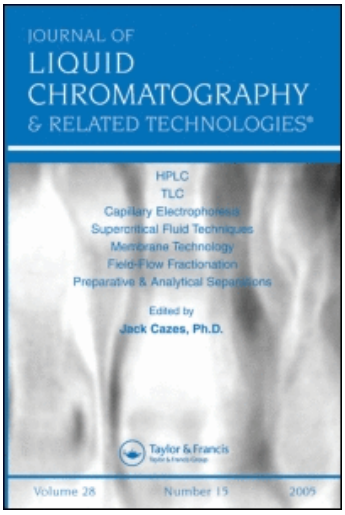

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Chromatographic Determination of Derivatives of p-Hydroxybenzoic Acid in *Melissa officinalis* by HPLC

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Abstract: This paper presents a fast method for the determination of phenolic compounds–benzoic acids in *Melissa officinalis.* Soxhlet extraction has been optimized for the isolation of benzoic acids (gallic acid, p-hydroxybenzoic acid, protocatechuic acid, gentisic acid, vanillic acid, and syringic acid) from plant material. The conditions of Soxhlet extraction, such as solvent composition and extraction time were studied. The efficient extraction of benzoic acids was achieved with a mixture of methanol–water (80 : 20, v/v) within 1 hour. Four RP-18 chromatographic columns were tested for the separation of the compounds. The complete separation of six benzoic acids was achieved on two analytical columns. The basic chromatographic characteristics were evaluated and an HPLC method using Alltima C18–Rocket, one of the suitable columns, was validated for determination of analytes. A mobile phase, which consisted of acetonitrile and formic acid (0.074 mol/L) with a linear gradient at a flow rate 1.5 mL/min was applied for the determination of benzoic acids in extracts of plant material.

Keywords: p-Hydroxybenzoic acid, *Melissa officinalis*, HPLC, Determination

INTRODUCTION

Benzoic acids comprise a class of phenolic acids that belong to a wide group of phenolic compounds. Phenolic compounds are widespread secondary plant

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metabolites. They constitute a large group of naturally occurring compounds, showing a broad spectrum of biological activities. Derivatives of benzoic and cinnamic acids (so-called phenolic acids) are one group of the many secondary metabolites implicated in allelopathy.[1] In addition, some phenolic acids are shown to exert some antifungal effects. Phenolic acids are widely distributed in the natural plants, e.g., fruits, vegetable, various medicinal, and other plants. Phenolic acids occur in plants in different concentrations and, of course, each plant sample could be specific enough for the presence of different phenolic acids and their derivatives in combination with the other groups of phenolic compounds.[2]

Melissa officinalis, which could be used for treatment of several medicinal conditions such as headaches, gastro-intestinal disorders, rheumatism, and nervousness,[3] belongs to the *Lamiacae* family, which is a rich source of plant species containing large amounts of phenolic acids.

Liquid extraction is a preferable preseparation technique for phenolic compounds. Commonly used extraction solvents are ethyl acetate, diethyl ether, methanol, and aqueous methanol, but the majority of the free phenolic compounds can be extracted with alcohols (methanol or ethanol) or alcohol–water mixtures.[4] When using alcohol–water mixtures, repeated extraction or reflux for 1 h are necessary to extract free phenolic acids.[5]

HPLC is the most commonly applied chromatographic method for the variety of phenolic compounds in foods and plants. Reversed–phase chromatography is the most popular mode. In most cases, the reported systems for the separation of phenolics utilize silica based C_{18} bonded-phase columns and organic-water (pH 2-3) or organic buffer mobile phases. Gradient elution is primarily used.[2,6–10]

This study develops a simple and effective method for the isolation and HPLC determination of six phenolic acids, belonging to benzoic acids, from the medicinal plant lemon balm (*Melissa officinalis*). The Soxhlet extraction for the isolation of benzoic acids was optimized and appropriate conditions for HPLC analysis were evaluated.

EXPERIMENTAL

Chemicals and Reagents

Phenolic compounds-gallic acid, p-hydroxybenzoic acid were purchased from Merck (Germany), protocatechuic acid was obtained from the Research Institute of Food Industry (Biocentrum Modra, Slovakia), gentisic acid, vanillic acid from MGP (Czech Republic) and syringic acid was supplied by Fluka (Switzerland). Acetonitrile and methanol (both HPLC grade) were obtained from Merck (Germany). Formic acid (p.a.) was supplied from Lachema (Czech Republic).

