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Journal of Chromatography A, 973 (2002) 221–227

JOURNAL OF CHROMATOGRAPHY A

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Short communication

Analysis of wine phenolics by high-performance liquid chromatography using a monolithic type column

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Received 9 November 2001; received in revised form 1 August 2002; accepted 1 August 2002

Abstract

A HPLC method involving direct injection was developed to determine phenolic compounds in wine. The separation was carried out on an RP C_{18} monolithic column using a binary gradient elution and diode array detection. In this way 17 monomeric compounds of different phenolic groups (hydroxybenzoic acids, hydroxycinnamic acids, hydroxycinnamyltartaric acids, flavanol, flavonol and stilbenes) could be separated and quantified in a single run and in a very short time.

Keywords: Wine; Monolithic columns; Phenolic compounds

1. Introduction

Wine polyphenols are regarded as a group of substances of extreme significance, contributing to wine colour, flavour, aroma and taste. Moreover, they can play a central role in human diet, acting as antioxidant and anti-mutagenic compounds [1,2]. Due to their physical–chemical characteristics, the analysis of wine monomeric phenolic compounds has been widely carried out with high-performance liquid chromatography (HPLC) equipped with reversed-phase (RP) columns, generally packed with spherical particles of silica bonded with octadecyl (C_{18}) chain. A suitable sample preparation step prior to HPLC injection may allow the extraction and concentration of many phenolic compounds of different nature, taking into account that run time, recovery and artifact formation must be optimised for polar and less-polar phenolic compounds. Several clean-up procedures reported in literature, generally based on liquid–liquid or solid-phase extraction [3–8], may not be considered totally effective and well-established for all the different classes of compounds [9]. On the other hand, when the wine sample is directly injected, a great column resolution and an appropriate gradient elution program are required to avoid interferences between the compounds of interest and the sample matrix [10–13]. Both clean-up procedures and long elution programs may result in an extended time of analysis, often inadequate to carry out a suitable wine quality control.

HPLC columns packed with monolithic supports, consisting of a single piece of porous material, have recently become the subject of extensive studies [14] because of their hydrodynamic advantages. Few separations with HPLC monolithic columns are

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0021-9673/02/$ – see front matter
PII: S0021-9673(02)01195-0

described in the literature in the field of food analysis, none of them dealing with wine phenolics [15–18].

The aim of this work was to develop a simple and rapid method to evaluate simultaneously several monomeric phenolic compounds in wines, using a commercial monolithic HPLC column and photodiode array detection.

2. Experimental

2.1. Wine samples

Five red wines (Sangiovese, Cabernet Sauvignon, Merlot) and nine white wines (Chardonnay, Albana, Sauvignon) were purchased in retail stores. Samples were diluted, if necessary, with eluent B, filtered through a 0.20 μm PTFE membrane filter (Millipore, Milan, Italy) and then injected.

2.2. Reagents and standards

Methanol and double-distilled water of HPLC grade were supplied by Merck (Darmstadt, Germany). *o*-Phosphoric acid was supplied by Carlo Erba (Milan, Italy). Standard solutions of gallic, protocatechuic, *p*-hydroxybenzoic, vanillic, caffeic, syringic, *p*-coumaric, ferulic acids and (+)-catechin, (−)-epicatechin, *trans*-resveratrol, myricetin, quercetin and rutin (Sigma–Aldrich, Milan, Italy) were prepared by dissolving known amounts of the analytical-reagent grade chemicals in ethanol–water (75:25, v/v). *cis*-Resveratrol was obtained after exposure to natural light of a solution of *trans*-resveratrol.

2.3. Instrumentation

Analyses were carried out with a HPLC Model 1100 system equipped with a diode array detector (Agilent Technologies, Palo Alto, CA, USA) and with Chemstation software (Hewlett-Packard, Waldbronn, Germany). LC separations were performed on a 100 mm×4.6 mm Chromolith Performance RP-18e column (Merck) controlled at 30±1 °C using an oven C-150 (Eldex Laboratories, Napa, CA, USA). The injection volume was 10 μl.

