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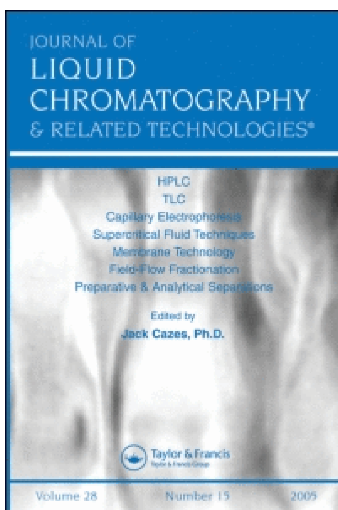

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Isolation and Determination of Phenolic Compounds in Fruit-Green Tea

Eva Blahová[a]; Eva Brandšteterová[a]; Adriana Fabulová[a]
[a] Department of Analytical Chemistry, Faculty of Chemical and Food Technology, Bratislava, Slovakia

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Isolation and Determination of Phenolic Compounds in Fruit-Green Tea

Eva Blahová,* Eva Brandšteterová, and Adriana Fabulová

Department of Analytical Chemistry, Faculty of Chemical and Food Technology, Bratislava, Slovakia

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ABSTRACT

Various HPLC columns with different length, particle size, sorbent properties, and carbon percentage were applied for the determination of main phenolic compounds present in mixed fruit-green tea. Separation characteristics were compared, and some of the tested columns were recommended for the developed HPLC assay. Yields of phenolic compounds were evaluated for water and organic-water mixtures as the extraction media. SPE, using Oasis HLB cartridges, was used for the cleaning up the liquid extracts of fruit-green tea samples.

Key Words: Phenolic compounds; HPLC; Fruit-green tea.

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*Correspondence: Eva Blahová, Department of Analytical Chemistry, Faculty of Chemical and Food Technology, Radlinského 9, 812 37, Bratislava, Slovakia; E-mail: eva.blahova@stuba.sk.

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INTRODUCTION

Phenolic compounds embrace a considerable range of substances that differ in the number of constitutive carbon atoms in conjunction with the structure of the basic phenolic skeleton plus the number and position of hydroxy substituents. They exhibit considerable diversity in terms of acidity, as well as polarity, ranging from hydrophobic to hydrophilic in character. The effect of structural diversity of the phenolics on physico-chemical behaviour such as solubility and partitioning process presents a challenging analytical problem. Due to the large number of closely related phenolic compounds and their structural variations, analytical procedures for the analysis of individual phenolic compounds have been relatively difficult and complicated.[1,2]

These natural antioxidants can exert considerable protection in humans, against aging and cancer caused by free radicals. Phenolic acids include benzoic acids, as well as cinnamic acids. Flavonoids form a diverse range of compounds and can be classified into many classes: flavone, flavonol, flavanone, flavanol, anthocyanin, chalcone, isoflavone, isoflavanone. Phenolic acids and flavonoids in plants may occur in the free form, but they are often glycosylated with various sugars, especially glucose.[3,4]

The main food sources of phenolic compounds are fruit and beverages (juice, wine, tea, coffee, chocolate, beer) and, to a lesser extent, vegetables, dry legumes, and cereals.[5] Tea (*Camellia sinensis*) confers great beneficial effects to the health of consumers, including the effects of reduction of cholesterol, depression of hypertension, antioxidation, antimicrobial, protection against cardiovascular disease, and cancer. Polyphenols, especially catechins and phenolic acids, have been considered the main players in these beneficial effects on the human health.[6]

Some papers describe determination of flavonols (myricetin, quercetin, kaempferol) in green and black tea,[7] in green tea infusions,[8] and in black tea.[9] Other authors developed an HPLC method for the simultaneous determination of some catechins, gallic acid, and caffeine, but without flavonols.[10,11] Many published papers[12–15] deal with the separation and the determination of catechins in tea, and tea infusions.

The aim of the presented work was to test more chromatographic columns in order to develop an HPLC method for the simultaneous determination of a great number of phenolic compounds including phenolic acids, flavanols (catechins), and flavonols, and apply it for the analysis of phenolic compounds present in the mixed fruit-green tea. Liquid–liquid extraction (LLE) and solid-phase extraction (SPE) were applied for the sample preparation before the HPLC assay.

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EXPERIMENTAL

Chemicals and Solutions

Methanol (gradient grade) was purchased from Merck (Slovakia) and formic acid (p.a.) from Lachema (Czech Republic).

Standards of rosmarinic (RA) and ferulic (FA) acids were obtained from the Research Institute of Food Industry, Biocentrum Modra (Slovakia), and sinapic acid (SinA) from Lambda Life, a.s. (Slovakia). Standards of gallic acid (GA), protocatechuic acid (PA), chlorogenic (ChA), *p*-hydroxybenzoic (*p*-HBA), caffeic (CA), syringic (SyrA), *p*-coumaric (*p*-CoA) acid, (+)-catechin (C), (−)-epigallocatechin gallate (EGCG), rutin (R), myricetin (M), quercetin (Q) and kaempferol (K) were supplied by MGP (Czech Republic).

