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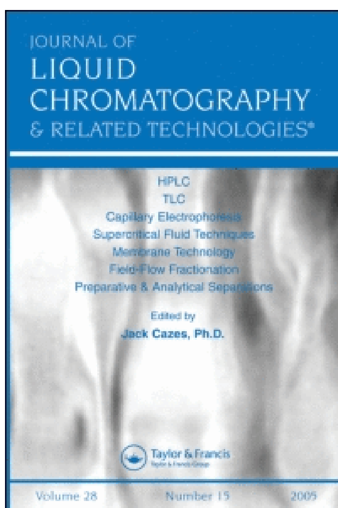

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GRADIENT RP-HPLC DETERMINATION OF FREE PHENOLIC ACIDS IN WINES AND WINE VINEGAR SAMPLES AFTER SPE, WITH PHOTODIODE ARRAY IDENTIFICATION

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ABSTRACT

The analysis of phenolic compounds in wines is of considerable commercial importance, since they are known to play the major role in defying the sensorial characteristics of wines, such as astringency, flavor, and color, as well as in the browning process, causing product deterioration. In this paper, an automated reversed phase high performance liquid chromatographic method, using a multistep binary gradient elution, is developed for the determination of five phenolic acids: caffeic, ferulic, vanillic, salicylic, and p-hydroxy-benzoic acid. The separation method was based on mobile-phase optimization and off-line solid-phase extraction (SPE) from wines and wine vinegar samples, using novel sorbent materials.

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The analytical column, an Inertsil C_8, 250 x 4 mm, 5 µm, used, was operating at ambient temperature. The elution solvents were classified as A: 5-95 (v/v) CH_3OH-H_2O and B: 90-10 (v/v) CH_3OH - H_2O, both A and B acidified with glacial CH_3COOH at pH 3-4. The samples were eluted with gradient starting with 10% in B and ending to 100% in B. Nicotinic acid was used as internal standard at a concentration of 10 ng/µL The flow rate of mobile phase was 0.8 mL/min and observed inlet pressure ranged from 270 to 345 kg/cm^2. A diode-array detector monitored the effluent and chromatograms were recorded at 290 and 256 nm. Comparing their retention time values and UV spectra in the 190-300 nm range with authentic standards stored in a data bank, we made identification of phenolic compounds.

The statistical evaluation of the method was examined performing intra-day (n=8) repeatability and inter-day (n=8) precision assays at three concentration levels and was found to be satisfactory, with high accuracy and precision results. High percentage recoveries from wines (98.7 ± 5.9, with RSD 6.0) and wine vinegar (100.8 ± 6.4, with RSD 6.4) samples were achieved using Nexus SPE cartridges with hydrophilic and lipophilic properties and solvent B as eluent.

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INTRODUCTION

Phenolic acids are hydroxycarboxylic acids with phenolic hydroxylic groups that occur widely in nature in their free forms or in the form of their esters and ethers. They are distributed in the plant kingdom and are present in wine, tea, fruits, juices, and various medical plants. The most frequent phenolic acids in plants are derivatives of benzoic and cinnamic acids, which occur as free acids and their depsides or glycosides. Their presence has biological effects as they influence their resistance and participate in defensive reactions against pathological processes, as well as against bacteria and viruses.[1-4]

Recently, several studies have pointed out that some low molecular phenolic acids, benzoic and cinnamic acid derivatives, show biological properties of related interest and have been reported to act as scavengers of free radicals, thus exerting potential health-promoting effects as antioxidant, antitumor, anti-mutagenic, and anticarcinogenic agents. They are also included in the list of Existing Food Additives as natural antioxidants in Japan. Among them, salicylic acid (2-hydroxy benzoic acid) has the widest pharmacological activity as disinfectant, keratolytic, and antibacterial activity. Caffeic (3,4-dihydroxy cinnamic acid), ferulic (4-hydroxy-3-methoxy cinnamic acid), and vanillic (4-hydroxy-3 methoxy

benzoic acid) acids have been reported to possess antibacterial, antivirus, antifever, and antirheumatic action.[3, 5-6]

Additionally, these molecules play a primary role in defying the sensorial characteristics of grapes and wines such as astringency, flavor, and color. Grapes contain a large amount of different phenolic compounds in skins pulp and seeds that are partially extracted during winemaking. Furthermore, they are responsible for the stability characteristics of wines. Changes in these compounds during processing and storage are also important for the quality of commercial products. Red wines may have very complex phenolic composition that changes over their shelf life, giving that oak wood taste typical of long aged products. The presence and abundance of these compounds are often related to the storage conditions and aging processes. Their concentration increase is probably due to extraction of aromatic compounds derived from lignin degradation.[7-10]

Once phenolic compounds are widespread in nature they have been successfully applied to quality control of plant foodstuffs, namely fruit derivatives. As it is well known, the exact identification of phenolic compounds requires mass spectrometry (MS). However, LC-MS is a very expensive technique not widely used in routine laboratories in wine industry. On the other hand, Diode Array Detection is respected as an indispensable tool for provisional identification of the main phenolic structures present in foods of different sources. Spectra from different phenolic classes allow speedy identification.[11-12]

Due to the importance of these substances for human health, accurate and precise methods for their determination in foodstuffs are required. The analytical methods must provide high resolution, preconcentration, and easy sample handling to avoid any evolution of the analysis.