2.4. LC procedure

Peak identification was made comparing retention times and spectra (200–450 nm) with those of pure compounds and spiking the wine samples with standard solutions. Quantification was made using calibration curves obtained by injecting known amounts of pure compounds as external standards. The number of theoretical plates for a single compound was calculated according with the formula depending from the half-height width, $W_{1/2.}$ The instrumental limits of detection (LODs) were calculated, for every single compound, considering a signal-to-noise ratio (S/N) of 3. Baseline noise was measured considering a peak-to-peak measurement within 3 min selected in three different parts of the chromatogram of the each standard phenolic compound.

Recovery performances were calculated injecting a red and a white wine spiked with approx. 1, 2, 4 mg/l of each phenolic compound and calculating the percent ratio between the concentrations observed and those expected. Intra-day and inter-day repeatability were estimated both for the retention time and concentration of each compound analysing the same wine sample by the same operator, for three times a day and for 3 consecutive days. Peak symmetry was evaluated for each compound using the Chemstation software Peak tailing was calculated for each substance according to the UPS method [19].

3. Method development

In order to optimise the elution, different tests were done, modifying the conditions previously experimented for the separation of wine phenolic with a conventional RP-C_{18} column [12]. The finally selected elution conditions consisted of a 2.1 ml/min flow-rate and a multistage gradient (Table 1), which was fully completed within 36 min, including the washing and the re-equilibration time. The separation achieved with the standard mixture is reported in Fig. 1 and shows a good resolution between the different compounds. The maximum pressure drop was 12 MPa, much lower than those previously observed [12] using a conventional RP-C_{18} column

Table 1
Gradient elution conditions

Time (min)	A (%)*	B (%)**	Elution
0	100	0	Isocratic
10	100	0	Isocratic
15	82	18	Linear gradient
20	75	25	Linear gradient
22	65	35	Linear gradient
30	0	100	Linear gradient
34	0	100	Isocratic
35	100	0	Linear gradient
36	100	0	Isocratic

*A: Methanol–double-distilled water (2.5:97.5, v/v) at pH 3 with H_3PO_4.
**B Methanol–double-distilled water (50:50, v/v) at pH 3 with H_3PO_4.

(100–120 MPa) packed with ultra-pure spherical silica, as a consequence of the higher macroporosity of monolithic columns [20].

Figs. 2 and 3 show the chromatographic profiles of a white and a red wine samples. The chromatograms are quite acceptable in terms of peak resolution [R_s ranging from 1.5 for two close peaks like caffeic acid and (+)-catechin to R_s=6.6 for myricetin and *cis*-resveratrol] allowing a good chromatographic separation of all the compounds of interest. $W_{1/2}$ was calculated for all the chromatographic peaks ranging from 0.31 for vanillic acid to 0.07 for *trans*-resveratrol. The number of theoretical plates, N was calculated for some compounds like (+)-catechin, syringic acid, *p*-coumaric acid, (−)-epicatechin, ferulic acid with capacity factor $k'>6$ for solutions at 10 mg/l and quantified in the range 43 800–93 600. Moreover two very intense peaks in the chromatogram at 324 nm were tentatively identified as *trans*-caffeoyltartaric acid (A) and *trans*-*p*-coumaroyltartaric acid on the basis of their retention times and UV spectra [21] and quantified considering the response of caffeic and *p*-coumaric acids, respectively.