Acidic water with pH = 2.5 was prepared by adding of 1.4 mL of formic acid to 0.5 L of deionized water. Stock solutions of standards (ca. 1 mg/mL) were prepared in methanol and stored in a freezer at −20°C. Working solutions were prepared by diluting the stock solutions with acidic water (pH = 2.5). Solutions were stored in the refrigerator at 5°C and were permanently stable.

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Samples

Samples of mixed fruit-green tea (Tango) were bought in the Slovak local market.

HPLC Equipment

The HPLC analyses were performed using an HP 1100 system (Hewlett-Packard, Waldbronn, Germany), which includes a pump with a degasser, a diode-array detector (DAD), and an HP ChemStation.

Chromatographic Conditions

The following analytical columns were tested for the separation of phenolic analytes: Develosil ODS (100 × 4.6 mm, 3 μm), Watrex (Slovakia), Symmetry Shield C18 (150 × 3.9 mm, 5 μm), Waters (USA), Reprosil C18 (250 × 4 mm, 5 μm), Watrex (Slovakia) and Platinum C18 (250 × 4.6 mm, 5 μm), Alltech (Belgium). Guard columns Symmetry Shield C18 (20 × 3.9 mm, 5 μm), Waters (USA) and Separon SGX C18 (10 × 4 mm,

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7 μm), Watrex (Slovakia) were used to protect analytical columns during analyses of sample extracts.

Gradient elutions with a mobile phase consisting of methanol and water (pH = 2.5) were used for the chromatographic separations. The mobile phase compositions during analysis for the particular columns are in Tables 1(a)–(d). The injection volume was 20 μL and the analyses were carried out at laboratory temperature (25°C). Diode array detector was working in the range of 200–400 nm and the chromatograms were acquired at wavelengths of 254, 260, 280, and 330 nm.

Table 1. Gradient elution systems.

Time [min]	Methanol [%]	Water (pH = 2.5) [%]
(a) Develosil ODS, F = 0.5 mL/min		
0	20	80
8	25	75
18	25	75
20	40	60
23	40	60
40	70	30
(b) Symmetry Shield C18, F = 0.5 mL/min		
0	20	80
10	31	69
17	31	69
32	55	45
40	80	20
(c) Reprosil C18 column, F = 0.5 mL/min		
0	25	75
8	25	75
23	45	55
30	45	55
40	75	25
(d) Platinum C18 column, F = 0.8 mL/min		
0	21	79
20	21	79
35	50	50
45	50	50
55	70	30

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Sample Preparation

Liquid–Liquid Extraction

Samples of tea were extracted in two ways. First, the tea was prepared using water to simulate brewing conditions for usual preparing of tea. The tea bags (1.5 g) were macerated with 250 mL of water with the temperature of 70°C and 100°C for 5 min. In the second method, sample of tea (1.5 g) was ground to powder and extracted with 15 mL of a mixture of methanol : water (80 : 20) in an ultrasonic bath Sonorex (Bandelin electronic (Germany). The number of the extraction steps was 5, and each step took 30 min. The extraction was performed at the temperature of 25°C. The extracts were filtered through nylon microfilters Tessek (Czech Republic) prior to injection into the HPLC column.

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Solid-Phase Extraction

Oasis HLB SPE cartridges (30 mg, 1 cc), Waters (USA) were applied for the clean-up procedure of the tea extracts. The column was conditioned with 1 mL of methanol and then with 1 mL of acidic water (pH = 2.5). Metanolic-aqueous extracts were diluted 1 : 3 with acidic water (pH = 2.5), and 0.5 mL of this solution was applied on the cartridge. Interferences were washed out with 1 mL of a mixture of methanol : water (pH = 2.5) (20 : 80, 10 : 90) or with water (pH = 2.5). The elution was performed with 1, 2, and 3 mL of methanol. The extract was evaporated to dryness and reconstituted in 0.5 mL of the mobile phase.

