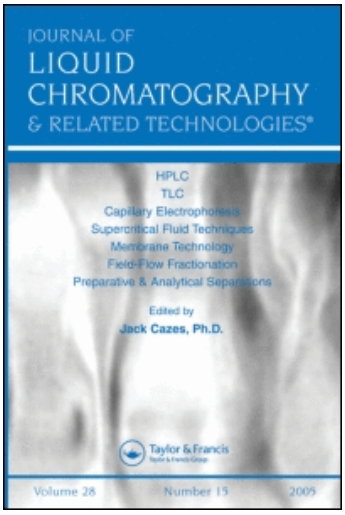

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Determination of Phenolic Acids by a Modified HPLC: Its Application to Various Plant Materials

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Determination of Phenolic Acids by a Modified HPLC: Its Application to Various Plant Materials

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Abstract: The determination of certain phenolic acids employing a gradient HPLC method is described, in this study. The analysis was performed by using a gradient program with the two solvents system (A: methanol:water:formic acid (10 : 88 : 2 v/v); B: methanol:water: formic acid (90:8:2 v/v)). The flow rate of 1 mL · min^{-1}, injection volume of 10 μL was used and signals were detected at 280 nm. Propylparaben was a suitable compound as an internal standard for this system. The method was validated and highly repeatable (between RSD values of 0.35–1.65) and linear results were obtained. The LOD and LOQ values of the phenolic acids are in the range of ($2.58 \times 10^{-6} - 9.69 \times 10^{-6}$ M) and ($7.83 \times 10^{-6} - 2.93 \times 10^{-5}$ M), respectively.

The applicability of the method was tested by using some plant source materials, and the phenolic acid contents were successfully determined via well-defined peaks. Therefore, the progressed method is suggested for the quantification of phenolic acids in food industry and laboratories.

Keywords: HPLC analysis, Wine, Sour cherry juice, Rose hip, Green tea, Tomato

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INTRODUCTION

In recent years, more clinical studies were focused on phenolic acids as a group of potentially immune stimulating compounds, i.e., plant inducers of fagocytosis, the synthesis of interferons, and antibodies in humans.[1] Additionally, they exhibit antiviral, antibacterial, anti-inflammatory, and antioxidant properties, so plants containing these compounds are widely used in phytotherapy and plant composed foods.[2]

Phenolic acids comprise a large group of organic compounds, and they are structurally simple phenolic compounds of the nonflavonoid family and synthesized through the shikimic acid pathway, and may occur in the bound or free form. There are two main groups of phenolic acids, both of which are hydroxyl derivatives of aromatic carboxylic acids: hydroxybenzoic acids and hydroxycinnamic acids.[3] The most widely distributed phenolic components in plant tissues are the hydroxycinnamic acids; p-coumaric, caffeic, and ferulic acids. They differ according to the number and position of hydroxylation and methoxylation of the aromatic ring.[4]

Several methods have been employed for the determination of phenolic acids. These are categorized as in the following: sophisticated methods such as LC-MS,[5] GC-MS,[6] PB-EI-MS,[7] and capillary electrophoresis studies.[8,9] There are some basic reverse phase high pressure liquid chromatographic (RP-HPLC) methods for the analysis of phenolic acids which have been proposed.[10–12] Many important HPLC applications were applied to plant materials to carry out their studies, including ultraviolet (UV) or photodiode array (PDA),[7,10–25] fluorescence,[26] electro-chemical (ED)[27,28] detection techniques.

The aim of this study is to develop a more repeatable, selective, precise, and accurate gradient HPLC method with its validation studies, and to apply this method to the extraction of plant based food samples, such as red wine prepared from Aegean grapes, sour cherry juice (*Cerasus vulgaris*, Mill.), rose hip (*Rosa canina*), green tea (*Camellia sinensis)*, and tomato (*Lycopersicon esculentum*), to ascertain the amount of phenolic acids after elution from the SPE column.