Stock standard solutions of each of the phenolic compounds (ca. 1 mg/mL) were prepared in methanol and stored in a freezer at −20°C. The stability of the stock solutions was controlled for one month and no change in concentrations was observed. Working solutions were prepared daily by mixing and diluting the stock solutions with a mixture of methanol and formic acid (0.074 mol/L), 50 : 50, v/v.

Plant Material

A plant sample of lemon balm (*Melissa officinalis*) was commercially available and it was purchased in a local pharmacy.

LC Instrumentations, Columns, and Conditions

An HP 1100 system (Hewlet-Packard, Germany), consisting of a pump with a degasser, a diode-array detector (DAD), a 20 μL injector and a HP ChemStation. Chromatographic columns tested: Alltima C18-Rocket (53 × 7 mm, 3 μm) (Alltech, Belgium), Reprosil C18 (250 × 4 mm, 5 μm), Develosil C18 (100 × 4.6 mm, 3 μm) and Separon SGX C18 (125 × 4 mm, 7 μm) obtained from Watrex (Slovakia). Gradient elution systems were developed utilizing different mobile phases. The gradient elution systems utilizing methanol with formic acid (0.074 mol/L) are listed in Table 1, and the systems utilizing acetonitrile and formic acid (0.074 mol/L) are summarized in Table 2. The flow rates are also shown in Tables 1 and 2.

The Alltima C18-Rocket (53 × 7 mm, 3 μm) (Alltech, Belgium) with a guard column Separon SGX C18 (10 × 4 mm, 7 μm) (Watrex, Slovakia) were finally used for the analysis of plant extracts. The mobile phase

Table 1. Gradient elution systems and flow rates using methanol and formic acid (0.074 mol/L) in the mobile phase

Column	Time (min)	Methanol (%)	Formic acid (0.074 mol/L), (%)	Flow rate (mL/min)
Alltima C18-Rocket, 53 × 7 mm, 3 μm	0	15	85	1.7
	8	35	65	
Reprosil C18, 250 × 4 mm, 5 μm	0	25	75	0.5
	8	35	65	
	20	35	65	
Develosil C18, 100 × 4.6 mm, 3 μm	0	20	80	0.5
	15	35	65	
Separon SGX C18, 125 × 4 mm, 7 μm	0	20	80	0.7
	15	30	70	

Table 2. Gradient elution systems and flow rates using acetonitrile and formic acid (0.074 mol/L) in the mobile phase

Column	Time (min)	Acetonitrile (%)	Formic acid (0.074 mol/L), (%)	Flow rate (mL/min)
Alltima C18-Rocket,	0	7	93	1.8
53 × 7 mm, 3 μm	8	11	89	
Reprosil C18,	0	10	90	0.7
250 × 4 mm, 5 μm	15	22	78	
	20	22	78	
Develosil C18,	0	7	93	0.7
100 × 4.6 mm, 3 μm	15	20	80	
Separon SGX C18,	0	7	93	0.7
125 × 4 mm, 7 μm	15	20	80	

consisted of acetonitrile and formic acid (0.074 mol/L) with linear gradient (from 5% to 15% of acetonitrile in 12 min). All analysis were carried out at laboratory temperature. The diode-array detector worked in the range of 200–400 nm and the chromatograms were acquired at wavelengths of 254, 280, and 340 nm.

Extraction Procedure

Extraction of plant material (*Melissa officinalis*) was performed by Soxhlet extraction. Plant material was ground to a powder. The powder (0.7 g) was extracted with 80 mL of the mixture of methanol–water (80 : 20, v/v) for 1 hour. The extracts were filtered through nylon microfilters (Watrex, Slovakia) prior to the injection into the LC system.

The powder (0.7 g) was spiked with 1 mL of the mixture of standards in order to determine recoveries of all analytes.

An aqueous extract of *Melissa officinalis* was prepared in accordance with the procedure recommended for its consumption. Boiling water, 200 mL (100°C) was poured over a tea spoon (0.8 g) of plant material and allowed to brew for 10 minutes. The aqueous extract was filtered through a nylon microfilter prior to the HPLC analysis.

