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Analysis of several phenolic compounds with potential antioxidant properties in grape extracts and wines by high-performance liquid chromatography–photodiode array detection without sample preparation

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Abstract

A RP-HPLC method that allows the separation of several types of phenolic compounds present in grapes and wines by direct injection of samples, using a binary gradient with solvents free of salts and photodiode array detection is described. Results show that more than 15 different phenolic molecules with antioxidant properties (flavan-3-ols, anthocyanins, cinnamic acid derivatives, flavonol derivatives and *trans*-resveratrol) may be separated in a single run by direct injection of red wine. The method is also valuable for the analysis of these compounds in white wine and in skins, seeds and pulp extracts of red and white grapes. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Wine; Grapes; Food analysis; Phenolic compounds; Antioxidants

1. Introduction

Grapes contain a large amount of different phenolic compounds in skins, pulp and seeds, that are partially extracted during winemaking [1]. Those molecules play an important role in some sensory properties of grapes and wines, such as astringency and color [2,3]. Furthermore, several studies have pointed out that many of them may show biological properties of interest, related to their antioxidant properties [4,5].

Red wines may have very complex phenolic composition that changes over their shelf-life [1]. The occurrence of these substances in wines is not only a consequence of their extraction from grapes

during winemaking. Once grapes are crushed before the beginning of alcoholic fermentation, several condensation reactions which involve some of those molecules (especially anthocyanins, catechins and procyanidins) take place, resulting in the formation of new polymeric pigments [6,7]. The complexity of wine phenolic composition led to the development of several methods with a view to obtaining fractions prior to high-performance liquid chromatography (HPLC) analysis; these fractions contain some of the different groups of phenolic compounds with specific chemical properties. Those procedures may involve the dealcoholization of wine, changes in its pH, extraction with non-polar organic solvents, or percolation through Polyclar AT columns or octadecylsilane cartridges [8–11]. Unfortunately, all these procedures can modify the phenolic composition of wines, due to oxidation, hydrolysis of esters,

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ethers and glycosidic bonds, and even isomerization [12].

Currently, the anthocyanins are the only group of phenolic compounds present in grapes and wines which is analyzed by direct injection of filtered samples in the chromatographic column, because these pigments absorb visible light in the range of 510–535 nm, as other phenolic compounds are colorless at these wavelengths [13]. Nevertheless, some attempts have been made to analyze any type of phenolic compound present in wine by direct injection of filtered samples in an HPLC system with a photodiode array detector [12,14–16]. These methods require complex solvent delivery systems, as they use ternary gradients, and sometimes solvents may contain salts [15]. Thus, we have tried to develop a simple method able to determine up to 15 different phenolic compounds simultaneously in grapes and wines, using a binary gradient and photodiode array detection.

2. Experimental

2.1. Reagents and standards

Acetonitrile of HPLC-grade was obtained from Merck (Darmstadt, Germany). Perchloric acid of analytical-reagent grade was obtained from Scharlau (Barcelona, Spain). All other chemicals (analytical-reagent grade) were obtained from Panreac (Mollet del Valles, Spain). Water was purified in a Milli-Q water purification system (Millipore, Bedford, MA, USA). Standards of gallic acid, (+)-catechin, (–)-epicatechin, (–)-epicatechin gallate, rutin and *trans*-resveratrol were purchased from Sigma (Alcobendas, Spain). Other standards were prepared by column chromatography and/or semi-preparative HPLC following procedures described in the literature: *trans*-caffeoyltartaric acid and *trans*-*p*-coumaroyltartaric acid from grape pulp [17], anthocyanins from grape skins [13], procyanidins B1 and B2 from grape seeds [18], and quercetin-3-*O*-glucuronoside from grapevine leaves [19]. The identity of *trans*-caffeoyttartaric acid, *trans*-coumaroyltartaric acid and quercetin-3-*O*-glucuronoside was established through hydrolysis followed by HPLC and thin-layer chroma-

tography (TLC) [17,19]. The structures of anthocyanins were elucidated by fast atom bombardment mass spectrometry (FAB-MS) and ¹H nuclear magnetic resonance (NMR) [13]. The identity of procyanidins was elucidated by complete acid hydrolysis, partial-acid catalysed degradation with phloroglucinol and phenylmethanethiol, FAB-MS and ¹H NMR [18].