RESULTS AND DISCUSSION

Chromatographic Conditions

Four reversed-phase C18 columns were tested in this work for the separation of 16 phenolic compounds: Develosil ODS, Symmetry Shield C18, Reprosil C18, and Platinum C18. Basic chromatographic characteristics (capacity factor, chromatographic resolution, and asymmetry factor, calculated in 10% of the peak height) were evaluated. The HPLC separation of phenolic standard compounds, performed by means of the Develosil ODS column, is demonstrated in Fig. 1 with conditions of gradient elution shown in Table 1, case a. This column allows separation of 15 phenolics. Analytes are separated with the resolution sufficient for the quantitative analysis

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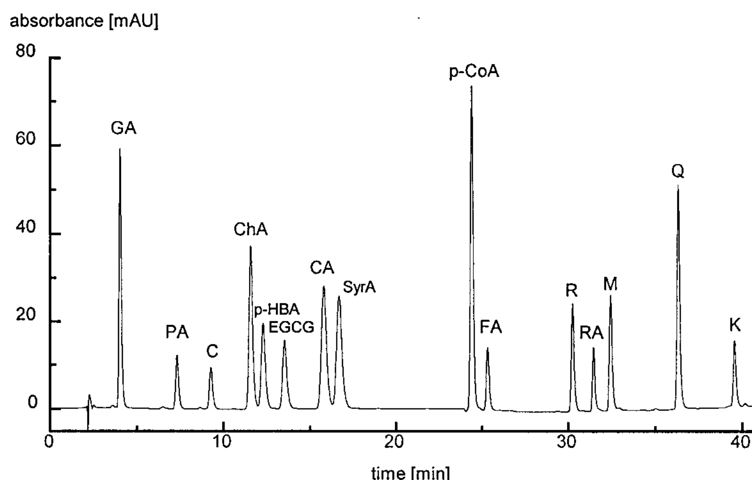

Figure 1. HPLC chromatogram of standard mixture of phenolics. HPLC column: Develosil ODS. Mobile phase: methanol : water (pH = 2.5), gradient elution, F = 0.5 mL/min. Detection: DAD, 280 nm. Injected volume: 20 μL. GA: 5.02 μg/mL, PA: 2.44 μg/mL, C: 4.99 μg/mL, ChA: 10.05 μg/mL, *p*-HBA: 4.93 μg/mL, EGCG: 5.05 μg/mL, CA: 4.99 μg/mL, SyrA: 500 μg/mL, *p*-CoA: 4.75 μg/mL, FA: 1.50 μg/mL, R: 10.04 μg/mL, RA: 2.50 μg/mL, M: 6.49 μg/mL, Q: 10.02 μg/mL, K: 2.99 μg/mL.

($R_{ij} > 1.5$), and from the results in Table 2, case a, it is obvious that peaks are symmetrical with As < 1.47.

Another column used for the separation of phenolic compounds was the Symmetry Shield column, which is capable of separating all 16 analytes with excellent resolution ($R_{ij} > 1.81$). The elution conditions are shown in Table 1, case b and the chromatogram is demonstrated in Fig. 2. Values of asymmetry factors are really excellent (0.96–1.2), and they are listed together with capacity factors and chromatographic resolution of peaks in Table 2, case b.

The next tested column was Reprosil C18. Figure 3 demonstrates the chromatogram with elution conditions listed in Table 1, case c. Chromatographic characteristics are shown in Table 2, case c. It is obvious from these results that syringic acid and rutin are not separated sufficiently ($R_{ij} = 0.78$), and symmetries of peaks are lower then in the previous cases.

The last tested column was Platinum C18 column. The mobile phase composition is listed in Table 1, case d, and the chromatographic separation is shown in Fig. 4. The separation was not successful in the case of chlorogenic and caffeic acid ($R_{ij} = 0.36$), but it was sufficient for the other 14 analytes.

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Table 2. Chromatographic characteristics (k, R_{ij}, As) for analytes using different analytical columns.

	k	R_{ij}	As
(a) Develosil ODS ($n = 2$)			
GA	0.89	10.96	1.47
PA	2.44	5.73	1.33
C	3.36	5.98	1.33
ChA	4.45	1.65	1.28
p-HBA	4.79	2.71	1.27
EGCG	5.38	4.35	1.28
CA	6.45	1.50	1.22
SyrA	6.87	16.38	1.28
p-CoA	10.50	2.86	1.32
FA	10.94	15.12	1.30
R	13.25	4.08	1.35
RA	13.83	3.24	1.32
M	14.29	11.98	1.41
Q	16.12	10.25	1.43
K	17.65		1.39
(b) Symmetry Shield C18 ($n = 2$)			
GA	1.46	13.15	1.20
PA	3.28	6.54	1.12
C	4.33	5.43	1.05
ChA	5.31	1.81	1.16
SyrA	5.66	2.25	1.14
p-HBA	6.11	5.32	1.09
CA	7.35	3.78	1.10
EGCG	8.55	4.00	0.96
SinA	9.74	4.30	1.06
FA	10.65	3.12	1.05
p-CoA	11.29	11.16	1.11
RA	13.32	2.52	1.11
R	13.74	12.73	1.09
M	15.73	10.28	1.15
Q	17.11	8.76	1.16
K	18.29		1.09
(c) Reprosil C18 ($n = 2$)			
GA	0.64	14.48	1.62
PA	1.94	3.51	1.19
C	2.37	6.67	1.25

