatment. Peaks numbered 1–5 correspond to the monoglucosides of the five anthocyanins found in grapes, peaks 6–8, 10 and 11 represent acetic-acid-acylated anthocyanins, peak 9 correspond to the petunidin-caffeoate and peaks 15–19 to the *p*-coumarates of delphinidin, cyanidin, petunidin, peonidin and malvidin, respectively. It was not possible to identify the other three chromatographic peaks pointed out in Fig. 2 according to their spectral properties because there were not found in sufficient amounts to obtain reliable spectra, but taking into account the obtained data we could assume that were probably caffeaotes derivates.

From the chromatograms obtained there were calculated diagrams in which the relative area values of each peak are represented; these diagrams represents the "fingerprint" of each cultivar.

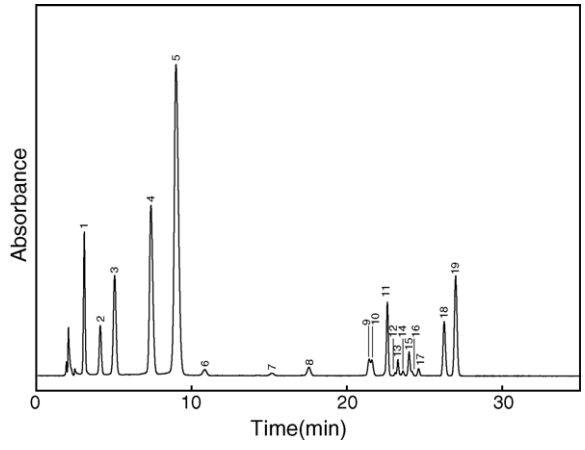

Fig. 2. Typical HPLC chromatogram of anthocyanin extracts captured at 546 nm. Peak identification is shown in Table 1.

3.2. Stability of anthocyanin composition along ripening

It is known that the synthesis of anthocyanins starts during ripening, increasing after it. Several authors have described the evolution of the anthocyanin during ripening [11–13]. Principally they observed an accumulation of malvidin derived compounds, which are formed at the end of the anthocyanin synthesis process (Fig. 1). However, cyanidin derivated pigments increased more slowly and their contribution to the total anthocyanin content declined along ripening.

In order to verify if the cultivar anthocyanin profile was stable along the process of commercial ripeness of the grape, we have gathered grapes of the cultivar "Mencía" from 17 August 2000 until 24 September 2000, analyzing later the anthocyanin profiles that were presented. Fig. 3 shows relative-area graph obtained for the grapes of cultivar "Mencia" gathered throughout this period of time. As we can observe, the variations showed by the chromatographic profiles are minimal. Only small variations were observed in the relative area of the peaks 4 and 5 corresponding to peonidin-3-monoglucoside and malvidin-3-monoglucoside. In our work, the first sample was harvested around 17 days after ripening, when the synthesis of anthocyanin was already advanced, as a result of that the anthocyanin content was high, and we did not observe the ripening-related evolution described by other authors. Our results are in agreement with those obtained by Arozarena et al. [9] who studied the anthocyanin composition of several cultivars and they did not detect ripening-related changes in anthocyanin patterns when working with samples of at least 20 days after véraison.