2.2. Preparation of samples

Extraction of phenolic compounds from the different parts of grape cluster (skins, seeds and pulp) was carried out following the procedure described by Bourzeix et al. [8]. Grape extracts and wines were filtered through a 0.45- μ m nylon membrane (Cole Palmer, USA) prior to analysis. Grape samples (Cabernet Sauvignon, red, and Chelva, white) were collected at El Encin Ampelographic Collection, Alcala de Henares, Spain. Wines were kindly supplied by INCAVI, Vilafranca del Penedes, Spain.

2.3. Equipment

Analyses were performed in a liquid chromatograph with Waters (Milford, MA, USA) M510 and M501 pumps, a Waters 680 gradient controller, a Rheodyne 7725 injection valve furnished with a 20- μ l loop, a Waters 996 photodiode array detector and a Millennium workstation ver. 2.15.01. Separation was carried out using a Waters Nova-Pak C₁₈ steel cartridge, 250 mm \times 3.9 mm, filled with 5 μ m particles, using a Waters Sentry Nova-Pak C₁₈ guard cartridge, 20 mm \times 3.9 mm, both thermostated in a water bath at 40°C.

2.4. Chromatographic conditions

The mobile phase was a linear gradient of water–acetonitrile (50:50) adjusted to pH 1.8 with perchloric acid (solvent B) in water–acetonitrile (95:5) adjusted to pH 1.8 with perchloric acid (solvent A), at a flow-rate of 0.6 ml/min, as shown in Table 1. Spectra were recorded each second between 250 and 600 nm, with a bandwidth of 1.2 nm.

Table 1
Linear gradient used for the separation of phenolic compounds present in grapes and wines

Time (min)	Solvent A (%)	Solvent B (%)
0	95	5
10	90	10
35	80	20
45	70	30
65	60	40
85	55	45
100	0	100
110	0	100

3. Results and discussion

Table 2 gives retention times and maximum absorbance wavelengths for the different standards. The compound numbers in this table correspond to their respective peaks in the chromatograms. Fig. 1 shows the chromatograms recorded at 280, 350 and 520 nm corresponding to a sample of red wine made with Tempranillo grapes. As can be noted, the three chromatographic profiles are quite different, and their complexity decreases as wavelength increases. At 520 nm, six anthocyanins were detected on the basis of their retention times and UV–visible spectra, compared with those of standards. At 350 nm, several other peaks appeared in the chromatograms. Some of these peaks, whose retention times were

over 40 min, were more intense at 350 nm than at 320 nm, probably meaning that they are flavonol derivatives. The UV–visible spectra of these peaks are quite close to those of flavonol glycosides available as standards and to the spectra of other flavonol glycosides previously reported [20]. One of them has been assigned to quercetin-3-*O*-glucuronoside on the basis of its retention time and visible–UV spectrum, and the others have been named FL1 to FL3. On the other hand, several other peaks with retention times below 40 min are more intense at 320 nm than at 350 nm. Two of them have been identified as *trans*-caffeoyltartaric acid and *trans-p*-coumaroyltartaric acid on the basis of their retention times and UV spectra. A third peak, which elutes just before *trans-p*-coumaroyltartaric acid, has been tentatively assigned to *trans*-feruloyltartaric acid, because its UV spectrum was quite close to that of ferulic acid. The chromatogram registered at 280 nm was much more complex, as other phenolic compounds, notably catechins, proanthocyanidins and polymeric molecules derived from flavan-3-ols, only absorb UV light significantly at wavelengths below 310 nm. The elution of oligomeric proanthocyanidins and polymeric molecules derived from flavan-3-ols causes a relatively intense baseline drift, especially after 40 min of elution, as it has been previously reported [21]. Of course, peaks corresponding to anthocyanins appeared in the chromato-

