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Determination of phenols in wines by liquid chromatography with photodiode array and fluorescence detection

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Abstract

A reversed-phase LC method, optimised for the separation of *trans*- and *cis*-resveratrol, catechin, epicatechin, quercetin and rutin, is reported. Analyses were performed on a reversed-phase column by gradient elution. Detection was carried out by photodiode array, although the use of a fluorimetric detector considerably lowered the detection limits for catechin, epicatechin and both resveratrol isomers. Identification by the two different detection systems was based on retention characteristics, UV spectra and peak purity index were compared with commercial standards. The procedures were applied to the determination of the phenolic compounds in different types of wines and musts.

Keywords: Wine; Food analysis; Phenols

1. Introduction

The chemical composition of a wine is influenced by the climatic and atmospheric conditions, soil type, vine cultivation and the treatment to which it is subjected. Phenolic compounds are responsible for colour and contribute to the bitter flavour of wines. They are also bactericidal agents and impart antioxidant properties [1], being especially found in the skin and seeds of the grapes. Only low levels occur in the must of white wine ($\leq$0.2 g/l), whereas red wines normally contain 1–2.5 g/l and even higher levels on occasions. Resveratrol (3,5,4′-trihydroxystilbene) is a compound found in many plants, including mulberries and grapes, and is thought to be effective in lowering serum lipids and inhibiting platelet aggregation [2]. Some recent findings suggest that resveratrol may also be anti-carcinogenic. Resveratrol exists in two isomeric forms with both the *trans*- and the *cis*-isomers being present in variable amounts in commercial wines. Other phenolic components of wine such as catechin, epicatechin, quercetin and its glucoside rutin also have antioxidant properties. Typical concentrations of epicatechin exceed 15 mg/l in white wine and 150 mg/l in red wine; quercetin shows a mean value of 25 mg/l in red wine and resveratrol concentrations are usually lower than 1 mg/l [3].

Atherosclerosis and coronary heart disease (CHD) are related to the excessive consumption of fatty foods, especially those containing saturated fatty acids and cholesterol. It has been suggested that the moderate intake of wine provides protection against CHD because the antioxidant properties of the phenolic compounds of wine delay the onset of atherogenesis and regulate thrombotic tendencies. The ‘‘French paradox’’ (apparent compatibility of a

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high fat diet with a low incidence of CHD) has been attributed to the regular consumption of red wine [4].

Some recent studies have determined polyphenolic compounds in wines principally using chromatographic techniques [5–10], with detection being carried out by spectrophotometry [7,8,10] or fluorimetry [9].

In the present study, separation of the phenolic components, catechin, epicatechin, rutin, quercetin and *trans*- and *cis*-resveratrol, was optimised using reversed-phase liquid chromatography (LC). Detection was performed using photodiode array or fluorescence detection because catechin, epicatechin and the resveratrol isomers are highly fluorescent. The fluorimetric detection procedure is more sensitive and specific than UV detection. The method permitted both the identification and determination of the phenols in different types of wines by direct injection, without any prior purification of the sample.

2. Experimental

2.1. Apparatus

The LC system consisted of a Kontron 325 liquid chromatograph operating at room temperature with a flow-rate of 1.0 ml/min. The spectrophotometric detector was a photodiode array Shimadzu SPD-M10Avp operating at wavelengths of 280 nm for catechin and epicatechin, 360 nm for rutin and quercetin and 300 nm for *trans*-resveratrol and *cis*-resveratrol. To increase the sensitivity of the procedure, a Kontron SFM 25 fluorescence detector set at 280/315 nm (excitation/emission) for catechin and epicatechin, 324/370 nm for *trans*-resveratrol and 260/370 nm for *cis*-resveratrol and a personal computer integration pack (Kontron) were also used. Aliquots of 50 μl were injected manually using a Model 7125-075 Rheodyne injection valve. The analytical column (Supelco) was 15×0.4 cm I.D., made of stainless steel and packed with Spherisorb ODS-2 with a particle size of 5 μm. A Supelco guard column packed with the same stationary phase was also used.