EXPERIMENTAL

Chemicals and Plant Materials

Gallic acid (GA), protocathechuic acid (protoCA), *p*-hydroxy benzoic acid (*p*-hydBA), vanillic acid (VA), caffeic acid (CA), chlorogenic acid (ChA), syringic acid (SA), *p*-coumaric acid (*p*-COU), ferulic acid (FA), *o*-coumaric acid (*o*-COU), *trans*-cinnamic acid (*tr*-CIN), propylparaben as internal standard (IS), methanol, and formic acids were analytical grade (Sigma Aldrich, St. Louis, MO, USA) and (Merck GmbH, Darmstadt, Germany).

The plant based food samples, which are red wine, sour cherry juice, rose hip, green tea, and tomato were supplied from a local supermarket.

Apparatus

An HPLC system consisting of a model 600 E HPLC pump, 717 plus Autosampler, 996 photodiode array detector (PAD), and data processor of a Millenium 32 was used for the HPLC analysis (all Waters Corp. Massachusetts, USA). Ultrapure water was supplied from Human UP 9000 System (18 mW) water purification system. Reverse phase C_{18} Ultrasphere column (Teknokroma, Barcelona, SP) (100×4.6 mm inner diameter $3\ \mu$) was employed for the analysis of phenolic acids.

Procedure

HPLC Analysis

The analysis was performed using a gradient program with a two solvent system (A: methanol:water:formic acid ($10:88:2$ v/v); B: methanol:water: formic ($90:8:2$ v/v)). Initial condition was 100% A; 0–15 min, changed to 100% A; 15–20 min, to 85% A; 20–30 min, to 50%; 30–35 min to 0% A; 36–42 min, then back to 100% A. The flow rate was always 1 mL $\cdot$ min^{-1} and the injection volume was 10 μL. The signals were detected at 280 nm. Standard phenolic acids were prepared in a solvent consisting of water:methanol ($50:50$; v/v). The IS technique was applied to the analysis to increase the repeatability; propylparaben was the suitable IS.

Solid Phase Extraction (SPE)

For cleaning up materials, a Superclean (C-18) (Sigma Aldrich, St. Louis, MO, USA) solid phase extraction cartridge was employed. Prior to the SPE, the cartridges were conditioned with 3 mL of methanol and 3 mL of aqueous solution of 2% HCl double distilled water, and they were kept in methanol when they were not used. The extraction procedure was as follows:

Red wine sample: A 10 mL wine sample was transferred to a beaker and left in a water bath at 80°C for half and hour to evaporate ethyl alcohol, 0.2 mL c-HCl (concentrated HCl) was added and shaken vigorously.

Sour cherry juice: A 10 mL sample was put into a beaker to which 0.2 mL c-HCl was added and then mixed vigorously.

Rose hip and green tea: Definite (about 2 g) amounts of samples were weighed and their infusions were prepared. They were centrifuged, the residues were discarded, and 0.2 mL c-HCl acids were added and mixed well.

Tomato: It was homogenized thoroughly and the same amount of sample was weighed. Double distilled water (10 mL) was pipetted into the sample and centrifuged for five min, and 0.2 mL c-HCl was added into the tube. A definite amount of clear supernatant was taken to apply the procedure as stated in the following:

Samples, which were processed as explained above, were passed through the cartridge. Impurities were washed out with 3 mL of 2% HCl. Retained phenolic acids of red wine, sour cherry juice, rose hip, green tea, and tomato were eluted with 5 mL methanol. Their appropriate dilutions were prepared, IS was added, and they were directly injected into the HPLC system.

RESULTS AND DISCUSSION

Optimization of the Method

Many gradient HPLC studies have been reported for the determination of phenolic acids.[10–28] Usually, a polar-non polar solvent system containing 2 mL acetic or 2 mL formic acid per 100 mL, or phosphate buffer at low pH, have been employed to separate the acids. We have tried acetic and formic acids for the resolution of relevant acids. In the use of formic acid instead of acetic acid, better resolution for chlorogenic and caffeic acids was observed. All the adjacent peaks were resolved from each other providing the resolution criterion ($R > 1.2$). The analysis was completed in about 30 min, but elution of the rest of the material in the column took almost 42 min.