There is extensive literature on the determination of phenolic acids in foodstuffs, including UV spectrophotometry, Gas Chromatography (GC), Thin Layer Chromatography (TLC), and High Pressure Liquid Chromatography (HPLC). Several HPLC methods are reported in literature concerning the determination of phenolic compounds by both gradient and isocratic elution. A poor resolution of caffeic, vanillic, and ferulic acid is noticed. Some of them propose direct injection of wine samples, but most authors propose sample preparation prior to the HPLC injection to purify and preconcentrate the samples. Liquid-liquid extraction using diethylether or ethyl acetate and Solid Phase Extraction methods are both proposed. The latter have numerous advantages over the former, such as high selectivity, speed, and ease of automation.[5, 13-18]

In this work, an HPLC method was developed for the determination of free phenolic acids, namely: caffeic acid (CA), ferulic acid (FA), vanillic acid (VA), salicylic acid (SA), and p-hydroxy-benzoic acid (4-hydroxy benzoic acid) (pHBA), by reversed phase HPLC, using UV-DAD detection. Wines and wine vinegar samples were analyzed after pretreatment by solid-phase extraction.

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EXPERIMENTAL

Instrumentation

A Shimadzu (Kyoto, Japan), quaternary low-pressure gradient system was used for chromatographic determination of phenolic acids. The solvent lines were mixed in a FCV-9AL mixer and an LC-9A pump was used to deliver the mobile phase to the analytical column. Sample injection was performed by an SIL-9A autosampler, and detection was achieved by an SPD- M6A Photodiode Array Detector equipped with Data acquisition software Class-M10A. Chromatograms were stored on the hard disk of a Function 486 PC and printed on a Hewlett-Packard LaserJet 4L Printer. Degassing of solvents was achieved by continuous helium sparking in the solvent flasks through a DGU-2A degassing unit.

The analytical column was an Inertsil C_8, 250 x 4 mm, 5 µm purchased by MZ-Analysentechnik (Mainz, Germany).

A glass vacuum-filtration apparatus obtained from Alltech Associates was employed for the filtration of the buffer solution, using 0.2 µm membrane filters obtained from Schleicher and Schuell (Dassel, Germany). A Glass-col, Terre Haute 47802 small vortexer and a Hermle centrifuge, model Z 230 (B. Hermle, Gosheim, Germany) were employed for the pre-treatment of wines and wine vinegar samples. The SPE study was performed on a Vac-Elut vacuum manifold column processor purchased from Analytichem International, a division of Varian (Harbor City, USA). All evaporations were performed with a 9-port Reacti-Vap evaporator (Pierce, Rockford, IL, USA).

UV spectra for selecting the monitoring wavelength of detection were provided by Diode Array Detector, while a Varian DMS 100S UV/Vis double-beam spectrophotometer was also used for UV spectra prior to chromatographic method development.

Samples, Chemicals, and Reagents

Ferulic acid, caffeic acid, and vanillic acid were obtained from Sigma, St. Louis, MO, U.S.A., while salycilic acid, p-hydroxybenzoic acid, and nicotinic acid from Merck (Darmstadt, Germany). Methanol HPLC grade was purchased from Riedel de Haen (AG, Seelze, Germany). Bis de-ionized water was used throughout analysis.

Glacial acetic acid and hydrochloric acid of analytical grade (p.a.) were supplied from Merck. Solid Phase Extraction cartridges ABSELUT-NEXUS with hydrophilic and lipophilic properties used for sample pretreatment, were supplied from Varian. The wines and wine vinegars were purchased from local stores.

Chromatography

Separation of phenolic compounds was achieved on an Inertsil C_8, 250 x 4 mm, 5 µm, analytical column. The elution solvents were classified as A: 5-95 v/v CH_3OH-H_2O and B: 90-10 v/v CH_3OH-H_2O, both acidified with glacial CH_3COOH at pH 3-4. The samples were eluted according to the following multi-step binary gradient: 10% in B as initial conditions, 15% in B during 15 min.; 20% in B during 3 min.; 25% in B during 2 min.; 30% in B during 2 min.; 65% in B during 2 min.; 70% in B during 2 min.; 75% in B during 2 min.; and finally, 100% in B during 2 min.; with an isocratic step of 10 min. Elution was performed at a solvent flow rate of 0.8 mL/min and observed inlet pressure ranged from 270 to 345 kg/cm^2. An equilibration time of 10 min. was required between runs.

A diode-array detector performed monitoring of phenolic acids at 256 nm, for p-hydroxybenzoic acid and vanillic acid and 290 nm, for caffeic, salicylic, and ferulic acids. Identification of phenolic compounds was made by comparing their retention time values and UV spectra in the 190-300 nm range with authentic standards stored in a data bank.