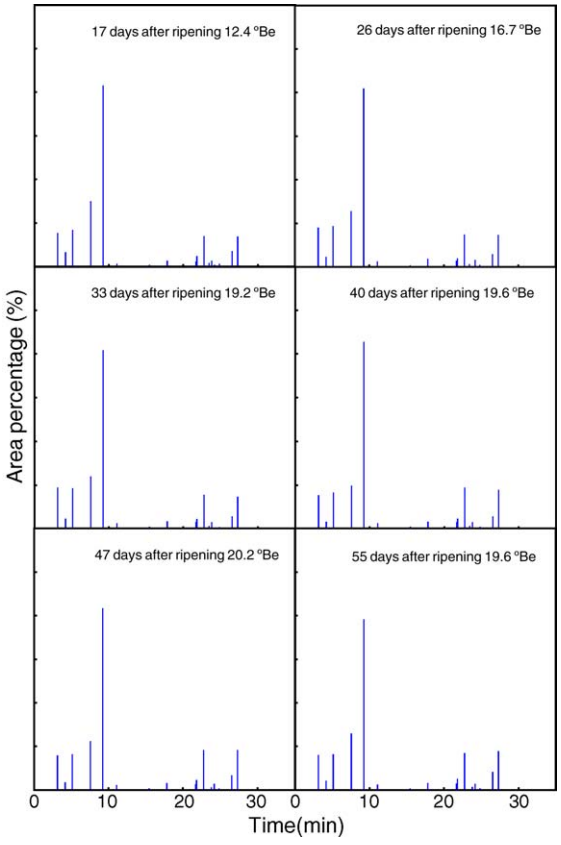

Fig. 3. Evolution of anthocyanin profile in cultivar "Mencía" during ripening.

3.3. Stability of anthocyanin composition along different vintages

Likewise there were studied the possible variations that the anthocyanin profile could present in different years. Fig. 4 shows relative-area graph obtained for the grapes of cultivar "Mencia" gathered in these different years. In this case the observed variations affect the majority of the peaks and are quantitatively greater that the observed ones along the comercial ripeness. For example, peak 1 correponding to delphinidin-3-monoglucoside ranges from 9.4% in 2000 to 4.0% in 2001, or peak 19 corresponding to malvidin-3-monoglucoside-p-coumarate ranges from 13.7% in 2001 to 8.0% in 2003.

Since these changes do not seem to be too important, we decided to analyze all the samples of the different cultivars gathered from 2000 to 2003 and use the mean values obtained to elaborate the varietal-typical anthocyanin profile.

3.4. Anthocyanin characterization of cultivars

Table 2 shows the mean contents (in peak area %) of the 19 anthocyanins isolated in each studied sample. From this Table, we can deduce that there is a high genetic heterogeneity within the accessions studied. Nevertheless, common characteristics exists to all the studied cultivars and

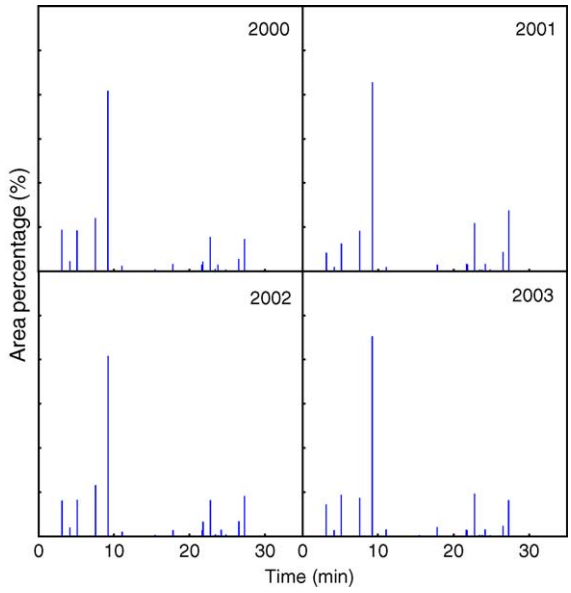

Fig. 4. Evolution of anthocyanin profile in cultivar "Mencía" along successive years.

we can affirm that the malvidin derivatives, and malvidin-3-monoglucoside especially, are the major anthocyanins in all the cases; the cyanidin derivatives are the less abundant anthocyanins. The major anthocyanin group in all these cultivars was 3-monoglucosides derivatives, ranged from 48.2% in "Couxo" to 94.6% in "Moscatel Negro", except in the cultivar "Bastardo Ruzo" in which the monoglucosides only represent 31.2%. In this case the majority group is the *p*-coumarate one that represents 60.7% of total anthocyanin content.

Data clearly shows that the anthocyanin fingerprint among grape cultivars is quite different at harvest, and this fact allows the use of this tool to differentiate them.