RESULTS AND DISCUSSION

Reversed phase chromatography is the most popular mode of analytical liquid chromatography for phenolic compounds. In most cases, the systems for the separation of phenolics in foods are carried out on reversed phase chromatography on silica-based C_{18} bonded-phase columns. At the beginning of the

study, a suitable analytical column, which would offer sufficient separation for a quantification of analytes, was estimated. Four reversed-phase C_{18} analytical columns with different properties (variety in their particle size, column length, and different sorbent endcapping) were tested for this purpose. The effectiveness of the HPLC separation was evaluated using standard solution containing 6 benzoic acids (ca. 10 μg/mL): gallic acid, p-hydroxybenzoic acid, protocatechuic acid, gentisic acid, vanillic acid, and syringic acid. The mobile phase consisting of methanol and formic acid (0.074 mol/L) was investigated. Different gradient elution systems were applied on the analytical columns (Table 1). Of course, differences in retention times and peak symmetries were observed (Figure 1). It is obvious that the separation of the analytes was not complete. The baseline separation of gallic acid, protocatechuic acid, vanillic acid, and syringic acid were achieved. But the separation of p-hydroxybenzoic acid and gentisic acid was not sufficient (R_{ij} was lower than 1.0) on all columns tested. Therefore, another mobile phase consisting of acetonitrile and formic acid (0.074 mol/L) was selected for the study. The gradient elution programs applied are shown in Table 2. The complete separation of six benzoic acids was achieved using Alltima C18-Rocket and

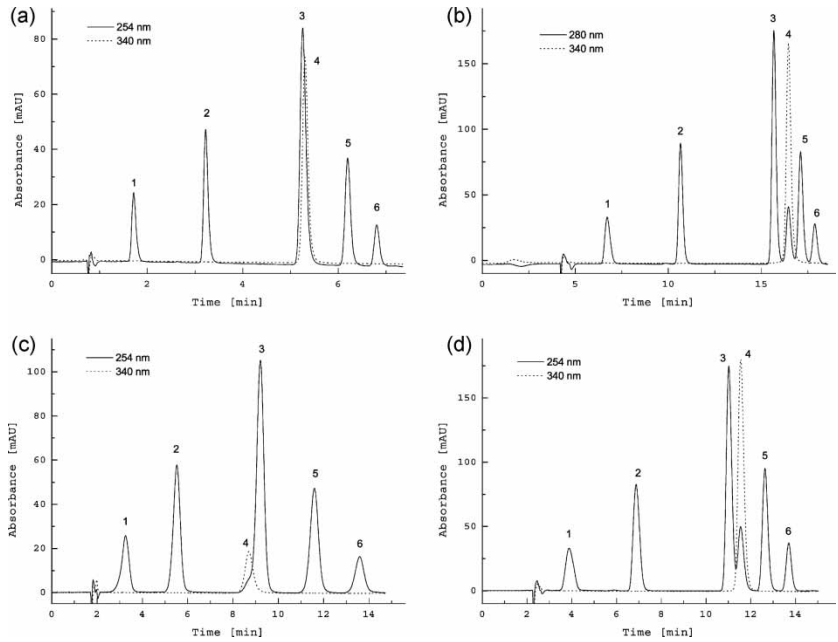

Figure 1. Chromatographic separation of benzoic acids using analytical columns: (a)–Alltima C18-Rocket, (b)–Reprosil C18, (c)–Separon SGX C18, (d)–Develosil C18. Mobile phase: methanol/formic acid (0.074 mol/L), gradient elution, see Table 1. 1-gallic acid, 2-protocatechuic acid, 3-p-hydroxybenzoic acid, 4-gentisic acid, 5-vanillic acid, 6-syringic acid.