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RESULTS

Chromatographic Separation

The chromatogram obtained under the experimental chromatographic conditions is illustrated in Figure 1. Resolution factors are 19.9 for nicotinic and p-hydroxybenzoic acid, 5.77 for p-hydroxybenzoic and vanillic acid, 0.96 for vanillic and caffeic acid, 6.92 for caffeic and ferulic acid, and 13.82 for ferulic and salicylic acid. Nicotinic acid was used as internal standard at a concentration of 10 ng/µL.

Calibration Data-Linearity Range

A calibration curve was constructed using standard solutions prepared by sequential dilution from stock (100 ng/µL) methanolic solutions at concentrations: 0.5, 1.0, 2.0, 3.0, 5.0, 10.0, 15.0, 30.0, and 50 ng/µL. All working standards were prepared in methanol containing the internal standard at a concentration of 10 ng/µL, and they were kept deep-frozen throughout analyses.

The minimum detectable concentration (LOD) was defined as a peak height that produces three times the baseline noise at 0.0005 AUFS. This was found to be 3.33 ng for all compounds, except for salicylic acid, which was 6.67

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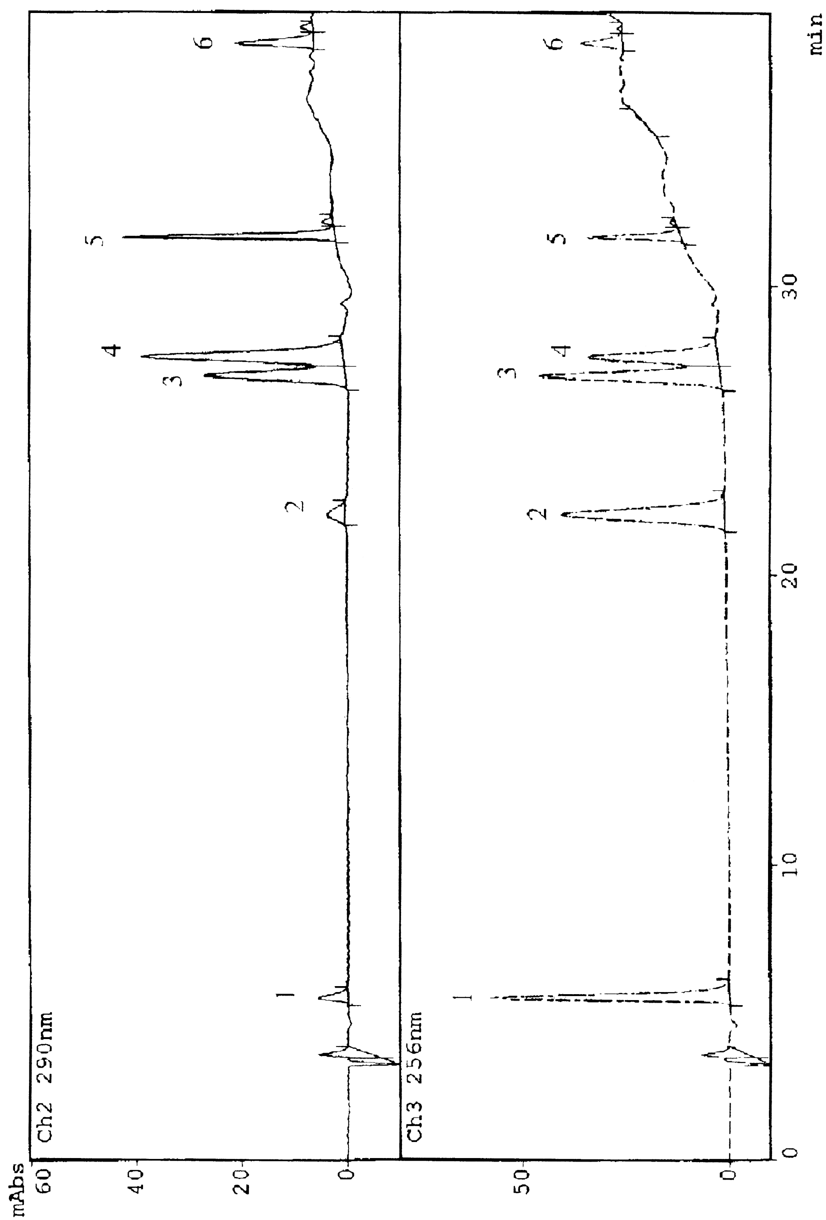

Figure 1. High performance liquid chromatogram of phenolic acids. 1 = nicotinic acid 5.44 min, 2 = p-hydroxybenzoic acid 22.08 min, 3 = vanillic acid 26.89 min, 4 = caffeic acid 27.54 min, 5 = ferrulic acid 31.66 min and 6 = salicylic acid 38.25 min. Chromatographic conditions are described in text.

ng. The LOQ was the lowest concentration of calibration standards with acceptable precision and accuracy and found to be 10.0 ng, except for salicylic acid that was 20.0 ng. The upper limit was found to be 50 ng/mL. Table 1 summarizes the obtained results.