Table 2
Index number, retention time and maximum absorption of standards

Compound	Number	Retention time (min)	Maximum absorption (nm)
Gallic acid	1	7.5–8.0	270.9
Procyanidin B1	2	19.0–20.0	278.0
<i>trans</i> -Caffeoyltartaric acid	3	20.0–21.0	329.0
(+)-Catechin	4	23.0–24.0	278.0
<i>trans</i> -Coumaroyltartaric acid	5	27.5–28.5	312.4
Procyanidin B2	6	30.0–31.0	278.0
(–)-Epicatechin	7	35.5–36.5	278.0
Delphinidin-3- <i>O</i> -glucoside	8	38.0–39.0	521.3
Cyanidin-3- <i>O</i> -glucoside	9	44.0–45.0	521.3
Petunidin-3- <i>O</i> -glucoside	10	47.0–48.0	523.7
Peonidin-3- <i>O</i> -glucoside	11	51.0–52.0	516.4
Malvidin-3- <i>O</i> -glucoside	12	52.5–53.5	527.3
Rutin	13	54.0–55.0	352.8
Quercetin-3- <i>O</i> -glucuronoside	14	55.5–56.5	354.0
Malvidin-3- <i>O</i> -glucoside-acetate	15	69.0–70.0	527.3
<i>trans</i> -Resveratrol	16	70.5–71.5	305.3
Malvidin-3- <i>O</i> -glucoside-(<i>p</i> -coumarate)	17	88.0–89.0	532.2

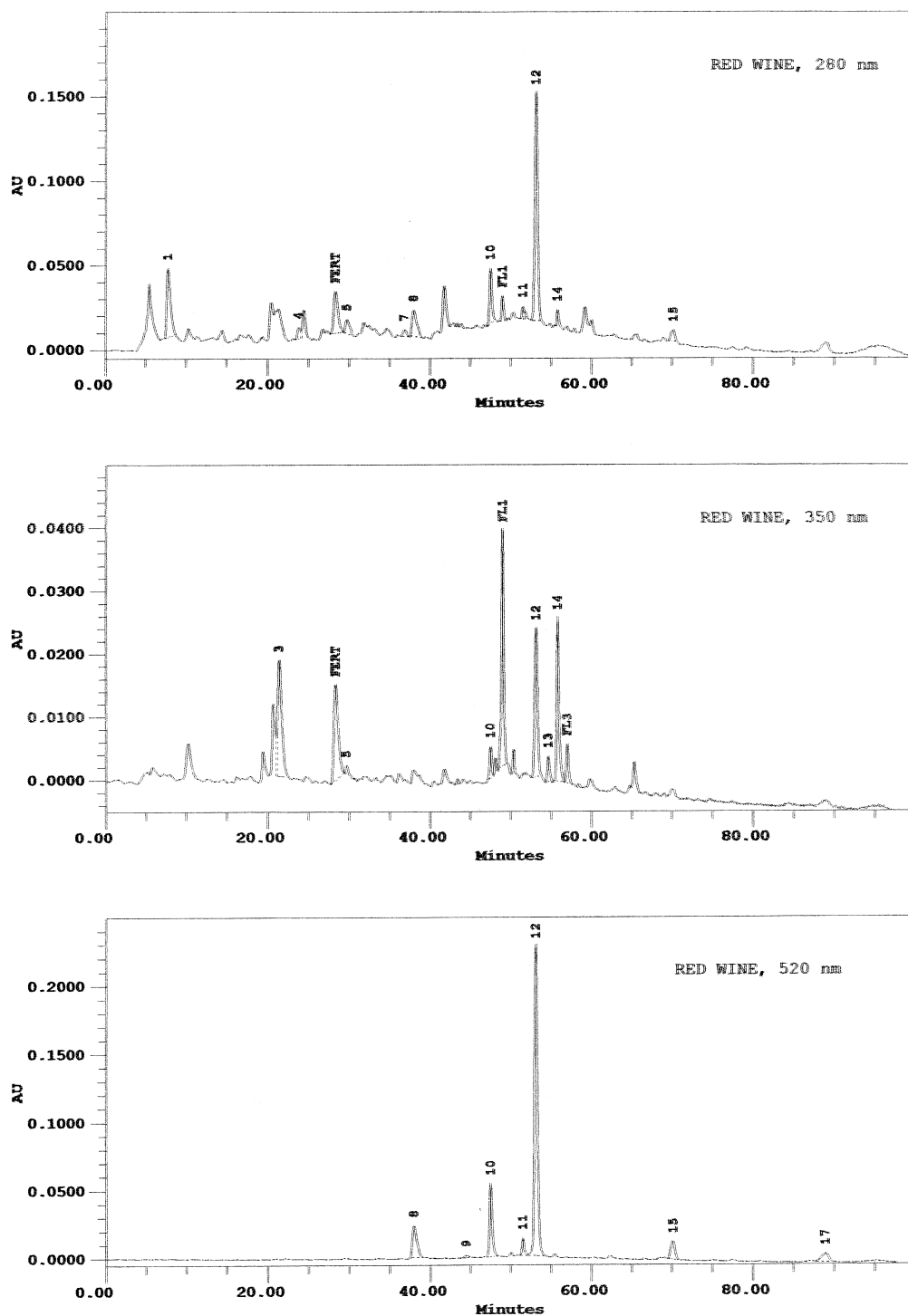


Fig. 1. Chromatograms registered at 280, 350 and 520 nm for a red wine.