2.2. Reagents

Acetonitrile (Romil, Loughborough, UK) and methanol (Riedel-de Haën, Seelze, Germany) were of liquid chromatographic grade. Doubly distilled water and acetic acid (Merck, Darmstadt, Germany) were purified using a Milli-Q system (Millipore, Bedford, MA, USA). The solvents were degassed by purging with helium gas. Stock solutions (500 μg/ml) of catechin, epicatechin, rutin, quercetin and *trans*-resveratrol were prepared by dissolving 25 mg of the commercial product (Sigma, St. Louis, MO, USA), without previous purification, in 50 ml of methanol. They were kept in dark bottles at 4°C. Working standard solutions were prepared by dilution with acetonitrile–5% aqueous acetic acid (10:90) mobile phase just before use. Stock solution of *cis*-resveratrol was prepared by UV irradiation of *trans*-resveratrol for 20 min at 254 nm [10] and dilution with the mobile phase. Thus, the irradiated standard contained two peaks, the first corresponding to *trans*-resveratrol and the second, which was *cis*-resveratrol, representing an amount identical to the decrease in the *trans*-isomer. The concentrations of the *cis*-resveratrol standards were based on the decrease in *trans*-resveratrol, the sum of the isomers being equal to the initial concentration of *trans*-resveratrol.

2.3. Wine samples

A group of commercially available wines and musts were analysed. Samples were opened, protected against sunlight and stored at 4°C. Analyses were carried out within a few days. An aliquot was filtered through a 0.2-μm nylon Millipore chromatographic filter, diluted when necessary (depending on the analyte, its level in the wine and the type of detector used) and analysed.

2.4. Chromatographic procedure

Samples of 50 μl of standard or wine were directly injected into the column. Elution was carried out with a mobile phase delivered at 1 ml/min according to the following gradient: the initial mixture was acetonitrile–5% aqueous acetic acid (9:91) for 10 min; linear gradient to (25:75) in 1 min, hold for 11 min; linear gradient to (70:30) in 1 min, hold for 5 min. Finally, the initial conditions were re-established in 1 min and hold for 15 min. Detection was carried out by monitoring the absorbance signals

at 280, 300 and 360 nm or using a fluorescence detector set at 280/315 nm for catechin and epicatechin, 324/370 nm for *trans*-resveratrol and 260/370 nm for *cis*-resveratrol. At the end of each day, the column was washed using a mixture of acetonitrile–water (50:50).

3. Results and discussion

3.1. Optimisation of the phenols separations using photodiode array detection

Reversed-phase chromatography was selected because polyphenols are insoluble in water but soluble in alcohols. The stationary phase was ODS (C_{18}), which permitted greater retention. The organic solvent selected for preliminary experiments was methanol (MeOH) due to the high solubility of phenols in this solvent. Thus, several experiments were carried out to resolve the phenol mixture using different MeOH–water mobile phases with a flow-rate of 1 ml/min. Values of p*K* for the phenols are around 9 (i.e., 9.449 for catechin) and, consequently, pH control was necessary for the separation [11]. The retention behaviour of phenols was studied in the presence of an acid, which prevented ionization of the hydroxyl groups. An initial mobile phase containing methanol–5% aqueous acetic acid was selected and there was a tendency for retention to increase when the concentration of acetic acid increased and the percentage of methanol decreased. The use of methanol in the mobile phase led to problems of high pressure and, consequently, acetonitrile (MeCN) was tried. The acetic acid concentration was again optimised and similar results were obtained using this organic solvent.

In summary, the isocratic elution of phenols was not possible since an optimal mobile phase for separating catechin and epicatechin (9% MeCN) did not elute the rest of the compounds. Similarly, when a higher concentration of organic solvent was selected (20% MeCN), the resveratrol isomers were separated, while catechin and epicatechin eluted near the void time and rutin and quercetin were retained. These latter phenols could be eluted with a stronger solvent (30–40% MeCN), although the other compounds eluted together at the void time. Consequently, a gradient elution technique was tried in an attempt to achieve good peak resolution and a shorter total analysis time. The gradient was started using a low concentration of MeCN (9%), which allowed the separation of catechin and epicatechin from the void time. Then, several gradients with different ranges and profiles were tried. A two-step gradient was necessary.