Method Validation: Accuracy, Precision, Stability

The precision of the method based on within-day repeatability, was performed by replicate injections (n=8) of three standard solutions covering different concentration levels: low, medium, and high, 3.0, 5.0, and 15.0 ng/μL, where peak areas were measured, in comparison to the peak area of the internal standard. Statistical evaluation revealed relative standard deviations, at different values. The inter-day precision (day-to-day variation) of the method was established using the same concentration range as above. A triplicate determination of each concentration was conducted during routine operation of the system over a period of eight consecutive days. Results are shown in Table 2.

Accuracy was determined by replicate analysis of three different levels (3.0, 5.0, and 15.0 ng/μL) and calculating the recoveries of actually found versus spiked values.

The stability of solutions was verified by storing sample solutions deep-frozen for one month. Concentrations were measured periodically (one, two, three and four weeks).

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Table 1. Regression Analysis, Sensitivity and Linearity Data for Phenolic Acids Determined Using the Developed Method

Analyte	Regression Analysis Data
p-HBA	A = (0.07956 ± 0.01808) + (0.35420 ± 0.00100) C r = 0.99998
Vanillic acid	A = (0.09437 ± 0.01811) + (0.12557 ± 0.00173) C r = 0.99962
Caffeic acid	A = (−0.10053 ± 0.00539) + (0.13275 ± 0.00039) C r = 0.99997
Ferulic acid	A = (0.06887 ± 0.01430) + (0.10875 ± 0.00153) C r = 0.99941
Salicylic acid	A = (−0.01252 ± 0.00280) + (0.02740 ± 0.00030) C r = 0.99964

A: Peak area ratio (analyte to internal standard).
C: Analyte concentration (ng/μL). r = correlation coefficient.

Table 2. Day-to-Day (Over a Period of 8 Consecutive Days) and Within-Day (n=8) Precision and Accuracy Study for Determination of Phenolic Acids

Analyte	Added (ng)	Found ± SD (ng)	RSD	R (%)	Found ± SD (ng)	RSD	R (%)
		Within-Day			Inter-Day		
p-HBA	60	55.7 ± 2.1	2.1	92.9	54.7 ± 4.4	8.1	91.1
	100	91.7 ± 0.5	0.5	91.7	92.1 ± 8.4	9.1	92.1
	300	286.3 ± 3.8	3.8	95.4	276.3 ± 5.5	1.9	92.1
VA	60	50.9 ± 4.4	4.4	84.8	55.5 ± 1 .6	2.9	92.5
	100	84.4 ± 7.7	7.7	84.4	98.1 ± 5.9	6.1	98.1
	300	264.6 ± 4.7	4.7	88.2	273.3 ± 10.6	3.9	91.1
CA	60	60.6 ± 2.8	2.8	101.0	62.1 ± 5.3	8.5	103.4
	100	103.9 ± 2.0	2.0	103.9	97.5 ± 5.6	5.8	97.5
	300	326.3 ± 4.3	4.3	108.8	294.6 ± 5.2	1.8	98.2
FA	60	53.3 ± 5.6	5.6	88.9	58.8 ± 1.2	1.9	98.1
	100	90.1 ± 0.2	0.2	90.1	97.3 ± 8.4	8.7	97.3
	300	265.5 ± 5.5	5.5	88.5	283.5 ± 9.6	3.4	94.5
SA	60	58.1 ± 5.3	5.3	96.8	56.2 ± 2.5	4.5	93.6
	100	98.1 ± 6.2	6.2	98.1	96.3 ± 1.4	1.5	96.3
	300	322.7 ± 2.6	2.6	107.6	284.4 ± 9.1	3.2	94.8

R=Recovery.

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Table 3. Set Up Parameters for Sample Preconcentration

Parameters	Conditions
Adsorbent	ABSELUT NEXUS
Conditioning	With 2mL CH_3OH and 2mL H_2O
Sample	Sample volume 200 µL of standard solution or 200 µL wine acidified with HCl (pH 2.5) or 200 µL wine acidified with HCl (pH 2.5) spiked with 200 µL of standard solution or 200 µL wine vinegar or 200 µL wine vinegar spiked with 200 µL of standard solution.
Wash	The cartridge is washed with water.
Drying	The cartridge is dried under vacuum.
Elution	2 mL 90-10 v/v CH3OH - H_2O acidified with glacial CH_3COOH pH 3-4.

