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Determination of some pharmacologically active phenolic acids in juices by high-performance liquid chromatography

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Abstract

Several phenolic acids, e.g. caffeic acid, chlorogenic acid, ferulic acid, gallic acid and ellagic acid, which occur naturally, are inhibitors of carcinogenesis. In this paper we present a new method for the simultaneous determination of all of these compounds, except ellagic acid, in juices by reversed-phase high-performance liquid chromatography (HPLC) using ultraviolet detection and involving isocratic elution, and we have devised an HPLC method for the determination of ellagic acid in juices. The experimental results showed that cherry juice contains a high concentration of chlorogenic acid and the content of bound gallic acid in black and green grape juices is high compared to that of other phenolic acids.

Keywords: Food analysis; Fruit juices; Phenolic acids; Gallic acid; Chlorogenic acid; Caffeic acid; Ferulic acid; Ellagic acid

1. Introduction

Phenolic acids (i.e. hydroxycarboxylic acids with phenolic hydroxyl groups) occur widely in nature in the forms of their esters, ethers or in their free forms [1]. Some phenolic acids, namely caffeic, chlorogenic, ferulic, gallic and ellagic acid, have been found to be pharmacologically active as antioxidant, antimutagenic and anticarcinogenic agents [2–10].

In view of the importance of these substances for health, accurate methods for their determination in foodstuffs are required. There is extensive literature on the determination of phenolic acids in foodstuffs, including UV spectrophotometry [11,12], gas chromatography [13–15], thin-layer chromatography [16–19] and high-performance liquid chromatography (HPLC) [15,17–25]. The HPLC technique is the most commonly used method for the determination of phenolic acids in different samples. Quantification, however, can often be difficult due to sample complexity or when studying certain substances simultaneously by a single HPLC method.

Simultaneous separation of some of these compounds in juices using gradient elution has been reported [18,25]. However, no HPLC method with isocratic elution has been described for the simultaneous determination of these pharmacologically active components of juices.

In this work, a direct method to determine simultaneously caffeic, chlorogenic, ferulic and gallic acids in various juices by reversed-phase HPLC using UV detection was developed. This method has some advantages in routine analysis because the method enables the separation of these compounds by isocratic elution using a mobile phase including 95.6% water.

This method is not suitable for the determination of ellagic acid. Charrier et al. [24] described an

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HPLC method for determining ellagic acid in oakwood and eucalyptus. Our initial attempts to use that method to determine this substance in juices were unsuccessful. The method described here for ellagic acid is a modification of the method of Charrier et al. [24].

We analysed three juices, namely green grape juice, black grape juice and cherry juice with the methods and for quantitative determination of free and bound phenolic acids in juices, we analysed the juices before and after alkaline hydrolysis [19,26]. Good resolution has been obtained in all cases.

2. Experimental

2.1. Equipment

A Gynkotek high-precision pump Model 300 B with a Gynkotek spectrophotometer SP-4 was used. Separation was carried out using a LiChrospher 100 RP-18 column (5 μm; 120×4 mm I.D., Merck, Darmstadt, Germany) with a precolumn (RP-18; 4× 4 mm, Merck). Chromatographic data were collected and recorded using a Shimadzu C-R3A Chromatopac.

2.2. Reagents and standards

Acetic acid and phosphoric acid (Merck) were used as pH modifiers. Solvents were of HPLC grade (Merck). Water was purified in a Milli-Q water purification system (Millipore, Bedford, MA, USA). Ellagic acid was obtained from Sigma (Deisenhofen, Germany) and other chemicals were purchased from Fluka (Neu-Ulm, Germany).

2.3. Sample preparation

2.3.1. Hydrolysis

Juice samples (150 ml) were hydrolyzed (2 *M* NaOH, pH ca. 12.5, under argon at room temperature) and then acidified to pH 3.4. The hydrolyses were completed after 48 and 62 h for cherry juice and the grape juices, respectively.

In order to determine of effect of air on phenolic acids during base hydrolysis, juice samples (150 ml) were hydrolyzed under air (2 *M* NaOH, pH ca. 12.5, room temperature). The times for hydrolyses were chosen as 48 and 62 h for cherry juice and grape juices, respectively.