(*continued*)

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Table 2. Continued.

	k	R_{ij}	As
EGCG	3.27	2.45	1.43
CA	3.55	3.19	1.25
ChA	3.87	5.92	1.18
p-HBA	4.46	1.94	1.23
p-CoA	4.64	15.18	1.22
FA	5.98	1.60	1.20
SyrA	6.11	0.78	1.07
R	6.18	11.84	1.28
RA	7.67	4.00	1.16
M	8.18	4.23	1.16
SinA	8.64	6.96	2.22
Q	9.55	4.84	3.03
K	10.14		2.27
(d) Platinum C18 ($n = 2$)			
GA	0.58	7.23	1.64
PA	1.42	6.20	1.52
C	2.46	2.41	1.41
p-HBA	2.92	9.53	1.45
ChA	4.86	0.36	0.00
CA	4.95	2.43	1.33
SyrA	5.72	1.72	1.28
EGCG	6.19	7.01	1.28
SyrA	6.11	0.78	1.07
FA	8.13	2.25	1.39
SinA	8.41	8.24	1.35
RA	9.31	2.58	1.35
R	9.59	3.03	1.30
M	10.08	6.11	2.13
Q	11.59	6.81	1.85
K	13.36		2.17

Sample Preparation

Liquid–Liquid Extraction

As it was mentioned in Experimental, two extraction methods were used for the isolation of phenolic compounds from the tea samples. The extraction using water as extraction solvent was performed at the temperatures of 70°C

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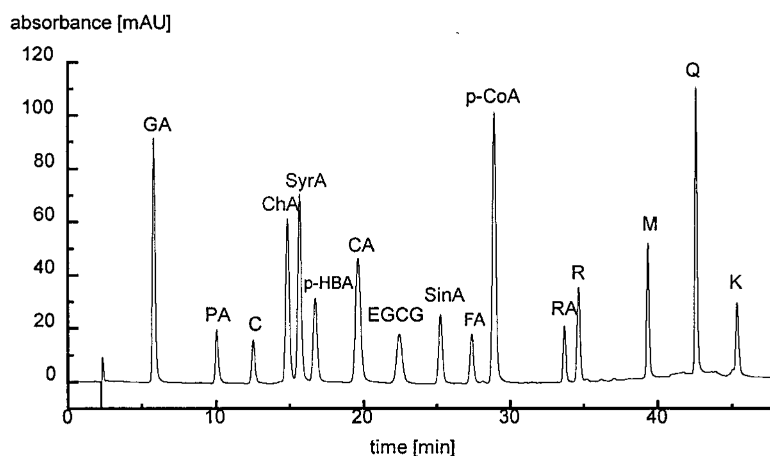

Figure 2. HPLC chromatogram of standard mixture of phenolics. HPLC column: Symmetry Shield C18. Mobile phase: methanol : water (pH = 2.5), gradient elution, F = 0.5 mL/min. Detection: DAD, 280 nm. Injected volume: 20 μl. GA: 10.04 μg/mL, PA: 4.88 μg/mL, C: 9.98 μg/mL, ChA: 20.1 μg/mL, *p*-HBA: 9.86 μg/mL, EGCG: 10.1 μg/mL, CA: 9.98 μg/mL, SyrA: 10.0 μg/mL, *p*-CoA: 9.5 μg/mL, FA: 3.0 μg/mL, R: 20.08 μg/mL, RA: 5.0 μg/mL, M: 12.98 μg/mL, Q: 20.04 μg/mL, K: 5.98 μg/mL.

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and 100°C. Platinum C18 and Symmetry Shield C18 columns were used for the analyses of these extracts and Fig. 5A, B show chromatograms of aqueous tea extract made at 100°C. It is obvious that the use of both columns is possible for the analysis. Gallic acid can be better quantified by means of Platinum C18 column than of the second one. On the other hand, catechin and syringic acid are better separated on Symmetry Shield C18 column. The separation of epigallocatechin gallate and rutin is similar using the both columns. The yields of phenolics at both temperatures are listed in Table 3, and it is obvious from the results that the yields increase with increasing temperature.