Well defined phenolic acid peaks, in turn, retention times as minutes GA (2.65), protoCA (4.87), *p*-hydBA (8.89), VA (13.59), CA (15.80), ChA (16.72), SA (18.57), *p*-COU (23.63), FA (25.54), *o*-COU (26.30), *tr*-CIN (28.31) appeared in the chromatogram.

Few studies have employed the IS technique for earlier reported phenolic acid determinations.[11,24] It is known that more repeatable results are obtained by using an IS technique. Some chemicals were tried to be used as IS, in the optimum analysis condition mentioned above, and propylparaben was the most suitable one. It was appropriate regarding some ways that the most important point is the plants do not include it because parabens are produced synthetically.

A standard solution of each of the phenolic acids in the range of $5.2 \times 10^{-5} - 6 \times 10^{-5}$ M) and a fixed amount, 5.59×10^{-5} M, of IS were prepared and they were injected into the HPLC and propylparaben appeared at 29.87 min. The chromatogram of the phenolic acids is demonstrated in Figure 1.

No interferences were observed in the mentioned conditions which provided the resolution.

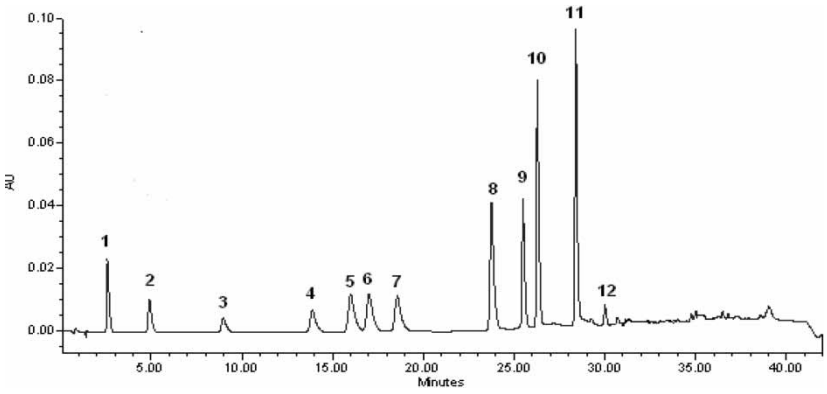

Figure 1. Chromatogram of certain phenolic acids in the optimum HPLC conditions presented in the experimental. Their symbols and retention times GA; 2.65 (1), protoCA; 4.87 (2), *p*-hydBA; 8.89 (3), VA; 13.59 (4), CA; 15.80 (5), ChA; 16.72 (6), SA; 18.57 (7), *p*-COU; 23.63 (8), FA; 25.54 (9), *o*-COU; 26.29 (10), *tr*-CIN; 28.31 (11), IS; 29.87 (12).

Validation Studies

Precision (Repeatability)

The precision tests (repeatability) were conducted by preparing a fixed concentration of phenolic acids and IS; they were injected into the HPLC ($n = 6$). The results were evaluated with regard to the areas of the peaks and peak normalizations (PN = peak area/peak retention time) and the rate of the peak normalizations ($R = PN_{acids}/PN_{IS}$). Injections were made, and the results were processed. The evaluation of repeatability as relative standard deviations (RSD) and standard deviation (SD) of the experiments are given in Table 1.

It was observed that the precision of the experiments increased by using the IS instead of simple areas of the peaks. This can be attributed to the fact that the employment of peak normalization and the processing of the internal standard become more repeatable by the use of the internal standard method. The results are very repeatable; the values for precision are in the 0.35–1.65 range. This shows that the method is highly reliable.