2.3.2. Extraction

All samples (hydrolyzed and non-hydrolyzed) were processed in the same way; each sample (containing 150 ml of juice) was subjected to extraction four times with 75 ml of ethyl acetate. Fractions were pooled and evaporated to dryness under vacuum with a rotatory evaporator, always keeping the bath temperature under 35°C. The residue was redissolved in 10 ml of ethanol and divided equally among five 25-ml round flasks. The flask contents were evaporated under vacuum with the rotatory evaporator, under 35°C, to dryness. Every extract sample was redissolved in a certain amount (depending on the amount of phenolic compounds in the juices) of mobile phase before the HPLC analysis.

2.4. Validation of extraction

Aliquots (0.05, 0.50, 5.00 and 10.00 mg) of each standard were added to 100 ml of juice, extracted and then analysed. This was repeated twice for each juice.

2.5. Chromatographic conditions

2.5.1. Determination of caffeic, chlorogenic, ferulic and gallic acid (method 1)

The chromatographic conditions were as follows: flow-rate, 0.5 ml/min; detection, UV absorption at 280 and 320 nm; volume injected, 20 μl; temperature, room temperature. The mobile phase composition was water–ethylacetate–acetic acid (95.6:4.1:0.3, v/v).

2.5.2. Determination of ellagic acid (method 2)

The chromatographic conditions were as follows: flow-rate, 0.5 ml/min; detection, UV absorption at 252 and 360 nm; volume injected, 20 μl; temperature, room temperature.

The mobile phase composition was optimized and the best composition obtained was water–methanol–phosphoric acid (62.40:37.45:0.15, v/v).

2.5.3. Identification and quantification of the compounds

Retention times and absorbance ratios (at two different wavelengths) against those of standards were used to identify the separated phenolic acids and to check their purity. Quantitative determinations were carried out by the external standard method.

3. Results and discussion

3.1. Chromatographic methods and validation of extraction

Simultaneous separation of some of these compounds using gradient elution has been reported [18,20,25]. In this work, a study was carried out to determine caffeic, chlorogenic, ferulic and gallic acids with isocratic elution (method 1). The mobile phase composition was optimized to obtain good resolution between the different peaks detected for juice samples by HPLC.

A study of the repeatability of the method and its reproducibility between days was performed. The results for repeatability showed a relative standard deviation ($n=7$) ranging from 1.5 to 5% and those for reproducibility between days ($n=10$, in one month) from 3.5 to 6%. Good response linearity was obtained for all of the compounds studied between 0.5 and 100 mg/l (mobile phase); (a linear response was obtained for chlorogenic acid between 1 and 100 mg/l) and the detection limit of the method was established as 0.5 mg/l.

The chromatograms obtained by method 1 of hydrolyzed and non-hydrolyzed juice samples are showed in Figs. 2–4. Fig. 1 shows a chromatogram of a mixture of the standard compounds obtained with this method.

Ellagic acid could not be determined by this method because it could not be eluted under the conditions adopted. Different methods have been developed for the determination of ellagic acid [22–24]. Our attempts to use some of the methods with isocratic elution [23,24] for the determination of ellagic acid in juices were unsuccessful. We modified the method of Charrier et al. [24] for this determination (method 2). The chromatograms obtained by

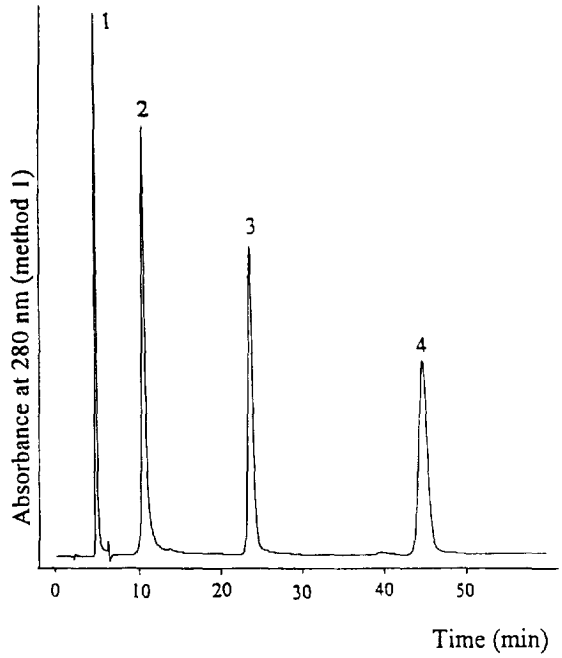

Fig. 1. Chromatogram of standards obtained by method 1. Concentrations are $4 \cdot 10^{-5}$ mol/l (in mobile phase) for all of the compounds. Peaks: 1, gallic acid; 2, chlorogenic acid; 3, caffeic acid; 4, ferulic acid.

method 2 of hydrolyzed and non-hydrolyzed juice samples are shown in Fig. 2, Fig. 3 and Fig. 4.