The second extraction procedure was performed using an organic-water mixture. Figure 6 shows the influence of volume of the extraction solvent (methanol : water, 80 : 20) on the percentage yield of each compound. It is obvious that four extraction steps are sufficient for the percentage yield >95% for all quantified analytes. The extracts were analysed by means of the same columns as in the previous case and the chromatograms are very similar. The total yields of phenolics after five extraction steps (75 mL of the extraction solvent) are listed in Table 4. It is possible to see from the results in Tables 3

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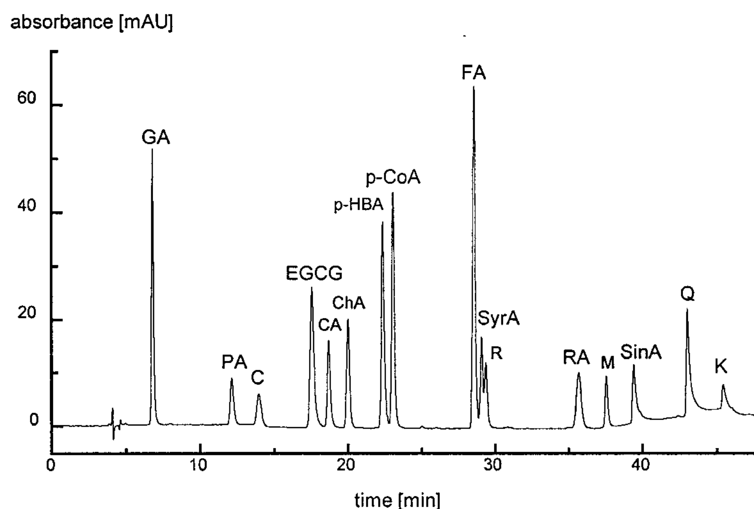

Figure 3. HPLC chromatogram of standard mixture of phenolics. HPLC column: Reprosil C18. Mobile phase: methanol : water (pH = 2.5), gradient elution, F = 0.5 mL/min. Detection: DAD, 280 nm. Injected volume: 20 μL. GA: 5.02 μg/mL, PA: 2.44 μg/mL, C: 4.99 μg/mL, ChA: 10.05 μg/mL, *p*-HBA: 4.93 μg/mL, EGCG: 5.05 μg/mL, CA: 4.99 μg/mL, SyrA: 5.00 μg/mL, *p*-CoA: 4.75 μg/mL, FA: 1.50 μg/mL, R: 10.04 μg/mL, RA: 2.50 μg/mL, M: 6.49 μg/mL, Q: 10.02 μg/mL, K: 2.99 μg/mL.

and 4, that ca 35% of the total amount of gallic acid and epigallocatechin gallate is available in the usual cup of tea ($t = 70°C$). Likewise, 19% of catechine, 56% of syringic acid, and 27% of rutin total content are present in the aqueous extract.

Solid-Phase Extraction

The methanolic, as well as aqueous, tea extracts have yellow colour and contain colored ballast compounds that could damage the analytical column. This is the reason for the further clean up of these extracts before injection into the HPLC system. The SPE method was chosen for this purpose in the present paper, and Oasis HLB cartridges were applied for the clean up of extracts and simultaneous isolation of analysed compounds.

A simple generic assay was modified in the washing and the elution step. First, a mixture of methanol : water (pH = 2.5) (20 : 80) was applied for the washing of interferences. All analytes except gallic acid were retained on the cartridge during washing. This mixture washed out 72.3% of gallic acid,

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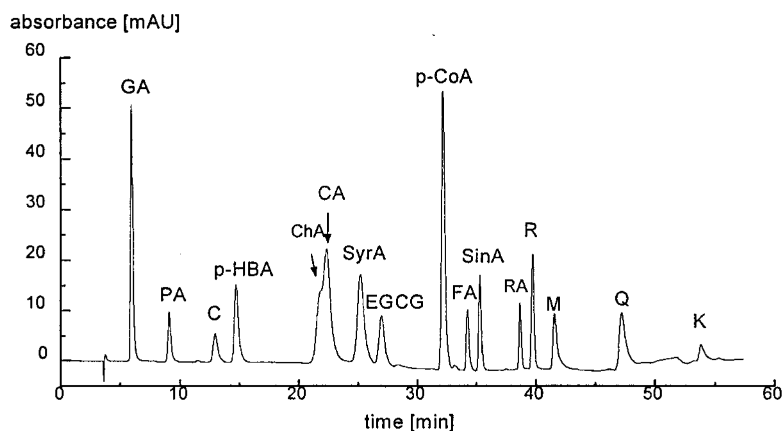

Figure 4. HPLC chromatogram of standard mixture of phenolics. HPLC column: Platinum C18. Mobile phase: methanol : water (pH = 2.5), gradient elution, F = 0.8 mL/min. Detection: DAD, 280 nm. Injected volume: 20 μL. GA: 10.04 μg/mL, PA: 4.88 μg/mL, C: 9.98 μg/mL, ChA: 20.1 μg/mL, *p*-HBA: 9.86 μg/mL, EGCG: 10.1 μg/mL, CA: 9.98 μg/mL, SyrA: 10.0 μg/mL, *p*-CoA: 9.5 μg/mL, FA: 3.0 μg/mL, R: 20.08 μg/mL, RA: 5.0 μg/mL, M: 12.98 μg/mL, Q: 20.04 μg/mL, K: 5.98 μg/mL.