grams registered at 280 and 350 nm, and peaks corresponding to flavonol and cinnamic acid derivatives in the chromatogram registered at 280 nm.

Fig. 2 shows the chromatograms registered at 280 and 320 nm for a white wine. Chromatograms are more simple than those obtained for red wine. At 320 nm, only three major peaks appear in the chromatogram. Other minor peaks appeared at 280 nm, whose UV spectra are quite close to those shown by catechins and dimeric procyanidins. However, as the amount of catechins, proanthocyanidins and their derivatives with tannic character is quite low in white wines [1], the chromatogram registered at 280

nm does not show a baseline drift as intense as that observed in red wine.

The chromatograms registered at 280 and 320 nm corresponding to extracts of grape skins, seeds and pulp present a large peak at about 8 min, which corresponds to a complex mixture of polar substances extracted from grapes and also to acetone used for preparing grape extracts. For a better comprehension of those chromatograms, the region between 0 and 10 min was not shown.

The chromatograms registered at 280 nm between 10 and 70 min for an extract of grape pulp and for an extract of white grape skins are displayed in Fig. 3.

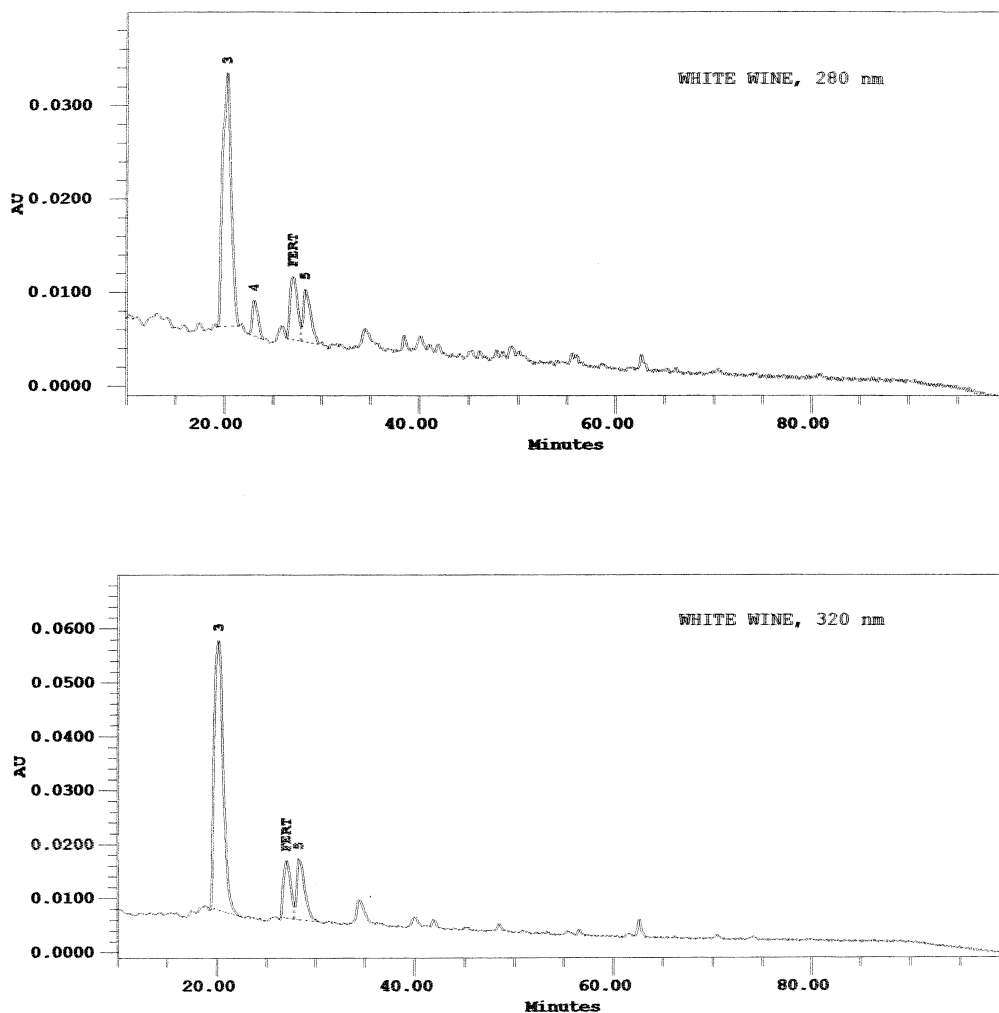


Fig. 2. Chromatograms registered at 280 and 320 nm for a white wine.

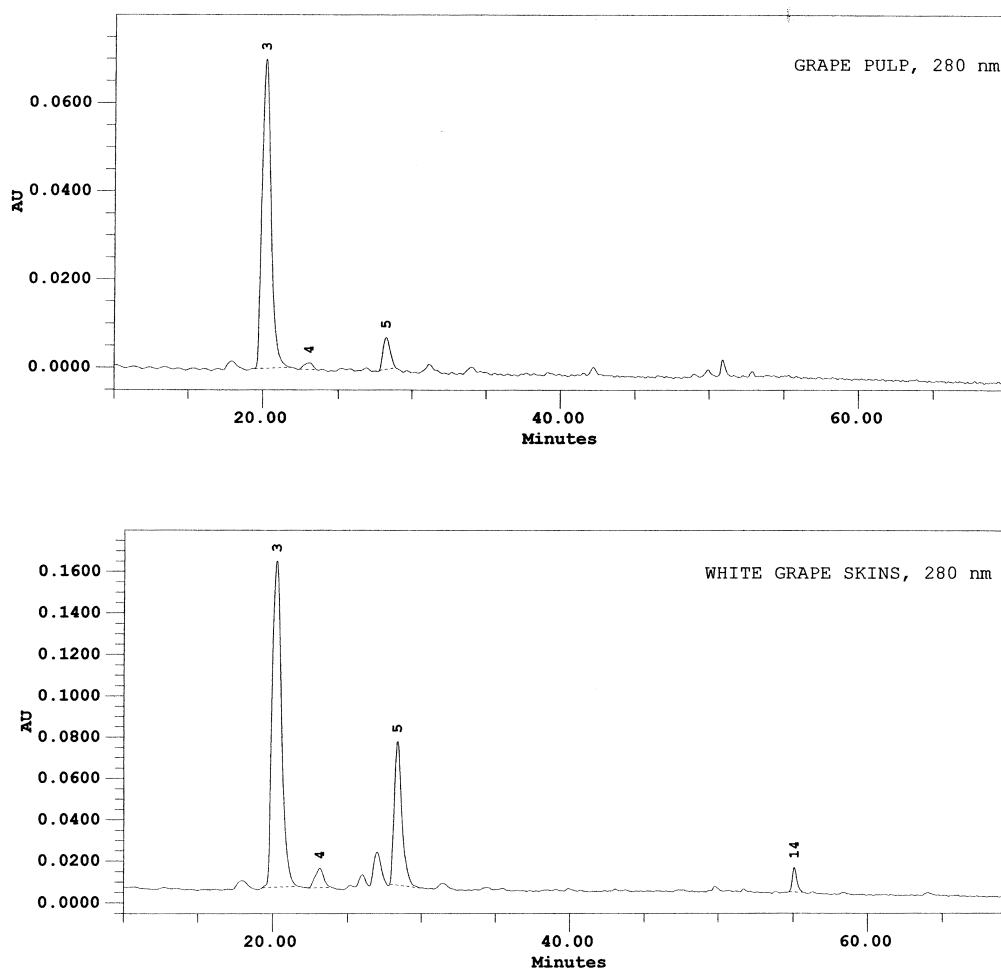


Fig. 3. Chromatograms registered at 280 nm for extracts of grape pulp and white grape skins.

Major components in both samples were cinnamic acid derivatives, and levels of (+)-catechin were quite low, as reported in the literature [8]. In addition, white grape skins contained a small amount of quercetin-3-*O*-glucuronoside.