These results were confirmed when a principal component analysis over the whole data set was performed. The first five principal components obtained, which explain the 85.7% of the cumulative variance, were selected (Fig. 5). In Table 3, we show the weights of the first five principal components

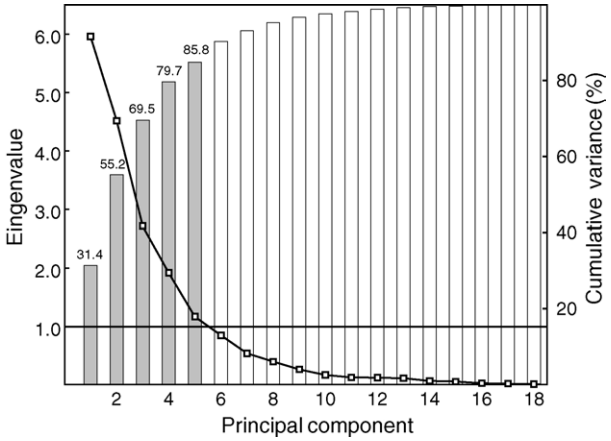

Fig. 5. Principal components screen plot.

Table 2
Anthocyanin mean values (in peak area %) and least significance difference for the 50 accessions studied ($n = 20$)

	Dpg	Cyg	Ptg	Png	Mvg	**Sum_g**	Dpac	Cyac	Ptac	Ptcf	Pnac	Mvac	**Sum_ac**	P-12	P-13	P- 14	Dppc	Cypc	Ptpc	Pnpc	Mvpc	**Sum_pc**
Albarello	16.6	4.9	14.4	6.4	45.4	**87.7**	0.8	0.1	0.6	1.5	0.2	2.1	**3.8**	0.0	0.3	0.1	1.2	0.0	0.2	0.5	4.8	**6.7**
Albarin Francés	12.3	4.7	12.0	10.3	32.4	**71.7**	2.8	0.7	2.6	3.2	1.2	5.9	**13.2**	0.0	1.2	0.1	2.3	0.0	0.4	2.2	5.9	**10.8**
Albarin Negro	8.8	3.2	10.2	17.6	44.0	**83.8**	0.3	0.0	0.5	1.1	0.5	2.1	**3.4**	0.1	0.4	0.2	1.2	0.1	0.4	2.6	6.7	**11.0**
Alicante	2.9	1.3	5.4	16.4	45.2	**71.2**	0.1	0.1	0.2	1.5	0.6	2.1	**3.1**	0.1	0.4	0.1	1.7	0.0	0.3	5.4	16.1	**23.5**
Bastardo Ruzo	0.6	0.7	1.8	7.6	20.5	**31.2**	0.0	0.0	0.0	1.4	1.8	4.9	**6.7**	1.7	1.8	3.7	3.0	1.8	2.8	14.0	31.9	**53.5**
Brancellao	3.8	3.9	5.5	25.3	39.8	**78.3**	0.1	0.1	0.3	0.5	1.4	3.1	**5.0**	0.2	0.7	0.2	0.8	0.3	0.6	4.7	8.7	**15.1**
Cabernet	7.3	1.7	6.4	6.6	37.3	**59.3**	2.3	0.5	2.4	0.6	2.5	21.2	**28.9**	0.1	0.2	0.3	0.5	0.1	0.4	1.5	8.1	**10.6**
Caiño Bravo	9.6	1.7	11.2	10.2	52.1	**84.8**	0.2	0.3	0.1	0.8	0.3	2.1	**3.0**	0.1	0.2	0.3	1.1	0.1	0.5	1.8	7.1	**10.6**
Caiño do Freixo	8.3	2.7	10.8	4.2	37.5	**63.5**	2.5	0.7	2.2	2.1	1.3	12.3	**19.0**	0.1	0.8	0.1	2.4	0.0	0.5	1.4	10.0	**14.3**
Caiño Gordo	6.6	1.4	10.0	10.9	54.5	**83.4**	0.1	0.0	0.2	0.7	0.5	1.6	**2.4**	0.1	0.2	0.3	1.1	0.1	0.6	2.6	8.6	**13.0**
Caiño Longo	1.0	0.3	2.3	2.6	53.0	**59.2**	0.0	0.0	0.0	0.3	0.3	4.7	**5.0**	0.1	0.1	0.5	1.1	0.1	2.8	1.5	29.7	**35.2**
Caiño MBG	6.2	1.2	9.5	8.3	55.3	**80.5**	0.2	0.0	0.4	1.0	0.4	2.4	**3.4**	0.2	0.2	0.6	1.5	0.0	0.5	2.0	10.3	**14.3**
Caiño Redondo	4.6	1.1	7.0	4.2	35.7	**52.6**	1.2	0.2	1.9	4.0	0.7	9.5	**13.5**	0.1	0.6	0.4	4.4	0.1	1.2	2.4	20.7	**28.8**