The optimal gradient selected was the following. First, an initial isocratic step with acetonitrile–5% aqueous acetic acid (9:91) for 10 min followed by a linear gradient to (25:75) lasting 1 min. This mixture was held for 11 min before another linear gradient was performed to (70:30) during 1 min, this mixture being held for 5 min. Finally, the initial conditions were re-established in 1 min and held for 15 min. The flow-rate was 1 ml/min. The chromatographic profile obtained using this programme is shown in Fig. 1. The elution order and the retention characteristics were: 1, catechin (t_R=4.1 min; K'=2.42); 2, epicatechin (t_R=8.5 min; K'=6.08); 3, rutin (t_R=14.4 min; K'=11.0); 4, *trans*-resveratrol (t_R=17.2 min; K'=13.3); 5, *cis*-resveratrol (t_R=19.5 min; K'=15.25); 6, quercetin (t_R=25.0 min; K'=19.83).

3.2. Calibration and precision using photodiode array detection

Calibration graphs were performed by plotting concentration (μg/ml) against peak area. Table 1 shows the equations obtained for the calibration graphs and the regression coefficients. The precision of the method was demonstrated by repetitive analyses, calculating the average relative standard deviation (RSD) for 10 replicate determinations. The detection limits were calculated on the basis of 3σ and the quantitation limits on the basis of 10σ, using the regression lines for the standards according to Miller and Miller [12]. Values are also given in Table 1.

3.3. Calibration and precision using fluorescence detection

As previously indicated, some of the phenols (catechin, epicatechin and the resveratrol isomers) are highly fluorescent. Consequently, the sensitivity of the procedure could be improved using a fluorescence detector. In this case, the chromatographic conditions were very similar but the gradient pro-