The repeatability ($n=7$), reproducibility ($n=10$, in one month) and detection limit of the method were 2%, 5% and 0.1 mg/l (mobile phase), respectively. Good response linearity was obtained for ellagic acid between 0.1–10 mg/l by using the wavelength of maximum absorption (252 nm).

For a validation of the extraction, four different known amounts of the free phenolic acids were added to the juices, extracted and then analysed. The values obtained after extrapolation of the curves to non-standard-added juices gave approximately the same values as obtained in the usual analysis. The extraction recoveries are given in Table 1.

3.2. Distribution

The distribution of bound and free phenolic acids in cherry juice, green grape juice and in black grape juice is presented in Table 2. To the best of our knowledge, the present results are the first to show

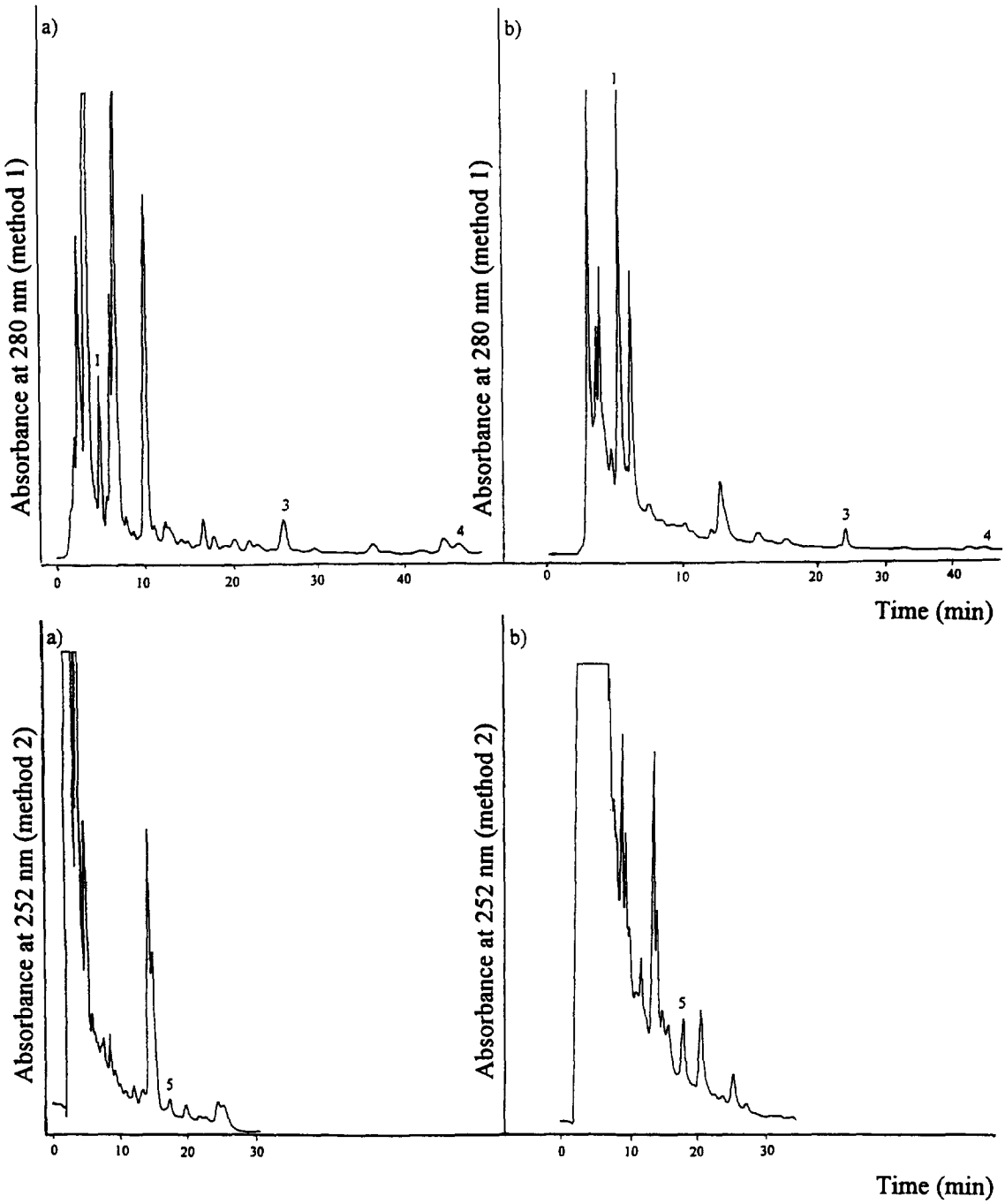

Fig. 2. Chromatograms of green grape juice extracts (a) before hydrolysis and (b) after complete hydrolysis. Peaks: 1=gallic acid; 2=chlorogenic acid; 3=caffeic acid; 4=ferulic acid; 5=ellagic acid. (Top) method 1; (bottom) method 2.