Table 1. Results of the precision test (as rate of PN) of certain phenolic acids ($n = 6$)

	GA	protoCA	*p*-hydBA	VA	CA	ChA	SA	*p*-COU	FA	*o*-COU	*tr*-CIN
SD	0.37	0.01	0.02	0.04	0.05	0.02	0.03	0.05	0.05	0.04	0.06
RSD	1.65	0.21	1.12	1.50	0.98	0.35	0.64	0.92	1.44	0.71	0.96

Linearity

The standard solutions of phenolic acids which were under the examination and fixed amount of IS were prepared and they were injected into the HPLC (n = 4). The calibration equations were constructed employing rate of peak normalization values against phenolic acid concentrations at 280 nm. The rate of peak normalizations of the phenolic acids and IS values were calculated as mentioned in the precision tests. LOD and LOQ were calculated by multiplying 3.3 and 10 with σ/a, respectively, where, σ is standard deviation of intercept, a is slope of the regression equation.

The calibration parameters of phenolic acids with their standard deviations, correlation coefficient (r), limit of detection (LOD), and limit of quantification (LOQ) values are given in Table 2.

Since the results of the repeatability and linearity are reliable for the purpose of the determination, it was decided that the method might be used for the phenolic acid analysis in some plant based materials.

Application of the Method to Some Plant Based Materials

Extracts of the plant based materials were prepared using SPE, which is detailed in the experimental section; their samples eluted by methanol from the SPE column were injected into the column.

Well-defined chromatograms were obtained, all the peaks were resolved from each other, and no interference was observed at the given conditions. The chromatogram of the retained compounds of red wine, sour cherry juice, rose hip, green tea, and tomato are presented in Figure 2.

Table 2. The calibration parameters of phenolic acids at 280 nm using rate of peak normalizations, with their correlation coefficient (r), LOD and LOQ values (n = 4)

Phenolic acids	a ± SD	b ± SD	r	LOD (molarity)	LOQ (molarity)
GA	555100 ± 1306	−1.30 ± 0.66	0.9972	3.94×10^{-6}	1.2×10^{-5}
protoCA	116700 ± 1244	−0.25 ± 0.09	0.9998	2.58×10^{-6}	7.83×10^{-6}
p-hydBA	36800 ± 724.8	−0.13 ± 0.06	0.9974	5.14×10^{-6}	1.56×10^{-5}
ChA	121900 ± 2362	−0.31 ± 0.10	0.9979	2.81×10^{-6}	8.53×10^{-6}
SA	102100 ± 3168	−0.14 ± 0.01	0.9999	9.69×10^{-6}	2.93×10^{-5}
VA	64280 ± 878.3	−0.09 ± 0.05	0.9987	2.49×10^{-6}	7.55×10^{-6}
CA	125700 ± 5639	−0.17 ± 0.26	0.9970	6.71×10^{-6}	2.03×10^{-5}
p-COU	119900 ± 2396	−0.26 ± 0.16	0.9973	4.51×10^{-6}	1.37×10^{-5}
FA	71770 ± 1358	−0.12 ± 0.09	0.9980	4.20×10^{-6}	1.27×10^{-6}
o-COU	113400 ± 2469	−0.22 ± 0.18	0.9969	5.40×10^{-6}	1.64×10^{-5}
tr-CIN	154300 ± 4711	−0.62 ± 0.26	0.9976	5.56×10^{-6}	1.68×10^{-6}

Abbreviations: a is slope; b is intercept; SD is standard deviation.