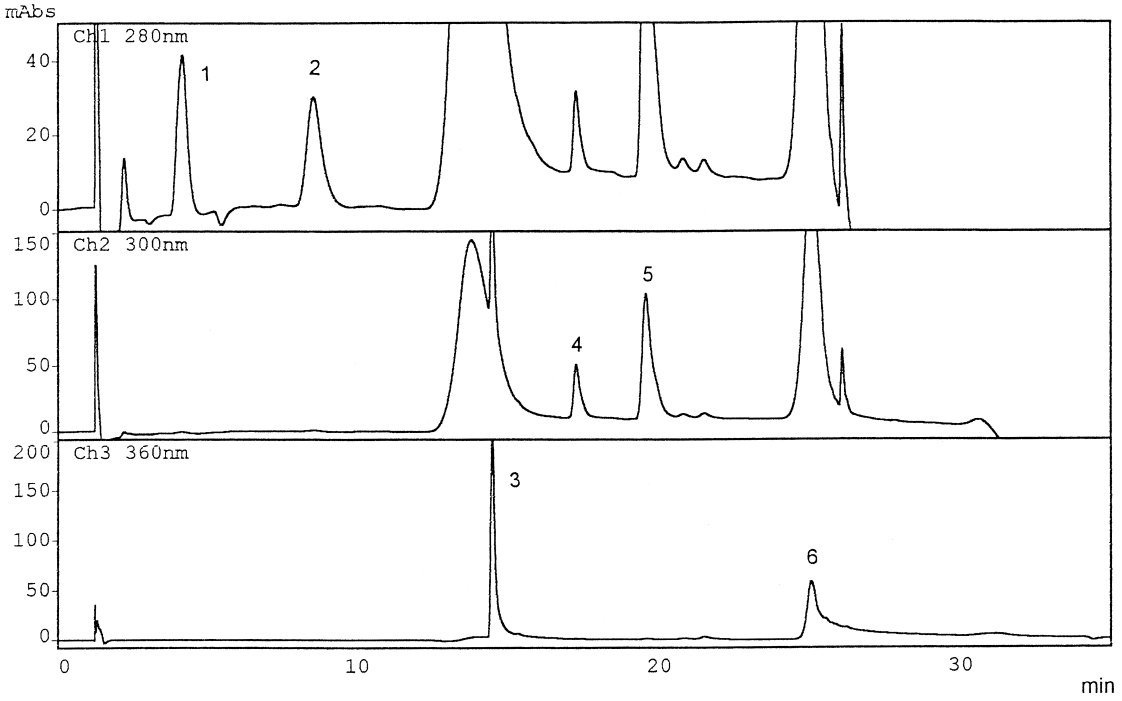

Fig. 1. Chromatographic profile using gradient elution. Flow-rate, 1 ml/min. The peaks correspond to: 1, catechin (25 μg/ml) and 2, epicatechin (25 μg/ml) measured at 280 nm; 3, rutin (25 μg/ml) measured at 360 nm; 4, *trans*-resveratrol (2 μg/ml) and 5, *cis*-resveratrol (18 μg/ml) measured at 300 nm and 6, quercetin (25 μg/ml) measured at 360 nm.

gramme was shorter because quercetin shows no fluorescence and the last step using a (70:30) mixture is not necessary. Thus, the optimal gradient when using the fluorimetric detector was: an initial isocratic step with an acetonitrile–5% aqueous acetic acid (9:91) mixture for 10 min followed by a linear gradient from (9:91) to (25:75) over 1 min, this mixture being held for 11 min. Finally, the initial conditions were re-established in 1 min and held for 15 min. The flow-rate was also 1 ml/min.Calibration graphs were performed by plotting concentration (μg/ml) against peak area. Table 2 shows the equations obtained for the fluorimetric calibration graphs and the regression coefficients, the RSDs for

Table 1
Calibration graphs using photodiode array detection

Compound	λ (nm)	Intercept	Slope	Correlation coefficient	Linearity interval	RSD (%)	Detection limit (μg/ml)	Determination limit (μg/ml)
Catechin	280	−1167	38 471	0.9998	1–25	4.7	0.20	0.67
Epicatechin	280	2313	36 556	0.9997	1–25	4.0	0.16	0.53
Rutin	360	−6628	84 271	0.9999	0.2–20	6.3	0.11	0.35
trans-Resveratrol	300	4142	513 122	0.9998	0.1–20	1.6	0.06	0.22
cis-Resveratrol	300	−9804	101 289	0.9999	0.5–20	8.6	0.21	0.72
Quercetin	360	169 851	117 868	0.9999	0.2–20	5.1	0.16	0.55

Table 2
Calibration graphs using fluorescence detection

Compound	$\lambda_{ex}/\lambda_{em}$ (nm)	Intercept	Slope	Correlation coefficient	Linearity range	RSD (%)	Detection limit (μg/ml)	Determination limit (μg/ml)
Catechin	280/315	0.272	74.9	0.9999	0.01–1	9.3	0.003	0.01
Epicatechin	280/315	−0.123	75.7	0.9999	0.03–1	6.6	0.005	0.02
trans-Resveratrol	324/370	0.430	93.3	0.9999	0.01–1	9.2	0.003	0.01
cis-Resveratrol	260/370	−0.072	161	0.9999	0.01–0.1	4.4	0.001	0.004

10 replicate determinations and the detection and quantitation limits calculated using the regression lines for the standards.

3.4. Analysis of wines and recovery study

Once the chromatographic conditions for the separation had been optimised, the procedure was applied to the determination of phenol components in wines, which required studying the possibility of a matrix effect. These studies were carried out using both photodiode array and fluorimetric detection and comparing the results obtained.