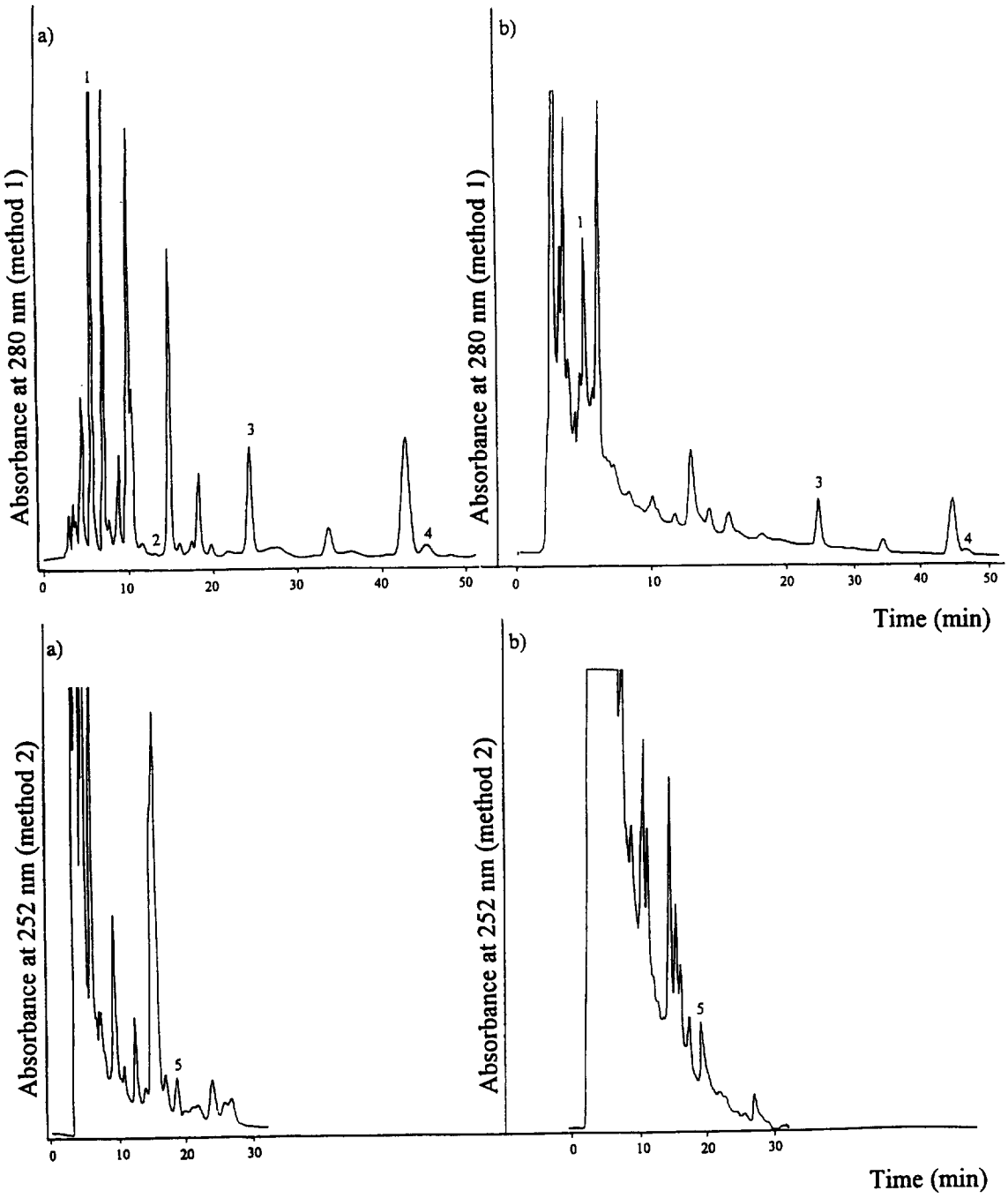

Fig. 3. Chromatograms of black grape juice extracts (a) before hydrolysis and (b) after complete hydrolysis. Peaks as in Fig. 2. (Top) method 1; (bottom) method 2.