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therefore, it was necessary to decrease the content of methanol in the washing solvent. Further, a mixture containing 10% of methanol was used but 23.7% of gallic acid was removed from the column using this solvent. The last procedure included water (pH = 2.5) without the presence of methanol, and this solvent washed out 9.6% of aforementioned acid.

The SPE elution step was performed with methanol. After elution with 1 mL of this solvent, all compounds except epigallocatechin gallate were fully eluted. However, this volume of methanol was not sufficient, as only 79% of epigallocatechin gallate was eluted. Therefore, volumes of 2 and 3 mL of methanol have also been tested, and it was found that 93% of retained epigallocatechin gallate was eluted with 2 mL and almost 100% with 3 mL of methanol. The recoveries of all analytes with RSD values are shown in Table 5.

As it was mentioned in the previous part of this work, five phenolic compounds were quantified in tea: gallic acid, catechin, syringic acid, epigallocatechin gallate, and rutin. So, it was not necessary to use complex elution conditions. Simpler gradient elution has also been developed for Platinum C18 and Symmetry Shield C18 columns and these conditions are listed in Table 6. Separation characteristics for such systems are shown in

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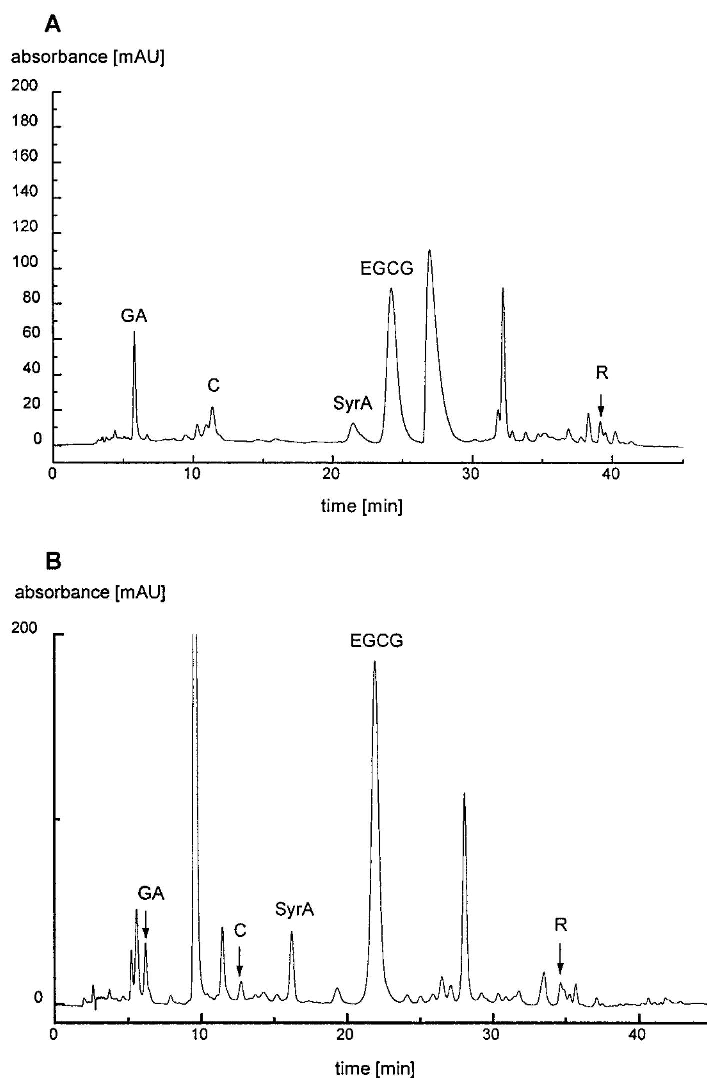

Figure 5. HPLC chromatogram of aqueous extract of tea (100°C). Mobile phase: methanol : water (pH = 2.5), gradient elution. Detection: DAD, 280 nm. Injected volume: 20 μL. A: HPLC column: Platinum C18, F = 0.8 mL/min. B: HPLC column: Symmetry Shield C18, F = 0.5 mL/min.

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Table 3. Yields and RSD values of GA, C, SyrA, EGCG and R from tea at different temperatures ($n = 3$).