Fig. 4 shows the chromatograms registered at 280, 350 and 520 nm between 10 and 100 min for an extract of red grape (Cabernet Sauvignon) skins. The chromatogram recorded at 520 nm was quite similar to that obtained for red wine at the same wavelength. However, the peaks corresponding to the acylated derivatives of malvidin-3-*O*-glucosides were more intense than in red wine. Chromatograms at 350 and 280 nm were more complex than at 520 nm, because other molecules, especially cinnamic acid derivatives

and flavonol glycosides, can present an intense response at those wavelengths. Like in the case of red wine, several unidentified flavonol derivatives have been named FL1 to FL3. However, the amount of catechins and oligomeric procyanidins in red grape skins was too low, and only a very small concentration of (+)-catechin was detected. As a consequence, the chromatograms from red grape extract do not show a baseline drift as intense as that observed in red wine at retention times over 40 min.

The chromatogram recorded at 280 nm between 10 and 60 min from an extract of grape seeds is displayed in Fig. 5. All the peaks shown in the chromatogram correspond to catechins and oligomeric procyanidins. Four of them have been assigned

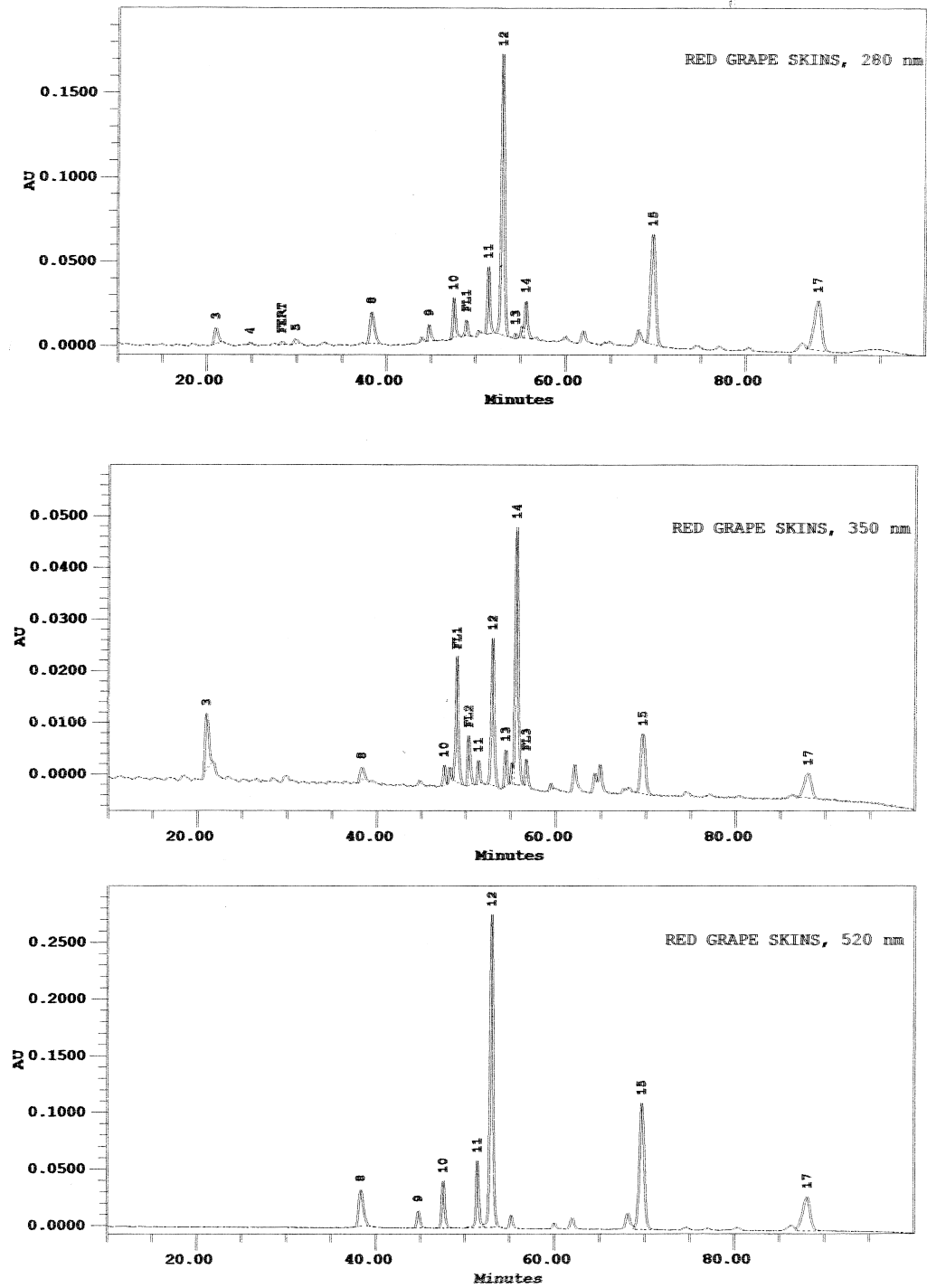


Fig. 4. Chromatograms registered at 280, 350 and 520 nm for an extract of red grape skins.

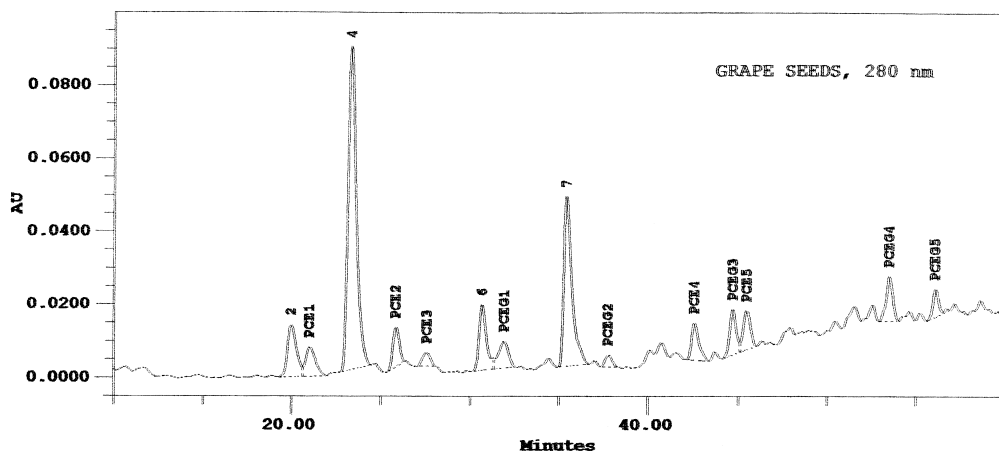


Fig. 5. Chromatogram registered at 280 nm for an extract of grape seeds.

to (+)-catechin, (–)-epicatechin, procyanidin B1 and procyanidin B2 on the basis of their retention times and UV spectra. Another four peaks, named as PCE1 to PC4 in the chromatogram, show similar UV absorbance spectra to those presented by (+)-catechin and (–)-epicatechin, and probably may be assigned to oligomeric procyanidins derived from (+)-catechin and/or (–)-epicatechin. Another