All the phenols showed lower retention times than those generally reported in the literature [3–5,10–

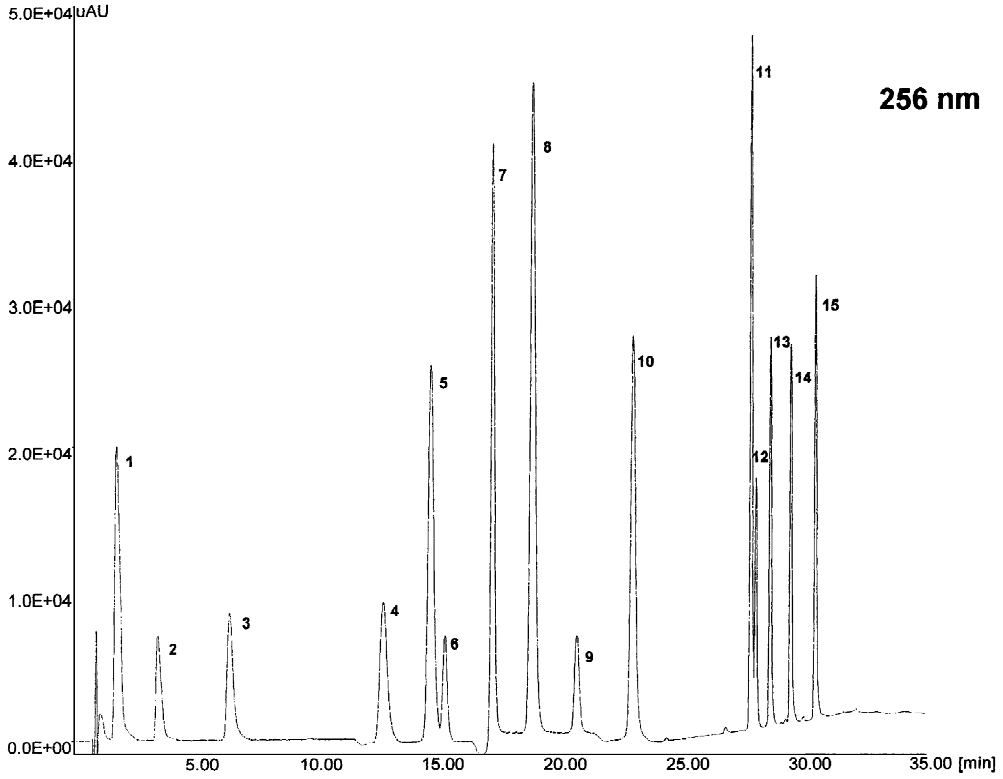

Fig. 1. Chromatogram of standard phenolic compounds (LC–DAD signal at 256 nm).