	100°C		70°C	
	Yield [mg/g]	RSD [%]	Yield [mg/g]	RSD [%]
GA	0.32	1.17	0.27	1.49
C	0.61	1.13	0.41	1.47
SyrA	0.48	0.63	0.37	0.46
EGCG	12.97	1.49	8.58	1.49
R	0.73	0.46	0.37	0.59

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Table 7, and Fig. 7A, B show chromatograms of standard mixtures of quantified analytes obtained on these two columns, using simpler gradient systems. It is obvious from the results (k values) in Table 7 and from Fig. 7A, B, that the separation order of syringic acid and epigallocatechin gallate is different for a particular column using these simpler gradient conditions. Figure 8 demonstrates chromatograms of tea extract before (a) and after (b) SPE obtained on a Platinum C18 column. It is possible to see that some

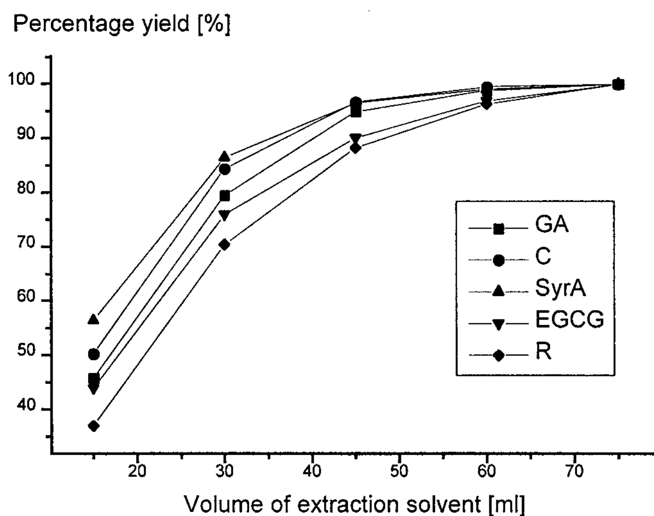

Figure 6. Relationship between volume of extraction solvent and percentage yield of phenolics. Extraction solvent: methanol : water (80 : 20), $t = 25$°C.

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Table 4. Yields and RSD values of GA, C, SyrA, EGCG and R from tea using methanol : water (80 : 20) as extraction solvent.

	GA	C	SyrA	EGCG	R
Yield [mg/g]	0.78	2.12	0.66	22.21	1.35
RSD [%]	0.80	1.35	0.73	1.63	0.96

Table 5. Recoveries of GA, C, SyrA, EGCG and R after SPE procedure.

	GA	C	SyrA	EGCG	R
Recovery (%)	90.1	100.2	99.2	99.0	100.1
RSD (%)	1.38	0.52	1.01	1.51	0.38

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Table 6. Simpler gradient elution system for Symmetry Shield C18 a Platinum C18, F = 0.8 mL/min.

Time [min]	Methanol [%]	Water (pH = 2.5) [%]
0	30	70
12	30	70
15	50	50

Table 7. Chromatographic characteristics (k, R_{ij}, As) for analytes using simpler gradient elution system for Symmetry Shield C18 a Platinum C18 column. ($n = 2$).

	Symmetry Shield C18			Platinum C18		
	k	R_{ij}	As	k	R_{ij}	As
GA	0.68	7.04	1.27	0.28	5.90	1.64
C	1.98	5.81	1.18	0.76	5.27	1.33
SyrA	3.56	4.28	1.11	2.11	12.20	1.43
EGCG	5.75	12.06	1.18	1.38	4.85	1.37
R	11.75	—	1.03	4.69	—	1.16

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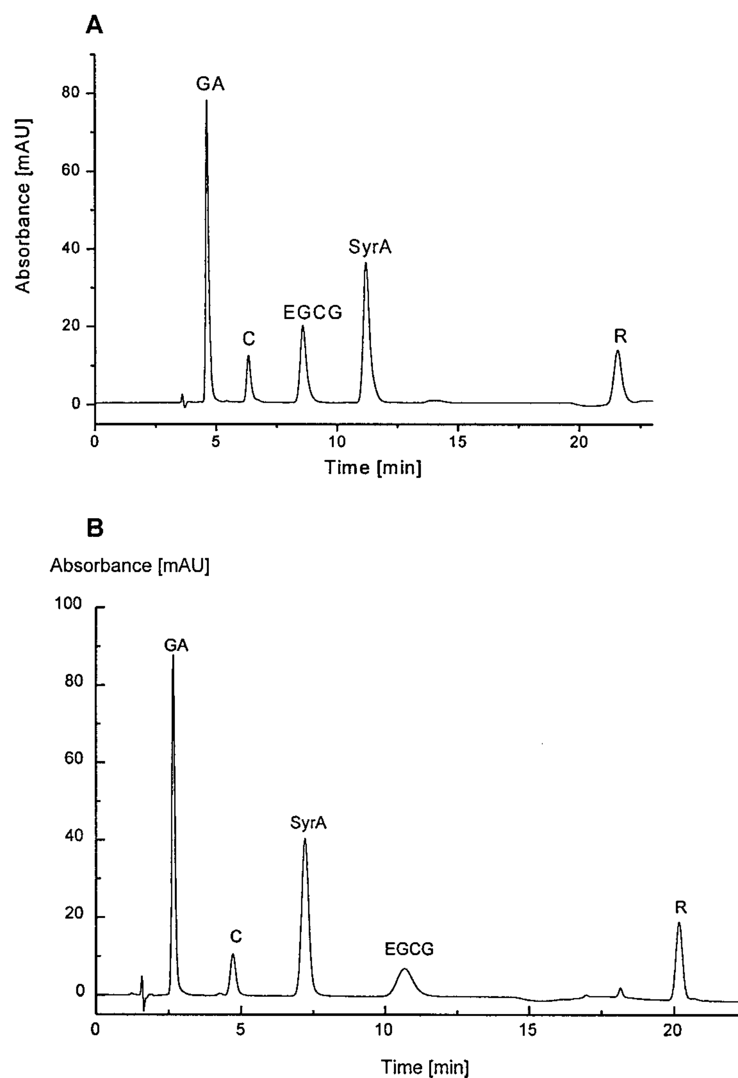