The chromatogram obtained for a red wine sample using the photodiode array detector is shown in Fig. 2. The peaks were identified by: (1) comparing the retention data obtained for the wine sample, the standards mixture and the wine spiked with the standards under identical conditions. (2) Using the photodiode array detector to continuously measure the UV–visible spectrum while the solute passed through the flow-cell and measuring the absorbance

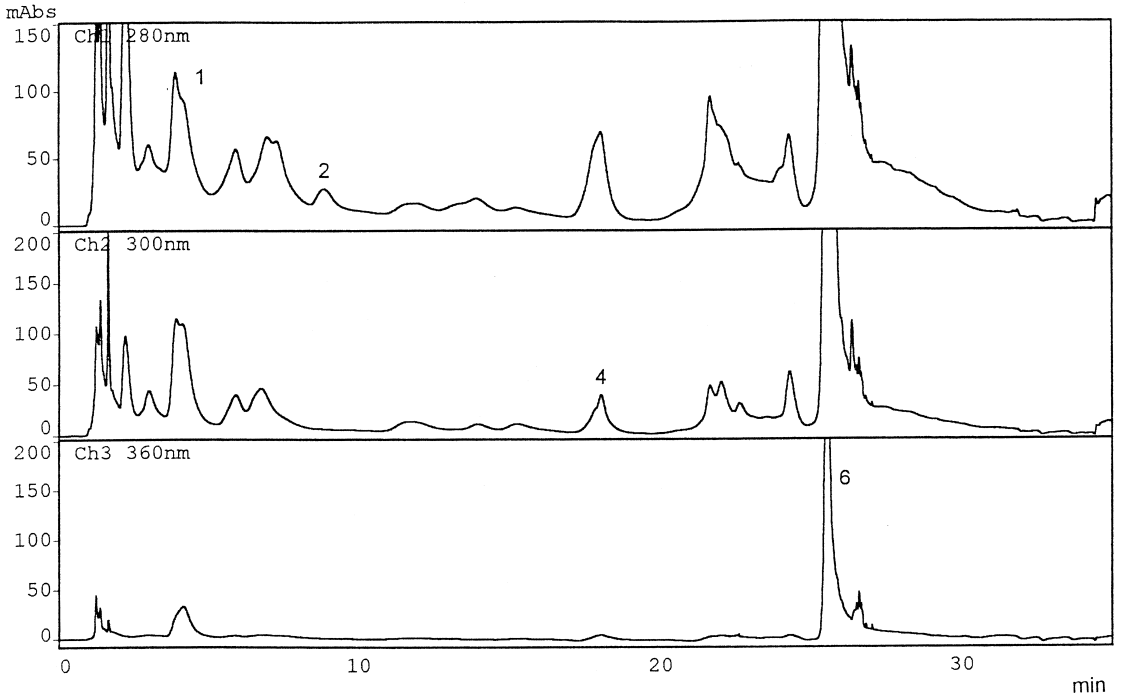

Fig. 2. Chromatogram for a red wine sample using gradient elution and photodiode array detection. Flow-rate, 1 ml/min. See Fig. 1 for identification of the peaks.

ratio at two wavelengths. (3) Comparing the information obtained using a fluorimetric detector.

When the absorption spectra of the different peaks obtained for the standards, the wine samples and the spiked samples of wine were compared, a good agreement was found. The following criteria were used to confirm the purity of the peaks: (a) peak purity curve, which is based on the similarity between the spectrum at the top of the target peak and the spectra at each point on that peak. If there is any part where this curve deflects toward the negative side, impurities are considered to be included at that