Table 4. Calibration Curves of Phenolic Acids in Wine Samples (C=ng/μL)

Sample	Compound	Regression Equation
White Wine	pHBA	$A = (-1.3581 \pm 0.1283) + (0.5069 \pm 0.0853)\ C$ $r = 0.97246$
	VA	$A = (0.5411 \pm 0.0049) + (0.1220 \pm 0.0068)\ C$ $r = 0.97643$
	CA	$A = (0.4829 \pm 0.0856) + (0.1435 \pm 0.0597)\ C$ $r = 0.96164$
	FA	$A = (-1.3093 \pm 0.3729) + (0.4864 \pm 0.0659)\ C$ $r = 0.9819$
Rosé Wine	pHBA	$A = (0.7491 \pm 0.1096) + (0.3679 \pm 0.0461)\ C$ $r = 0.9846$
	VA	$A = (0.6709 \pm 0.2266) + (0.1249 \pm 0.0398)\ C$ $r = 0.9078$
	CA	$A = (1.3220 \pm 0.3869) + (0.1733 \pm 0.0292)\ C$ $r = 0.9723$
	FA	$A = (1.9221 \pm 0.4643) + (0.2965 \pm 0.0351)\ C$ $r = 0.9862$
Red Wine	pHBA	$A = (-0.1080 \pm 0.0533) + (0.1421 \pm 0.0191)\ C$ $r = 0.9822$
	VA	$A = (0.4510 \pm 0.0744) + (0.1408 \pm 0.0462)\ C$ $r = 0.9667$
	CA	$A = (0.2109 \pm 0.0216) + (0.1199 \pm 0.0137)\ C$ $r = 0.9299$
	FA	$A = (1.1142 \pm 0.2108) + (0.1363 \pm 0.0159)\ C$ $r = 0.9865$

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Solid Phase Extraction-Application to Real Samples

The SPE protocol used in this assay involves the use of a polymeric adsorbent ABSELUT-NEXUS cartridges (acidic basic screen) with a sorbent designed to extract a wide range of organic compounds from different matrices using non-conditioned SPE (NC-SPE) technique, as stated by the manufacturer. It is a highly cross-linked spherical polymeric sorbent with a combination of hydrophilic and lipophilic moieties.

To optimize the HPLC conditions for the analysis of phenolic compounds in wines and wine vinegars, an artificial mixture was prepared containing the five phenolic compounds.

The repeatability and intermediate precision of the method was assayed. The optimized HPLC methodology was then applied to the analysis of red, white, and rosé wine, and vinegars from red, white and rosé wine.

Table 5. Calibration Curves of Phenolic Acids in Wine Vinegar Samples (C= ng/μL)

Sample	Compound	Regression Equation
White Wine Vinegar	pHBA	A = (0.1426 ± 0.0196) + (0.3874 ± 0.0375) C r = 0.9880
	VA	A = (0.1537 ± 0.0976) + (0.1798 ± 0.0300) C r = 0.9728
	CA	A = (−0.2959 ± 0.0739) + (0.2169 ± 0.0434) C r = 0.9615
	FA	A = (−0.3651 ± 0.0223) + (0.2735 ± 0.0773) C r = 0.9261
Rosé Wine Vinegar	pHBA	A = (−0.9161 ± 0.1846) + (0.5892 ± 0.0895) C r = 0.9774
	VA	A = (0.4061 ± 0.0491) + (0.1751 ± 0.0269) C r = 0.9802
	CA	A = (−0.7452 ± 0.1535) + (0.3018 ± 0.0191) C r = 0.9967
	FA	A = (0.3759 ± 0.8418) + (0.2802 ± 0.0636) C r = 0.9509
Red Wine Vinegar	pHBA	A = (−2.8653 ± 0.4038) + (0.8915 ± 0.0305) C r = 0.9988
	VA	A = (−0.9129 ± 0.1066) + (0.3028 ± 0.0080) C r = 0.9993
	CA	A = (−1.6482 ± 0.5581) + (0.4466 ± 0.0422) C r = 0.9912
	FA	A= (−0.0198 ± 0.0060) + (0.3612 ± 0.0877) C r = 0.9443

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An aliquot of 200 μL of wine acidified with HCl (pH 2.5) diluted with water and 200 μL of standard solution was applied to the NEXUS SPE cartridge, which was preconditioned with 2 mL CH_3OH and 2 mL water. This step was necessary for enhanced analyte recovery. After washing the cartridge with water, phenolic acids were eluted with 2 mL 90-10 v/v CH_3OH-H_2O, acidified with glacial CH_3COOH, pH 3-4. The samples were subsequently evaporated to dryness under gentle nitrogen steam in a 45°C water bath, and the residue was reconstituted to 200 μL, with nicotinic acid (internal standard) 10 ng/μL. Solutions were filtered through membrane filters (0.2 μm pore size) prior to injection onto the HPLC system.

Similar extraction protocol was followed for the analysis of wine vinegars without the addition of hydrochloric acid. Blank wine and wine vinegar samples

were also treated in the same way as described above. Table 3 describes the sample preparation scheme.