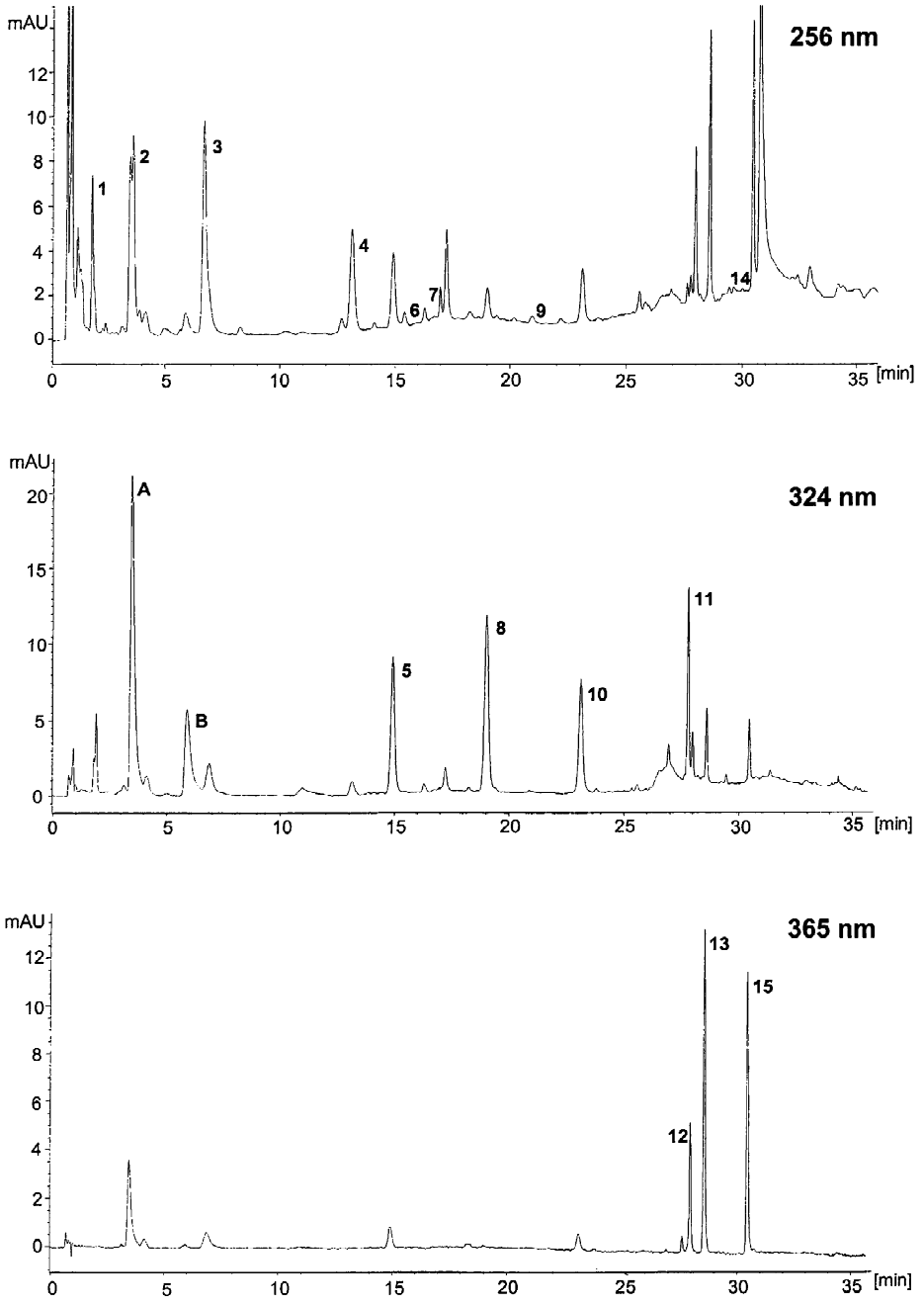

Fig. 2. Chromatograms of white wine sample (LC–DAD signals at three different wavelengths: 256, 324, 365 nm).

13,22–24]. Only Dominguez et al. [7] and Malovanà et al. [8] have recently developed a HPLC separation of 17 phenolic compounds within 35 min, even if a previous liquid–liquid extraction of the sample is required. Anyway, in a conventional RP column more time is required between two injections to wash