Figure 7. HPLC chromatogram of standard mixture of phenolics. Mobile phase: methanol : water (pH = 2.5), gradient elution, F = 0.8 mL/min. Detection: DAD, 280 nm. Injected volume: 20 μL. GA: 10.04 μg/mL, C: 9.98 μg/mL, EGCG: 10.1 μg/mL, SyrA: 10.0 μg/mL, R: 20,08 μg/mL A: HPLC column: Platinum C18. B: HPLC column: Symmetry Shield C18.

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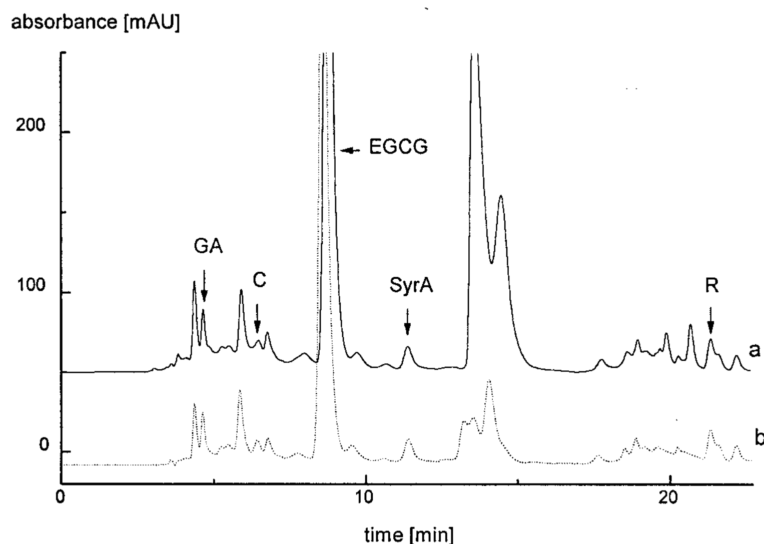

Figure 8. HPLC chromatogram of tea extract. HPLC column: Platinum C18. Mobile phase: methanol : water (pH = 2.5), gradient elution, F = 0.8 mL/min. Detection: DAD, 280 nm. Injected volume: 20 μL. (a) Before SPE, (b) after SPE.

interfering compounds were partly removed after the SPE procedure. The increase of SPE clean-up effect would be possible with the use of additional methanol into the washing solution. But an increased content of organic modifier during the washing step causes the decrease of recovery of gallic acid. So, if the analytes are present in high concentration, it is possible to also choose the assay with lower recoveries but better removing for interfering compounds.

The calibration curves for all five determined analytes were obtained from the peak areas [mV s] vs. concentrations [μg/mL], and showed good linearity (gallic acid (1–21 μg/mL): $y = -14 + 69.36x$, $r = 0.9998$, SD = 11.04; catechin (3–40 μg/mL): $y = -27.12 + 18.15x$, $r = 0.9992$, SD = 12.26; syringic acid (2–17 μg/mL): $y = 5.10 + 69.88x$, $r = 0.9997$, SD = 11.68; epigallocatechin gallate (45–500 μg/mL): $y = 10.12 + 45.08x$, $r = 0.9999$, SD = 7.26; rutin (4–32 μg/mL): $y = -4.17 + 16.99x$, $r = 0.9999$, SD = 1.82). The limits of detection were the following: GA: 12 ng/mL, C: 21 ng/mL, EGCG: 10 ng/mL, SyrA: 23 ng/mL, R: 13 ng/mL.

The developed HPLC method could be applied for the simultaneous determination of 16 phenolic compounds. Two different preparation techniques were developed: LLE for the isolation of phenolics from tea

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samples and SPE for the clean up of extracts obtained by LLE. The simple LLE with hot water and with methanolic-aqueous mixture were compared. Solid phase extraction with novel polymer sorbent, Oasis HLB, was recommended as the effective and simple isolation and clean-up technique.

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