Calibration curves are presented in Tables 4 and 5. The recovery efficiency was determined by adding measured amounts of standards to the samples, prior to extraction from sample matrix. Table 6 summarizes the results from recovery experiment at two concentration levels, 5.0 and 10.0 ng/μL. Values represent the mean of triplicate measurements. Controls from all studied samples were prepared and subjected to the same extraction procedure. The recoveries were determined by subtracting the values obtained for the control matrix preparation from

Table 6. Recovery Study of Phenolic Acids in Wine and Wine Vinegar Samples After PE

Compound	Added (ng)	Measured (ng)	R (%)	Measured (ng)	R (%)
		White Wine		White Wine Vinegar	
pHBA	100	99.4	99.4	101.0	101.0
	200	190.8	95.4	198.4	99.2
VA	100	96.7	96.7	119.3	119.3
	200	196.4	98.2	191.0	95.5
CA	100	95.0	95.0	103.1	103.1
	200	197.2	98.6	185.4	92.7
FA	100	95.7	95.7	102.6	102.6
	200	196.4	98.2	191.0	95.5
		Rosé Wine		Rosé Wine Vinegar	
pHBA	100	95.5	95.5	117.5	117.5
	200	221.6	110.8	193.6	96.8
VA	100	93.2	93.2	92.7	92.7
	200	195.2	97.6	191.8	95.9
CA	100	98.5	98.5	107.3	107.3
	200	209.2	104.6	189	94.5
FA	100	113.7	113.7	106.2	106.2
	200	199.4	99.7	180.6	90.3
		Red Wine		Red Wine Vinegar	
pHBA	100	83.5	83.5	103.9	103.9
	200	176.6	88.3	194.0	97.0
VA	100	90.2	90.2	96.9	96.9
	200	193.0	96.5	204.6	102.3
CA	100	93.4	93.4	110.9	110.9
	200	199.4	99.7	183.6	91.8
FA	100	113.5	113.5	98.0	98.0
	200	199.8	99.9	197.8	98.9

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Table 7. Control Sample Analysis

Sample	Compound	Concentration (ng/μL)
White Wine	pHBA	3.6
	FA	3.4
Rosé Wine	pHBA	2.7
	FA	2.5
Red Wine	pHBA	1.8
	FA	2.7
Red Wine Vinegar	pHBA	1.6
	FA	1

those of the samples prepared with the added standards. Results are tabulated in Table 6. From the examined phenolic acids only pHBA and ferulic acid were found to be present in wine samples and in red wine vinegar. Initial concentrations of identified phenolic acids in control samples are tabulated in Table 7.

Representative chromatograms of wine and wine vinegar samples are illustrated in Figures 2, 3, and 4.

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DISCUSSION

Phenolic compounds have gained enormous attention in the last years as they exhibit antioxidant activity and important quality properties. The phenolic compounds in wine contribute to the characteristic taste, changing with age and also possessing bacteriological effects.

An automated reversed phase high performance liquid chromatographic method, using a multistep gradient elution, is described for the simultaneous analysis of phenolic acids: caffeic, ferulic, vanillic, salicylic, and p- hydroxybenzoic acid. Although initially well separated from the rest of the phenolic acids, salicylic acid was not possible to be identified, due to column deterioration throughout analyses.

The separation method was based on mobile-phase optimization and off-line solid-phase extraction (SPE) from food samples: wines and wine vinegars, using novel sorbent materials. A diode-array detector was used to monitor the eluted compounds.

The optimized methodology is useful for the phenolic compounds determination in red, white, and rosé wine and wine vinegars, after pretreatment using SPE. The method is accurate and precise. High extraction rates are achieved.