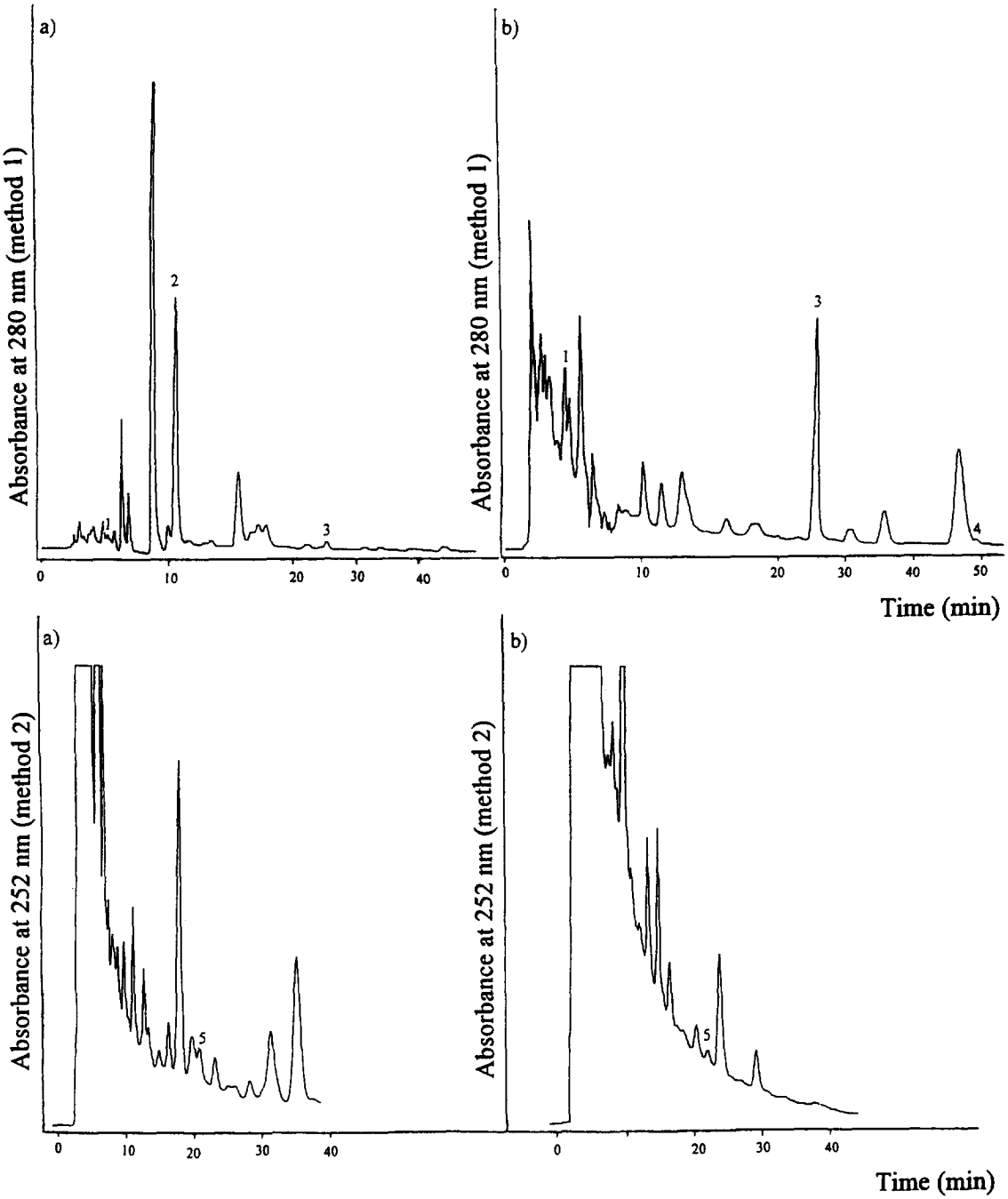

Fig. 4. Chromatograms of cherry juice extracts (a) before hydrolysis and (b) after complete hydrolysis. Peaks as in Fig. 2. (Top) method 1; (bottom) method 2.