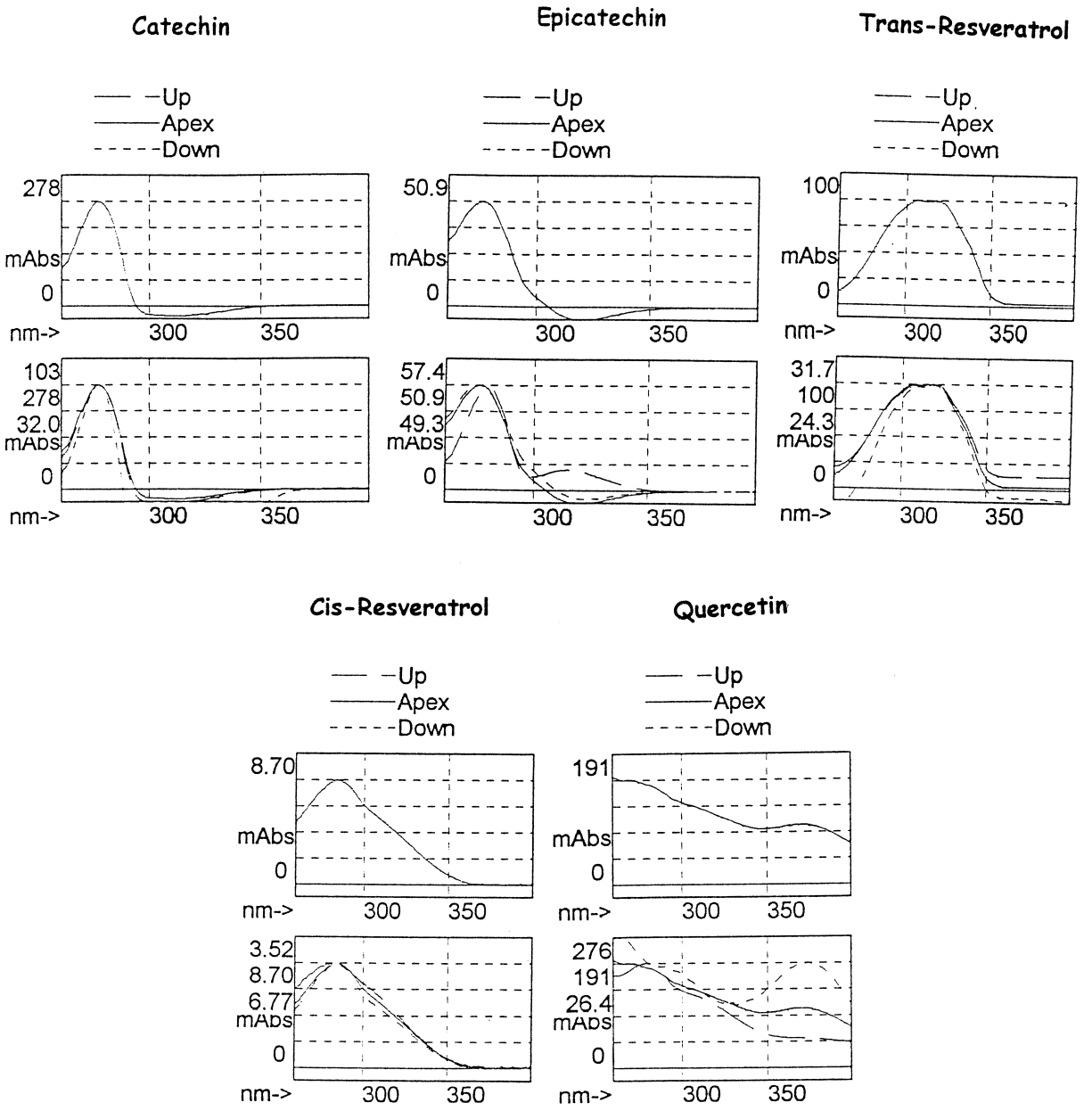

Fig. 3. Peak purity curves obtained for the peaks corresponding to the phenolic components present in a sample of red wine.

part. (b) The ratio between chromatograms obtained using two wavelengths, the purity of the target peak being displayed as a chromatogram ratio. The purity of a peak is considered greater as the shape of the chromatogram ratio becomes closer to that of a rectangle. Fig. 3 shows the peak purity curves