Carnaz	1.7	0.4	3.9	5.4	42.5	**53.9**	0.1	0.0	0.6	0.9	0.6	7.1	**8.4**	0.2	0.3	1.3	2.0	0.1	2.2	3.9	26.7	**34.9**
Carrasco	4.9	0.7	8.5	4.3	52.5	**70.9**	0.2	0.0	0.4	1.0	0.9	3.4	**4.9**	0.2	0.3	0.9	2.5	0.0	0.5	1.5	17.3	**21.8**
Cascon	4.6	1.5	7.2	6.9	39.8	**60.0**	1.6	0.3	2.3	1.5	2.5	12.4	**19.1**	0.2	0.5	0.7	1.8	0.0	0.5	2.7	12.9	**17.9**
Castañal	16.7	3.4	16.2	11.5	38.7	**86.5**	0.5	0.0	0.6	1.0	0.8	1.9	**3.8**	0.0	0.3	0.1	1.4	0.1	0.3	1.5	5.2	**8.5**
Castellana	15.3	4.2	14.9	8.2	38.2	**80.8**	0.6	0.1	0.7	3.0	0.1	1.6	**3.1**	0.1	0.9	0.2	2.3	0.1	0.2	1.8	7.4	**11.8**
Catalán Negro	7.5	3.0	13.2	9.1	42.7	**75.5**	0.3	0.1	0.5	2.5	0.2	1.6	**2.7**	0.1	1.4	0.3	3.1	0.0	0.3	3.5	10.6	**17.5**
Corbillon	5.8	1.7	7.8	7.6	41.4	**64.3**	1.7	0.3	2.3	1.5	1.6	11.2	**17.1**	0.1	0.4	0.5	1.7	0.1	0.5	2.6	11.5	**16.4**
Couxo	3.6	0.5	6.4	3.6	34.1	**48.2**	1.1	0.2	1.8	0.5	4.2	11.3	**18.6**	0.1	0.6	1.1	4.3	0.0	1.3	2.2	22.9	**30.7**
Domingos Pérez	4.2	1.9	5.7	13.5	45.0	**70.3**	0.3	0.5	0.9	0.5	0.5	5.4	**7.6**	0.2	0.6	0.5	1.3	0.1	1.0	4.2	13.8	**20.4**
Espadeiro	9.4	2.6	9.6	6.2	31.5	**59.3**	2.6	0.5	2.7	2.4	2.7	9.1	**17.6**	0.1	1.0	0.5	3.6	0.1	0.3	2.1	13.2	**19.3**
Ferrol	11.6	4.3	12.8	5.2	30.1	**64.0**	4.0	1.2	4.5	2.4	1.5	10.5	**21.7**	0.0	1.1	0.1	2.1	0.1	0.2	1.5	7.0	**10.9**
Folla Redonda	19.8	9.3	25.2	6.6	23.9	**84.8**	1.5	1.9	1.4	1.5	0.5	1.0	**6.3**	0.0	0.0	1.7	3.3	0.0	0.6	0.1	1.1	**5.1**
Follajeiro	4.7	0.8	6.5	4.4	39.7	**56.1**	1.5	0.2	2.2	1.5	1.6	13.9	**19.4**	0.1	0.3	0.6	2.1	0.0	0.9	2.2	16.6	**21.8**
Garnacha Tintorera	2.2	0.6	4.1	11.9	43.6	**62.4**	0.1	0.0	0.2	1.5	0.3	2.3	**2.9**	0.0	0.4	0.1	1.9	0.1	0.4	6.2	24.1	**32.7**
Gran Negro	1.5	0.5	2.6	20.9	45.5	**71.0**	0.0	0.0	0.0	0.6	1.2	1.4	**2.6**	0.2	0.3	0.6	0.5	0.1	1.1	5.3	17.9	**24.9**
Loureira	15.1	3.5	14.1	4.7	44.9	**82.3**	1.0	0.1	1.0	2.0	0.5	2.8	**5.4**	0.0	0.3	0.1	1.8	0.1	0.1	0.6	7.4	**10.0**
Mencia	6.4	1.4	7.9	9.7	42.8	**68.2**	1.1	0.2	1.6	1.0	2.5	9.5	**14.9**	0.1	0.3	0.3	1.5	0.0	0.3	3.3	10.2	**15.3**
Mencia Asturiana	5.3	1.6	7.2	12.2	39.3	**65.6**	1.1	0.6	2.0	2.0	3.0	9.8	**16.5**	0.1	0.5	0.3	1.5	0.0	0.5	3.7	9.3	**15.0**
Mencia Pata Perdiz	8.2	2.5	10.5	15.7	45.2	**82.1**	0.3	0.1	0.5	1.5	0.3	2.2	**3.4**	0.1	0.5	0.3	1.4	0.1	0.3	2.9	7.7	**12.4**