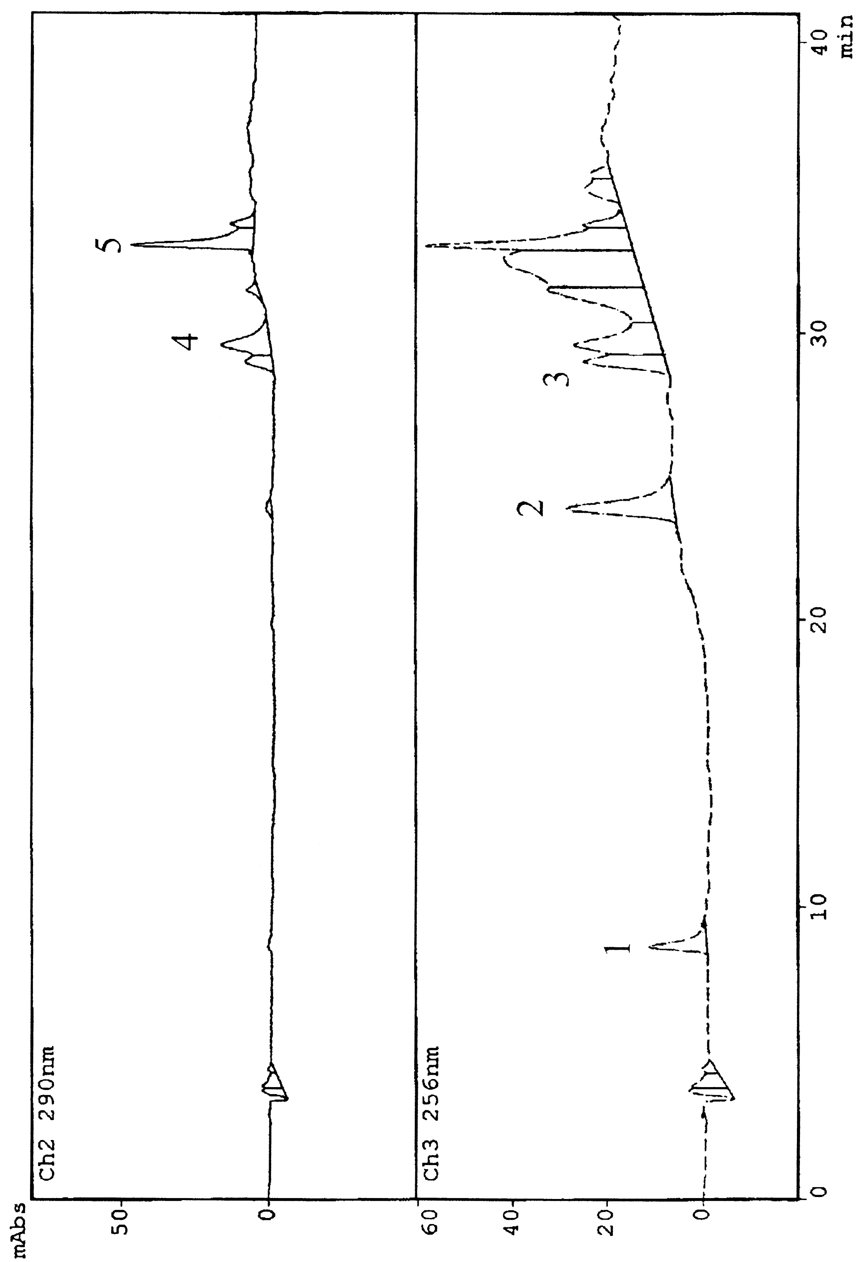

Figure 2. Chromatogram of phenolic acid analysis in white wine: 1 = nicotinic acid, 2 = p-hydroxybenzoic acid, 3 = vanillic acid, 4 = caffeic acid, 5 = ferrulic acid, 6 = salicylic acid. Chromatographic conditions are described in text.

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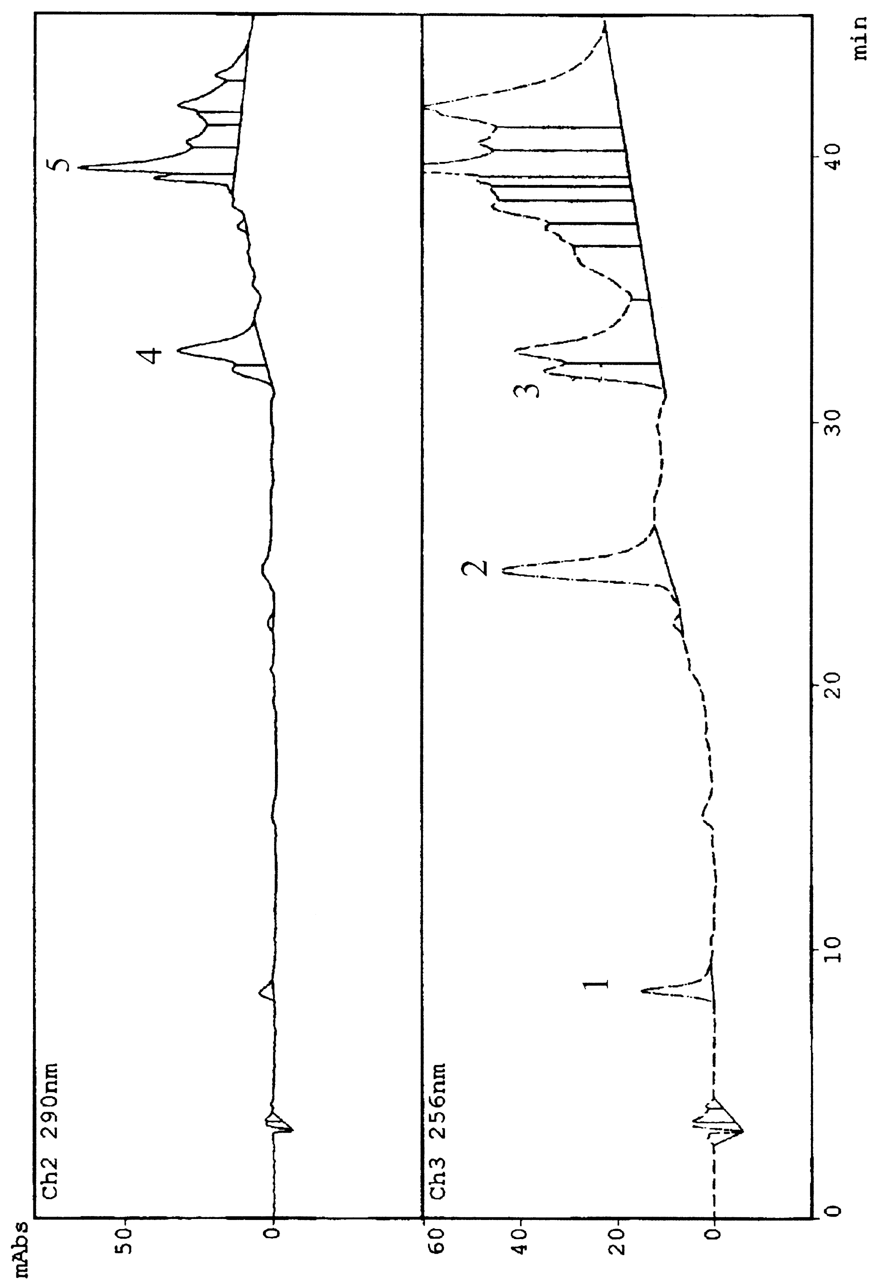