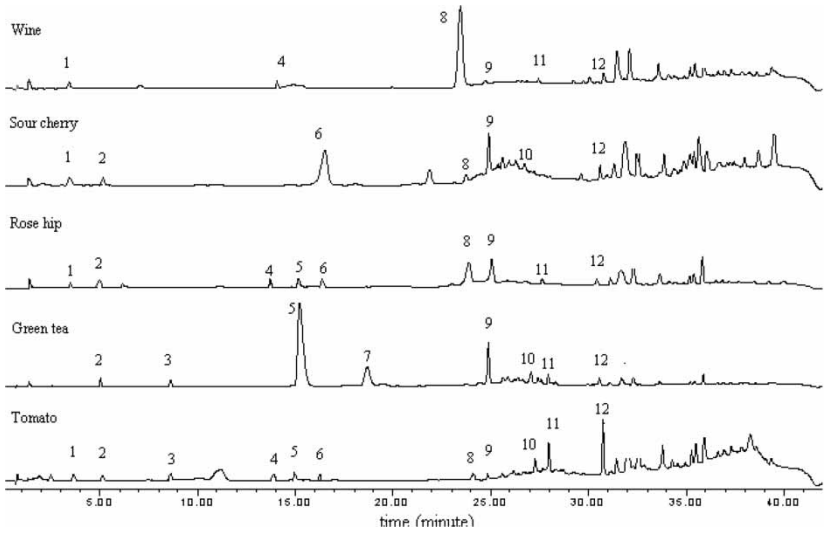

Figure 2. The chromatograms of red wine, sour cherry juice, rose hip, green tea and tomato extracts after processing by SPE. (attenuations are different from each other).

The relevant peaks of the phenolic acids were detected by a standard addition technique and their quantifications were realized using calibration equations via the rate of peak normalization values. Their concentrations were calculated to be, for red wine and sour cherry juice as mg/L, for rose hip, green tea, and tomato as mg/100 g. These values are summarized in Table 3.

The analyses of phenolic acids indicated the applicability of the method to the plant derived materials. Various studies have been performed to determine the phenolic acids in the mentioned samples using HPLC and most applications have been done with the various wine samples.[11,13–17] These have been performed by RP-HPLC,[11,13,14] LC-MS,[15] and CZE.[16,17] The extraction material was cleaned by using the SPE technique. In this study, some phenolic acids have been determined in wines and the major peaks are usually GA, CA, p-COU, and FA. Besides; p-hydBA and tr-CIN are not encountered in the studies involving wine analysis. Here, we have determined tr-CIN, but have not observed any CA.

There are some reports of tests performed by HPLC for the determination of phenolic acids in various fruit juices, but there has not been any study involving sour cherry juice.[18–20] Usually, CA, ChA, p-COU, and FA have been quantified in these studies, but we have determined, as the main phenolic acids, ChA, GA, and FA in sour cherry juice. It can be concluded that there is some parallelism between the results of the studies.

To the best of our knowledge, no reports have appeared dealing with the constituents of rose hip. The main peaks determined in the related extract were

Table 3. Phenolic acid contents of some plant sources material

Phenolic acids	Wine (mg/L)	Sour cherry juice (mg/L)	Rose Hip (mg/100 g)	Green Tea (mg/100 g)	Tomato (mg/100 g)
Samples analyzed					
GA	3.18	9.85	2.3	—	0.11
protoCA	—	6.97	1.4	21.8	0.08
p-hydBA	—	—	—	8.7	7.2
VA	1.59	—	6.9	—	0.19
CA	—	—	3.1	287.3	0.22
ChA	—	50.78	8.5	—	0.16
SA	—	—	—	162.6	—
p-COU	33.69	17.14	24.9	—	0.06
FA	1.38	9.25	23.9	39	0.21
o-COU	—	2.78	—	1.9	0.24
tr-CIN	0.69	—	1.7	1.5	0.20

p-COU, FA, and ChA. It is observed that rose hip is very rich in phenolic acids.

Some reports have appeared on phenolic acids in green teas, employing an HPLC-DAD system[21,22] and high-speed countercurrent chromatography (HSCCC).[23] The main phenolic acids were found to be CA, SA, FA, and protoCA in green tea.

A detailed HPLC-DAD study has been reported on the phenolic acids of tomato; it is stated that CA, p-COU, and ferulic acids were identified.[24] The amounts of phenolic acids were found to be very low in tomato in this study.

It has been already thought that, to elucidate the true level of organic acids, their free and bound forms have to be examined.

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