Merenzao	3.2	1.2	4.6	7.0	47.0	**63.0**	0.3	0.0	0.4	1.0	0.6	7.2	**8.5**	0.1	0.6	0.6	1.6	0.0	1.6	3.1	19.8	**26.1**
Moscatel Bago Miudo	2.6	0.7	5.0	6.4	50.7	**65.4**	0.2	0.0	0.5	0.9	1.0	6.8	**8.5**	0.3	0.2	0.4	1.5	0.1	1.1	2.7	19.0	**24.4**
Moscatel Negro	4.3	7.4	5.9	42.4	34.6	**94.6**	0.0	0.0	0.0	0.2	0.2	0.4	**0.6**	0.0	0.1	0.2	0.2	0.1	0.2	2.1	1.8	**4.4**
Mouraton	4.3	1.3	6.9	9.8	40.5	**62.8**	0.3	0.1	0.4	1.5	1.0	2.9	**4.7**	0.3	0.6	0.9	2.7	0.1	0.3	4.0	22.4	**29.5**
Negreda	4.9	1.0	7.2	7.3	42.1	**62.5**	0.3	0.0	0.4	2.0	0.7	2.8	**4.2**	0.2	0.6	0.9	2.9	0.1	0.5	3.9	22.4	**29.8**
Negrona	6.6	2.3	9.4	19.2	42.9	**80.4**	0.4	0.0	0.4	1.0	0.8	2.2	**3.8**	0.2	0.5	0.3	1.5	0.1	0.4	4.0	7.9	**13.9**
Pecho	2.8	1.5	4.9	11.4	42.2	**62.8**	0.2	0.1	0.5	0.9	0.9	5.3	**7.0**	0.4	0.6	1.0	1.8	0.0	1.4	5.4	18.8	**27.4**
Prieto Picudo	10.5	4.6	10.8	11.6	34.4	**71.9**	0.9	0.4	1.3	3.0	0.7	4.3	**7.6**	0.1	1.2	0.2	2.8	0.0	0.5	2.7	10.1	**16.1**
Retinto	8.4	2.0	10.7	8.6	52.2	**81.9**	0.2	0.0	0.4	1.0	0.4	2.0	**3.0**	0.0	0.2	0.2	1.3	0.0	0.4	1.7	10.4	**13.8**
Serradelo	2.5	9.4	3.5	50.0	20.9	**86.3**	0.0	0.4	0.0	1.0	1.2	1.1	**2.7**	0.0	0.0	1.0	0.6	0.0	0.2	5.7	2.6	**9.1**
Souson	17.3	2.0	15.3	3.4	45.3	**83.3**	0.7	0.0	0.7	2.0	0.3	2.7	**4.4**	0.0	0.2	0.2	1.8	0.1	0.3	0.6	7.4	**10.2**
Tinta da Zorra	3.6	1.5	4.3	22.1	44.7	**76.2**	0.0	0.0	0.0	0.7	0.4	1.3	**1.7**	0.2	0.4	0.2	0.8	0.1	0.5	7.0	12.3	**20.7**
Tinta Femia	6.2	1.2	8.7	7.5	57.2	**80.8**	0.2	0.0	0.3	1.0	0.4	2.1	**3.0**	0.2	0.2	0.6	1.5	0.1	0.7	1.9	10.2	**14.4**
Tinta Pais	27.8	7.8	17.5	7.6	30.2	**90.9**	0.8	0.2	0.7	1.0	0.4	1.4	**3.5**	0.0	0.4	0.1	0.9	0.1	0.1	0.6	2.5	**4.2**
Tintilla	18.2	2.9	15.5	4.6	44.8	**86.0**	0.9	0.0	0.7	1.5	0.5	2.5	**4.6**	0.0	0.3	0.1	1.4	0.1	0.1	0.6	5.4	**7.6**
Verceiruda	5.1	2.3	6.6	10.4	39.8	**64.2**	1.1	0.2	1.7	1.5	1.7	10.6	**15.3**	0.1	0.5	0.5	1.5	0.1	0.8	3.6	12.0	**18.0**
Verdejo	2.2	0.3	3.6	6.4	43.6	**56.1**	0.3	0.1	0.4	0.9	0.9	8.0	**9.7**	0.2	0.5	1.0	1.8	0.1	2.1	4.4	23.4	**31.8**
Verdello Tinto	19.7	8.1	14.8	11.1	36.5	**90.2**	0.8	0.1	0.6	1.0	0.5	1.7	**3.7**	0.0	0.5	0.1	0.9	0.1	0.0	0.7	3.0	**4.7**
LSD	4.14	1.87	2.94	6.05	5.70		0.57	0.39	0.67	0.60	0.57	2.79		0.15	0.26	0.32	0.62	0.09	0.56	1.11	6.15	