Figure 3. Chromatogram of phenolic acid analysis in rosé wine: 1 = nicotinic acid, 2 = p-hydroxybenzoic acid, 3 = vanillic acid, 4 = caffeic acid, 5 = ferrulic acid, 6 = salicylic acid. Chromatographic conditions are described in text.

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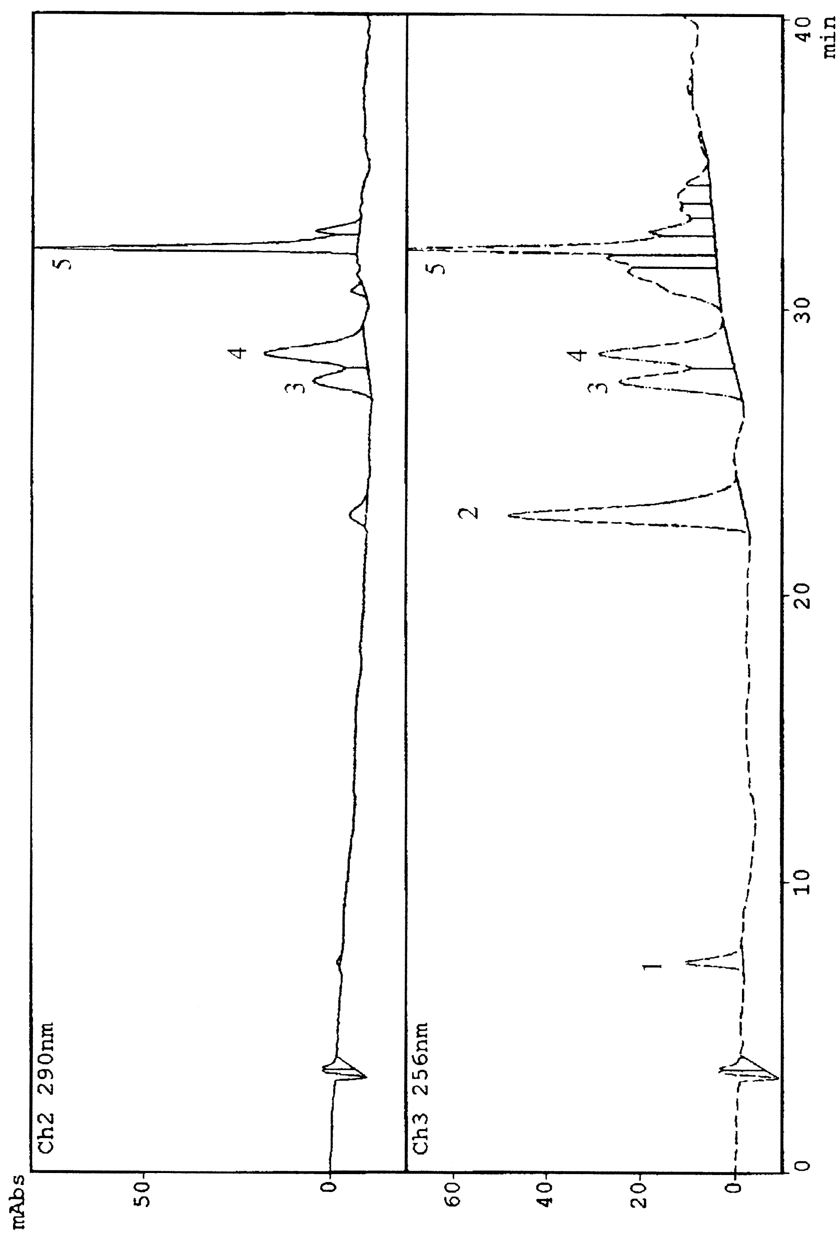

Figure 4. Chromatogram of phenolic acid analysis in white wine vinegar (spiked sample): 1 = nicotinic acid, 2 = p-hydroxybenzoic acid, 3 = vanillic acid, 4 = caffeic acid, 5 = ferrulic acid, 6 = salicylic acid. Chromatographic conditions are described in

No matrix interference was noticed. Conditioning of the sorbent bed was required to get higher recovery rates in spite of manufacturer's instructions, as this sorbent was initially designed to function without conditioning.

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