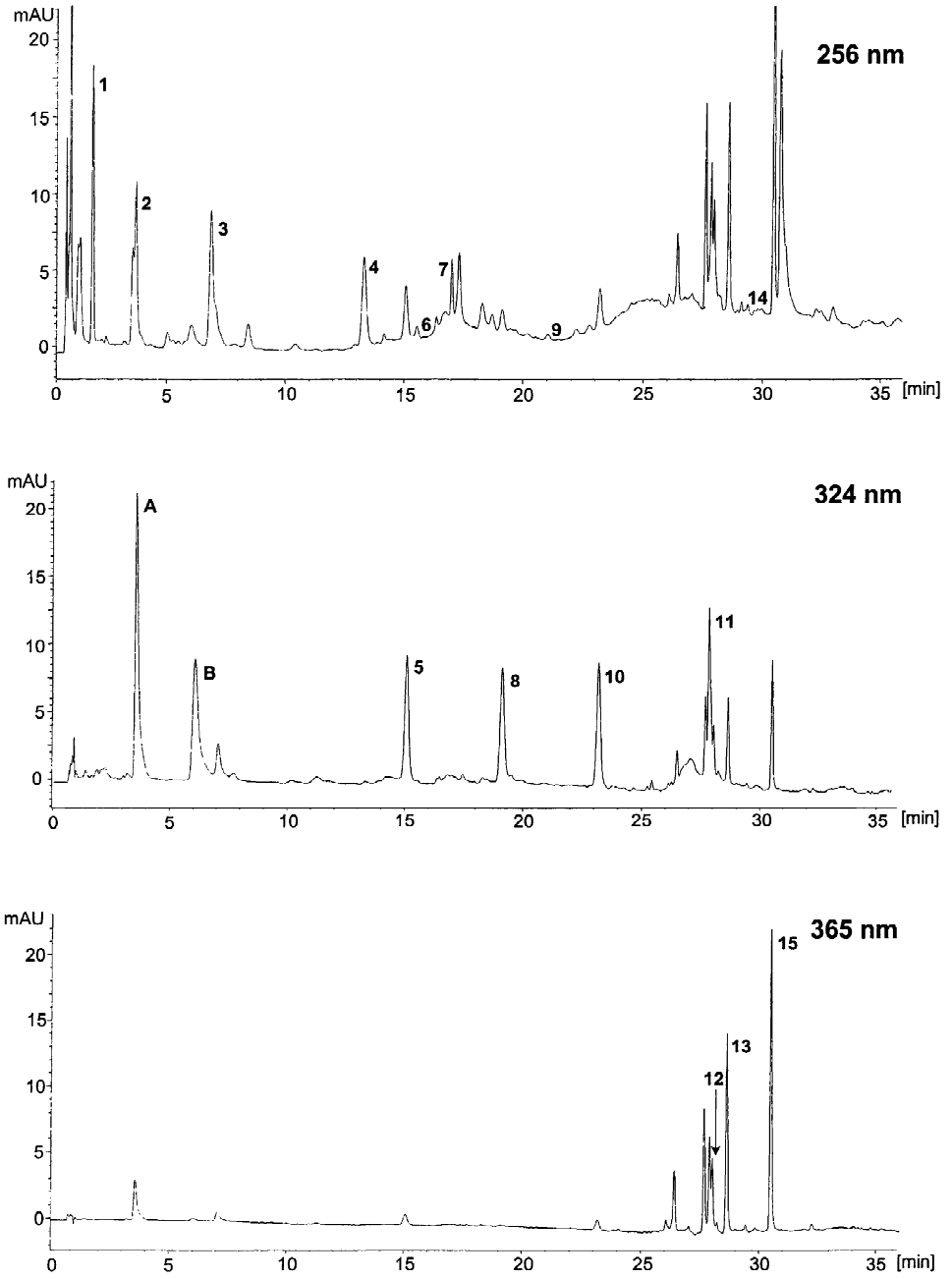

Fig. 3. Chromatograms of red wine sample (LC–DAD signals at three different wavelengths: 256, 324, 365 nm).

and re-equilibrate the stationary phase. The rigidity of the monolithic column structure allows rapid washing and a virtually instantaneous re-equilibration. Consequently, a consistent reduction of the total time of analysis (25–50%) could be obtained referring to the better-published separations on conventional RP columns with direct sample injection [9,13,26].

Table 2
Analytical data

	Compound	λ[a] (nm)	k'	Calibration curve parameters[b]			$LOD_{(S/N=3)}$ (μg/l)
				a (±SD)	b (±SD)	r^2	
1	Gallic acid	280	1.02	12.349±0.001	0.06±0.01	0.998	20
2	Protocatechuic acid	256	3.05	17.15±0.02	−0.10±0.06	0.999	15
3	*p*-Hydroxybenzoic acid	256	6.47	24.60±0.02	−0.03±0.01	0.998	15
4	Vanillic acid	256	13.7	16.14±0.01	−0.31±0.03	0.998	35
5	Caffeic acid	324	15.8	25.36±0.01	0.01±0.03	0.998	20
6	(+)-Catechin	280	16.4	3.574±0.004	−0.26±0.03	0.999	110
7	Syringic acid	280	18.5	13.54±0.03	1.30±0.03	0.999	25
8	*p*-Coumaric acid	308	20.4	36.57±0.01	−0.35±0.06	0.998	20
9	(−)-Epicatechin	280	22.6	3.331±0.002	−0.02±0.01	0.998	160
10	Ferulic acid	324	25.1	26.01±0.01	0.18±0.07	0.999	20
11	*trans*-Resveratrol	308	30.5	35.71±0.02	0.30±0.04	0.998	10
12	Rutin	365	30.7	7.40±0.01	0.12±0.03	0.999	55
13	Myricetin	365	31.4	19.72±0.01	−0.56±0.05	0.998	40
14	*cis*-Resveratrol	280	32.4	18.26±0.01	0.32±0.03	0.999	30
15	Quercetin	365	33.5	17.572±0.002	0.16±0.01	0.998	20
A	Caftaric acid	324	2.78##	/	/	/	/
B	Coutaric acid	308	11.03##	/	/	/	/