Table 3
Weights of first five principal components

Peak	Principal component weights				
	I	II	III	IV	V
1-Dpg	0.314	0.020	0.208	−0.236	−0.248
2-Cyg	0.256	−0.022	0.404	0.180	0.073
3-Ptg	0.324	0.045	0.191	−0.287	0.241
4-Png	−0.003	−0.214	0.261	0.479	−0.319
5-Mvg	−0.033	−0.280	−0.379	−0.254	0.016
6-Dpac	0.198	0.371	−0.097	0.111	0.007
7-Cyac	0.183	0.276	0.115	0.161	0.232
8-Ptac	0.157	0.387	−0.167	0.144	−0.029
9-Ptcf	0.119	0.271	0.067	−0.308	−0.480
10-Pnac	−0.066	0.296	−0.163	0.352	0.073
11-Mvac	−0.031	0.315	−0.329	0.247	0.108
12-?	−0.331	0.113	0.235	−0.068	0.118
13-?	−0.107	0.291	0.209	−0.174	−0.393
14-?	−0.285	0.149	0.245	−0.013	0.353
15-Dppc	−0.050	0.322	0.003	−0.331	−0.123
16-Cypc	−0.266	0.111	0.331	−0.063	0.147
17-Ptpc	−0.321	0.062	−0.067	−0.067	0.297
18-Pnpc	−0.337	0.026	0.217	0.136	−0.226
19-Mvpc	−0.345	0.079	−0.184	−0.155	−0.003

The symbol '?' in the table is used for 'not identified' anthocyanins.