Table 1
Extraction recovery

	Amount recovered mean (%)	S.D. (%)
Gallic acid	96.9	3.8
Chlorogenic acid	94.1	4.7
Caffeic acid	98.5	2.1
Ferulic acid	97.9	2.5
Ellagic acid	99.2	1.8

Values are mean and S.D. of 48 findings [four different amounts of each standard added to the three juices (twice) and two injections for each one].

the composition of the bound phenolic acids in all of the juices.

In order to determine the contents of bound phenolic acids, the juices were hydrolyzed under argon. The times for completion of hydrolyses were 48 h for cherry juice and 62 h for green and black grape juices. The rates of hydrolyses were monitored by HPLC by sampling of reaction mixtures after different times of hydrolyses. Fig. 5 Fig. and 6 show the effect of the time of hydrolyses on the concentrations of the phenolic acids for green grape juice and cherry juice, respectively. The curves of black grape juice were similar to those of green grape juice.

The content of chlorogenic acid was very high compared to that of the other phenolic acids in non-hydrolyzed cherry juice and this substance was hydrolyzed rapidly to caffeic acid (Fig. 6).

In the green and black grape juices only minor amounts of phenolic acids occur in the free state, while most are present in conjugated forms that can be liberated by hydrolysis. Complete liberation of

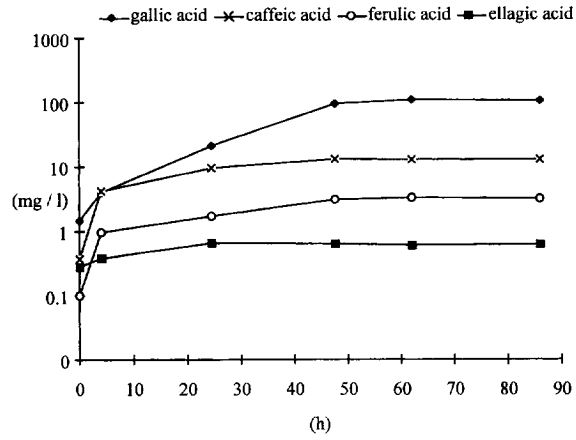

Fig. 5. Experimental dependence of the time of hydrolysis on the concentrations of the phenolic acids for green grape juice.

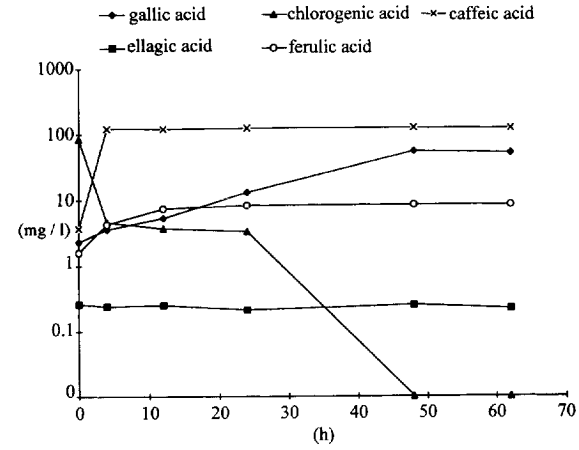

Fig. 6. Experimental dependence of the time of hydrolysis on the concentrations of the phenolic acids for cherry juice.

Table 2
Phenolic acids in juices (mg/l)

	Cherry juice		Green grape juice		Black grape juice	
	Free	Hydrolyzed	Free	Hydrolyzed	Free	Hydrolyzed
Gallic acid	2.3±0.2	54±4	1.45±0.05	110±6	5.24±0.08	79±7
Chlorogenic acid	85±2	0.0	0.0	0.0	0.1±0.05	0.0
Caffeic acid	3.7±0.2	124±3	0.37±0.04	12.9±0.5	1.05±0.04	22±1
Ferulic acid	1.6±0.1	8.4±0.3	0.7±0.03	3.3±0.1	0.1±0.04	5.0±0.3
Ellagic acid	0.26±0.04	0.25±0.08	0.28±0.05	0.6±0.1	0.41±0.05	0.6±0.1

Values are mean±S.D. of eight replicates (two extractions and four injections of each one).