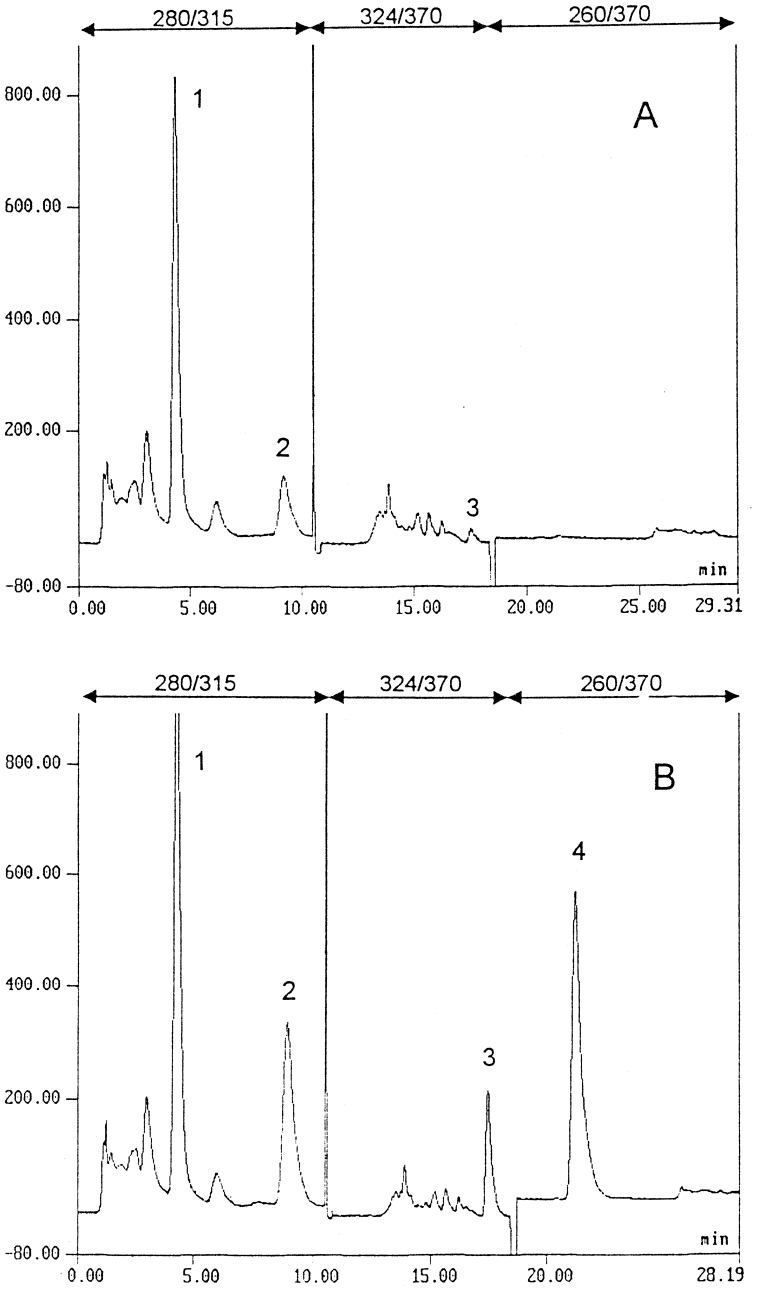

Fig. 4. Chromatographic profiles using fluorescence detection for a red wine sample (A) and a red wine sample spiked with the standards (B). The peaks correspond to: 1, catechin (1 μg/ml); 2, epicatechin (2 μg/ml); 3, *trans*-resveratrol (0.9 μg/ml) and 4, *cis*-resveratrol. The wavelength changes are shown at the top of the figure.

obtained for the peaks corresponding to the phenolic compounds present in wine. The results showed that no impurities coeluted with the analytical peaks and the small shoulders which appear in some peaks could be due to the ionization of the hydroxyl groups of the phenols.

Finally, the fluorimetric detector confirmed the identity of the compounds which show fluorescent properties by providing chromatographic profiles corresponding to a sample of wine and to the same sample spiked with the standards (Fig. 4). Again, no interfering peaks were observed in the wine samples investigated. As can be seen, this detection system showed a very higher sensitivity than that of the diode array detection system.