and it can be observed that the first principal factor depends mainly on the contribution of delphinidin, cyanidin and petunidin monoglucosides, and the *p*-coumarates. Fig. 6 shows the distribution of the 50 cultivars studied along the first two principal components (54.9% of total variability) and clearly confirm the dispersion of the samples studied. Stands out the cultivar "Bastardo Ruzo", which turns out to be clearly distanced from the rest, this result agrees with the showed ones in Table 2. However, not all the cultivars could be differentiated applying the two principal components plot, this is the case of the couples: "Mouratón"/"Negreda", "Alicante"/"Brancellao", and "Caiño Bravo"/"Retinto". At this respect, Table 4 shows the values of principal components

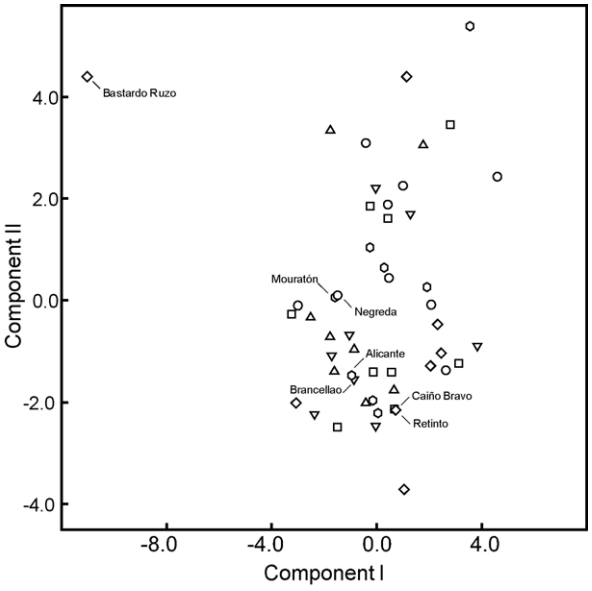

Fig. 6. Two-dimensional plot of the two first principal components.

Table 4
Principal components values for the 50 accessions studied

Variety	Principal component				
	I	II	III	IV	V
Albarello	2.632	−1.377	0.456	−1.081	0.566
Albarín Francés	2.795	3.459	0.826	−0.125	−1.603
Albarín Negro	0.654	−1.758	0.521	0.119	−0.296
Alicante	−0.869	−1.548	−0.304	0.093	−1.479
Bastardo Ruzo	−11.019	4.403	6.233	−0.932	1.246
Brancellao	−0.957	−1.479	1.156	1.846	−0.700
Cabernet	0.998	2.252	−2.420	3.030	1.501
Caiño Bravo	0.676	−2.136	−0.244	−0.549	0.819
Caiño do Freixo	1.765	3.052	−0.938	0.175	−0.229
Caiño Gordo	−0.038	−2.463	−0.628	−0.485	0.427
Caiño Longo	−3.071	−2.012	−2.514	−0.758	1.705
Caiño MBG	−0.153	−1.961	−0.870	−0.903	0.428
Caiño Redondo	−0.416	3.096	−0.994	−1.991	−1.836
Carnaz	−3.254	−0.265	−1.220	−0.465	1.081
Carrasco	−0.857	−0.956	−1.234	−1.286	0.497
Cascon	−0.042	2.214	−1.563	1.307	0.174
Castañal	2.046	−1.282	0.801	−0.539	0.676
Castellana	1.913	0.262	1.629	−2.169	−1.259
Catalán Negro	0.461	0.435	1.117	−2.254	−2.194
Corbillon	0.421	1.610	−1.348	0.915	0.155
Couxo	−1.767	3.349	−2.094	0.904	0.996
Domingos Pérez	−1.040	−0.676	−0.164	0.659	0.268
Espadeiro	1.134	4.400	−0.368	0.201	−0.864
Ferrol	3.548	5.399	−0.051	1.038	−0.342
Folla Redonda	4.585	2.430	3.707	−0.369	3.775
Follajeiro	−0.261	1.854	−2.159	0.668	0.405
Garnacha Tintorera	−1.614	−1.391	−0.647	−0.402	−1.581
Gran Negro	−2.370	−2.237	−0.375	1.250	−0.295
Loureira	2.315	−0.464	0.050	−1.412	0.155
Mencia	0.280	0.641	−1.462	1.409	0.185
Mencia Asturiana	0.418	1.885	−1.072	1.765	−0.449
Mencia Pata Perdiz	0.558	−1.410	0.491	−0.368	−0.597
Merenzao	−1.779	−0.717	−1.341	−0.373	0.111
Moscatel Bago Miudo	−1.714	−1.082	−1.643	−0.181	0.495
Moscatel Negro	1.040	−3.712	2.140	3.165	−0.623
Mouraton	−1.589	0.060	0.126	−0.768	−0.640
Negreda	−1.489	0.095	−0.093	−1.460	−0.888
Negrona	−0.141	−1.400	0.588	0.345	−0.605
Pecho	−2.518	−0.338	−0.110	0.156	0.093
Prieto Picudo	1.277	1.707	1.257	−1.056	−1.889
Retinto	0.718	−2.156	−0.803	−0.784	0.252
Serradelo	0.453	−2.212	3.622	4.818	−1.052
Souson	2.073	−0.857	−0.080	−1.967	0.490
Tinta da Zorra	−1.499	−2.487	0.437	0.989	−1.099
Tinta Femia	−0.416	−2.017	−0.871	−1.038	0.575
Tinta Pais	3.828	−0.886	2.479	−0.552	1.513
Tintilla	2.425	−1.029	0.171	−1.413	0.631
Verceiruda	−0.260	1.042	−0.855	1.100	−0.125
Verdejo	−3.004	−0.100	−1.203	−0.147	0.706
Verdello Tinto	3.121	−1.232	1.955	−0.095	0.718