Table 3
Phenolic acids in juices (mg/l) after base hydrolysis under air and under argon

	Cherry juice		Green grape juice		Black grape juice	
	Air	Argon	Air	Argon	Air	Argon
Gallic acid	0.9±0.5	54±4	4.1±0.3	110±6	3.4±0.4	79±7
Caffeic acid	19.9±0.7	124±3	9.0±0.5	12.9±0.5	17.4±0.6	22±1
Ferulic acid	5.2±0.3	8.4±0.3	1.9±0.1	3.3±0.1	3.1±0.3	5.0±0.3
Ellagic acid	0.23±0.08	0.25±0.08	0.4±0.1	0.6±0.1	0.5±0.1	0.6±0.1

Values are mean±S.D. of eight replicates (two extractions and four injections of each one).

caffeic acid and gallic acid in grape juices took place after 48 and 62 h, respectively.

These phenolic acids, especially gallic acid, were unstable when treated with 2 *M* NaOH (pH ca. 12.5) under air and it was necessary to hydrolyze the juices under argon. The data on the comparison of hydrolysis under air versus under argon are given in Table 3. The times of hydrolyses were 62 and 48 h for grape juices and cherry juice, respectively.

For example, Fig. 7 shows the chromatograms of hydrolyzed green grape juice (62 h hydrolysis) under air (a) and under argon (b).

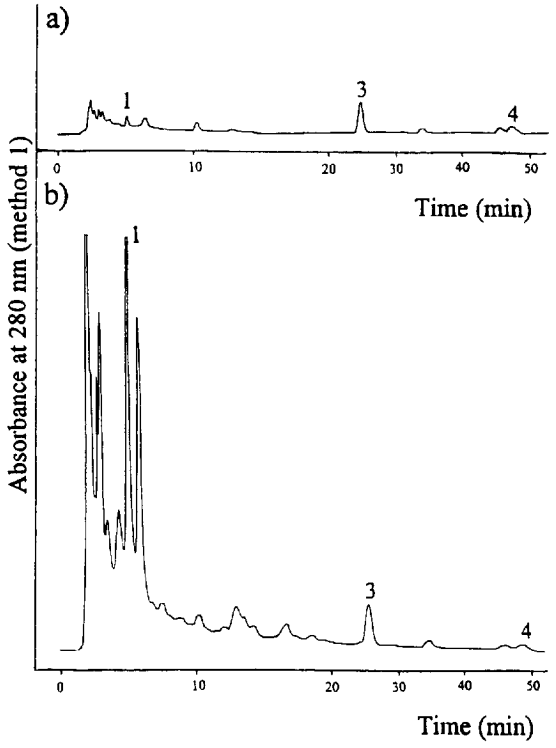

Fig. 7. Chromatograms of green grape juice extracts after 62 h hydrolysis (a) under air and (b) under argon. Dilution of juice extracts and adjustment of the HPLC system were similar for (a) and (b). Peaks as in Fig. 1.

4. Conclusions

Two methods for determining five pharmacologically active phenolic acids in juices were developed. The methods showed good separation of the phenolic acids of cherry juice, black grape juice and green grape juice.

The experimental results indicated that cherry juice contains a high concentration of chlorogenic acid (85 mg/l), which can be hydrolyzed to caffeic acid. It was shown also that the phenolic acids occur mainly in bound forms in green and black grape juices and that their bound gallic acid contents were high (110 and 79 mg/l, respectively) compared to those of the other phenolic acids.

The phenolic acids, components of human foods, have shown interesting activities as inhibitors of mutagenic and carcinogenic processes. However, these studies were performed with pure crystallin substances, while in foods the substances are usually present as bound phenolic acids and little is known about the fate of most plant phenolics after ingestion [4]. In order to utilize the anticarcinogenic properties of the phenolic acids for reduction of risk of human cancer, further studies are necessary. However, we know that chlorogenic acid, a bound caffeic acid (5′-caffeoylquinic acid), is similar to caffeic acid, an inhibitor of carcinogenesis in animal studies and in vitro [2,5–7].

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