Absolute recoveries were evaluated by comparing the concentrations found in two wine samples (white and red wine) spiked with known amounts of each phenol. These were submitted to the chromatographic procedure and the concentrations were obtained using the calibration graphs. When all spike and recovery data were combined, an average recovery±SD (n=12) of 98.5±1.5% was obtained.

Once the phenolic compounds were identified and the absence of matrix effect had been proved, the quantification of phenols in different wines was carried out. Table 3 shows the results obtained using both photodiode array (column 1) and fluorescence (column 2) detectors. As can be seen, catechin and epicatechin were found in practically all the wine samples, the levels of the former being considerably higher. High levels of quercetin were also found in many samples, while rutin was only detected in the must sample. Resveratrol appeared in lower concentrations than the other phenols, the *trans*-isomer predominating. A statistical study to compare the results obtained using both detection systems was carried out. The paired t-test was used and the values obtained for t and the associated P values (95% confidence interval) were: for catechin, t=0.361 (P=0.726); epicatechin, t=−1.631 (P=0.137) and *trans*-resveratrol, t=−0.379 (P=0.730). The conclusion reached was that the change that occurred was not sufficiently great to exclude the possibility that the difference was due to chance.

The purity of the peaks was tested by using the peak purity curves and the averaged values for the purity index were: catechin, 0.987±0.0085 (n=11); epicatechin, 0.993±0.0084 (n=10); rutin, 0.990 (n=1); *trans*-resveratrol, 0.991±0.0103 (n=4); *cis*-re-

Table 3
Phenol content in different types of wines

	Catechin (μg/ml)		Epicatechin (μg/ml)		Rutin (μg/ml)		*trans*-Resveratrol (μg/ml)		*cis*-Resveratrol (μg/ml)		Quercetin (μg/ml)	
Red wine												
Cabernet-97	124.8	124.3	15.0	16.8	ND[a]	–	ND	0.25	ND	0.039	15.6	–
Syram-97	71.3	71.4	21.9	21.2	ND	–	2.35	2.36	ND	ND	43.1	–
Sabatacha-97	55.9	55.3	6.79	6.93	ND	–	ND	ND	ND	ND	ND	–
Merlot-97	108.2	108.3	24.4	24.8	ND	–	1.64	1.58	ND	ND	34.3	–
Tempranillo-97	65.8	65.6	13.6	13.6	ND	–	ND	ND	ND	ND	30.0	–
Rodrejo-97	52.0	53.7	10.8	11.4	ND	–	ND	ND	ND	ND	22.7	–
Gran Noval-97	30.5	31.6	2.18	2.74	ND	–	0.38	0.48	0.16	0.021	ND	–
Sabatacha-95	77.8	76.0	6.15	6.30	ND	–	ND	ND	ND	ND	ND	–
Jumilla-95	55.3	56.0	3.69	3.80	ND	–	0.50	0.50	ND	ND	12.6	–
White wine												
Jumilla-97	33.1	32.0	ND	0.99	ND	–	ND	0.06	ND	0.08	ND	–
Must												
Red	11.6	10.9	3.90	4.10	3.90	–	ND	0.16	ND	ND	0.71	–

[a] ND, Not detected.

sveratrol, 0.999 ($n=1$) and quercetin, 0.993±0.0084 ($n=7$). These average values indicate that the purity of the peaks was satisfactory.

4. Conclusion

A method for analysing six phenolic constituents of wine by LC in a single chromatogram is reported. The sample was directly injected into the column without a previous treatment and the analysis was carried out by gradient elution. A comparison of two detection modes using a photodiode array and a fluorescence detector was performed. Diode array detection allowed the identification of the peaks using the peak purity curve and the ratio of the chromatogram at two wavelengths. On the other hand, the use of the fluorimetric detection system led to a decrease in the detection limits, confirming the identity of the peaks. The procedures could be applied to the determination of the phenolic compounds in various types of wine.

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