for all the cultivars studied, and as we can see the first two principal components for the above-mentioned cases are very similar. The separation of those couples of cultivars was finally achieved with the help of principal component III. The use of the third principal component, increases the accumulated variance used up to 69.4%, and allows to differentiate the cultivars that with the exclusive use of the first two principal components seemed to be equal (Table 4).

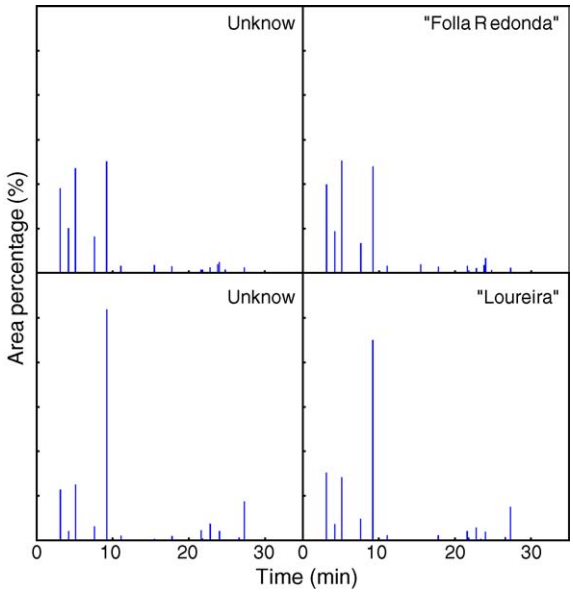

Fig. 7. Test for method validation.

Therefore, from the statistical analysis we may conclude that the amounts and distribution of different anthocyanins depend directly on cultivar. This fact allows to use the anthocyanin fingerprints to determine the assignation of unknown samples to a determinated cultivar.

In order to test the validity of this method, several unknown samples have been analysed to compare their anthocyanin profile with the fingerprint database created from all the 50 studied cultivars. In any case the unknown samples analysed were correctly assigned. Fig. 7 shows the relative-area graph for two of these unknown samples and the assigned cultivar using the fingerprint database.

4. Conclusions

From the results obtained, we can conclude that the chromatographic method proposed is suitable for routinary analysis of red grape cultivars identification, and therefore for to distinguish between them.

With this method it was possible to classify all the red grape accessions from the germplasm collection at the “Misión Biológica de Galicia”.

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