Average of nine readings on wine samples.
[a] Wavelength of detection.
[b] Regression equation $y=ax+b$; $n=7$ points in the range of concentrations indicated (typically 0.3–20 mg/l).

4. Analytical data

The calibration curves (Table 2) indicate a good linearity in the considered range of concentration.

The LODs are in the range from 8 μg/l for *trans*-resveratrol to 161 μg/l for (−)-epicatechin. These values are lower than those reported by other authors, working with conventional RP-C_{18} columns and diode array detectors [8,9,25], with the exception of Ho et al. [23], who declared values 20–30-times better than the others. Our results can probably be explained by the good separation on the monolithic column, which originated narrower and higher peaks than those observed on conventional columns, hence contributing to increase the S/N.

Peak tailings (ranging from 1.0 to 1.2) and symmetries (ranging from 0.8 to 0.9) indicate that all the peaks have good chromatographic characteristics, even if the use of ultra pure spherical silica column could give slightly better results [12,20]. Intra-day and inter-day relative standard deviations (RSDs) for retention times and concentrations were always better than 3 and 6%, respectively. The recovery values ranged from 103 to 95% (RSD always lower than 5%), a better result than those generally obtained when a sample clean up was carried out [3–5,8]. Hence, our results using this monolithic type column seem to confirm that a limited manipulation and the direct injection of the sample, could improve the repeatability and the accuracy of the wine phenolic analysis [26].

5. Application

The concentrations of polyphenolic compounds found in red and white wines are included in Table 3. Taking into account the differences between analytical methods, wines origin and specific varietal patterns, the levels of the different compounds are extremely comparable to those reported by other authors [8,9,21,23–25,27].

6. Conclusions

It can be concluded that, under our conditions, the monolithic column could operate at a higher flow-rate than a conventional RP column with a reduced pressure drop and shorter washing and re-equilibra-

Table 3
Polyphenolic constituents (mg/l) in red and white wine

	Red wine (n=5)		White wine (n=9)	
	Max	Min	Max	Min
Gallic acid	61	39	13	1.6
Protocatechuic acid	8.9	3.4	1.2	0.5
p-Hydroxybenzoic acid	0.3	nd*	0.07	nd*
Vanillic acid	2.2	1.6	0.4	nd*
Caffeic acid	8.7	2.2	7.7	0.6
(+)-Catechin	40	23	13	3.1
Syringic acid	2.2	1.3	0.98	0.41
p-Coumaric acid	3.1	0.5	1.4	nd*
(−)-Epicatechin	23	18	4.4	2.0
Ferulic acid	4.0	nd*	0.47	nd*
trans-Resveratrol	1.5	0.2	0.32	nd*
Rutin	10	3.6	2.7	nd*
Myricetin	4.4	1.7	0.26	nd*
cis-Resveratrol	0.2	nd*	nq#	nd*
Quercetin	6.2	3.5	1.7	nd*
Caftaric acid	75	20	79	12
Coutaric acid	6.2	2.0	18	1.2

*nd, Not detected. Concentration lower than LOD.
#nq, Not quantified. Concentration lower then LOD×10.

tion times. This produces a faster separation of the phenolic compounds, if compared to conventional RP columns, and contributes to improve the S/N.

In addition the direct injection allows a rapid and accurate separation of the main wine polyphenolic compounds, whose evaluation could be useful for technological and nutritional studies.

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