five peaks, named as PCEG1 to PCEG5, presented UV spectra that were intermediate between those of catechins [(+)-catechin and (–)-epicatechin] and (–)-epicatechin gallate. These compounds may be assigned to oligomeric procyanidins that contain at least one (–)-epicatechin gallate moiety. These results are quite similar to those reported in the literature [21].

Recently, the biological activity of *trans*-resveratrol and their derivatives, that may be relatively abundant in grapes and wine, was reported [22–24]. For this reason, the detection of *trans*-resveratrol in the different samples has been tested. This substance was not detected in wines used in this study. Furthermore, the levels of *trans*-resveratrol in grape skins extracts were extremely low. This fact may be easily explained, because *trans*-resveratrol and other stilbenes are produced by grapes in response to mold infections and physiological stresses, and their levels in grapes and wines may be low if those phenomena do not appear.

The described procedure allows the separation of different types of phenolic compounds in grapes and wines by direct injection of samples. As the mobile

phase pH is too low, flavylum cation is the only chemical species of anthocyanins that can be detected [25]. For red wines, other chemical species of anthocyanins are present at wine pH, and also an important amount of free anthocyanins may be combined with sulfites. Thus, the results obtained with this chromatographic procedure should not be a real picture of the actual state of anthocyanins in red wines, and need to be complemented with fluorimetric, colorimetric or mass spectra analysis to give an accurate description of the different types of chemical species of anthocyanins present in wines, including those combined with sulfites [26].

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