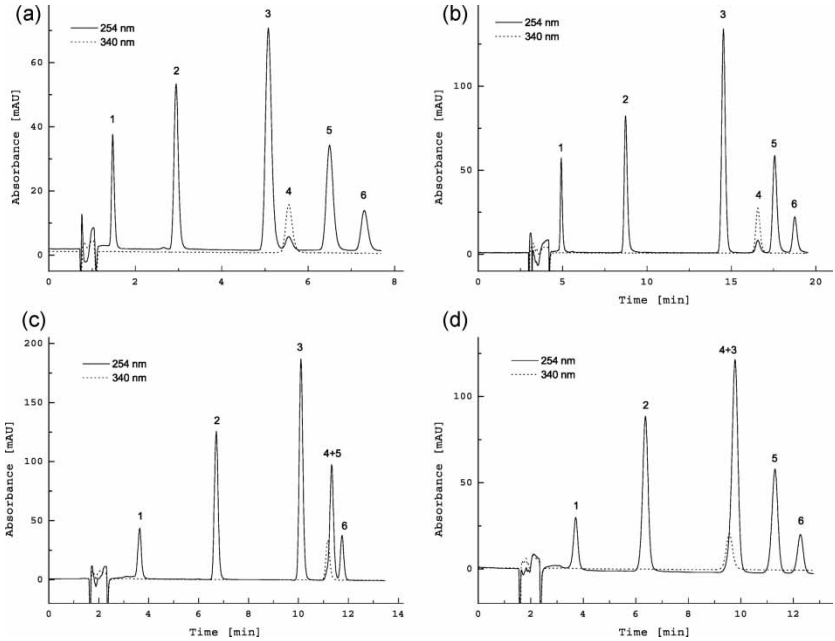

Figure 2. Chromatographic separation of benzoic acids using analytical columns: (a)–Alltima C18-Rocket, (b)–Reprosil C18, (c)-Separon SGX C18, (d)–Develosil C18. Mobile phase: acetonitrile/formic acid (0.074 mol/L), gradient elution, see Table 2. 1-gallic acid, 2-protocatechuic acid, 3-p-hydroxybenzoic acid, 4-gentisic acid, 5-vanillic acid, 6-syringic acid.

Reprosil C18 columns. It is shown in Figure 2a, 2b. The improvement of the separation was not observed in the cases of Develosil C18 and Separon SGX C18 columns (Figure 2c, 2d). The chromatographic characteristics for Alltima C18-Rocket and Reprosil C18 columns are summarized in Table 3.

As can be seen in Figure 2a, fast separation can be performed using an Alltima C18-Rocket. Therefore, the Alltima C18-Rocket column was chosen for the next experiments, for the analysis of plant extracts. The mobile phase consisting of acetonitrile and formic acid (0.074 mol/L), with a linear gradient (from 5% to 15% of acetonitrile in 12 min) and the flow rate of 1.5 mL/min were applied. The main validation parameters (listed in Table 4), in order to check the suitability of the chromatographic assay for the determination of 6 benzoic acids using the Alltima C18-Rocket column, were evaluated. The repeatability of the retention times was determined (from 10 injections of standards at different concentrations). The repeatability of retention times for all standards was under 1.3%. The linearity of the method was evaluated by the parameters of the calibration lines. The calibration lines were constructed by plotting peak area and standard concentration values in the range of 1.0–10.0 μg/mL for each analyte (6 con-

Table 3. Capacity factor (k), chromatographic resolution (R_{ij}), asymmetry factor (A_s) values, and number of theoretical plates (n) for all analytes using 2 different analytical columns

	k	R_{ij}	A_s	n/m
Alltima C18-Rocket, 53 × 7 mm, 3 μm				
Gallic acid	0.94	8.70	1.3	39,067
Protocatechuic acid	2.86	8.71	1.2	61,027
p-Hydroxybenzoic acid	5.65	1.66	1.2	96,957
Gentisic acid	6.28	3.06	1.2	106,737
Vanillic acid	7.51	2.52	1.1	126,059
Syringic acid	8.56		1.1	157,306
Reprosil C18, 250 × 4 mm, 5 μm				
Gallic acid	0.59	12.51	1.3	34,002
Protocatechuic acid	1.82	13.84	1.3	33,870
p-Hydroxybenzoic acid	3.68	4.33	1.1	69,436
Gentisic acid	4.34	1.92	1.1	67,212
Vanillic acid	4.66	2.31	1.2	73,191
Syringic acid	5.05		1.2	84,123

centrations, each measured in triplicate) and evaluated by linear regression. The limits of quantification (LOQs) were calculated from the peak height based on signal-to-noise ratio of 10.

The extraction of benzoic acids from plant material was examined. The duration of Soxhlet extraction was investigated at first. Plant powder, 0.7 g, was extracted by Soxhlet extraction with 80 mL of methanol. The time of extraction was 1 h, 4 h, and 8 h, and the extraction yields of analytes were compared (Table 5). It can be seen that the extension of the extraction time

Table 4. Parameters of calibration lines $y = a + bx$, limit of quantification LOQ, and the repeatability of retention times on column Alltima C18-Rocket (53 × 7 mm, 3 μm)

	Slope (a)	Intercept (b)	Correlation coefficient (r^2)	LOQ (μg/mL)	Repeatability of retention times[a] (%)
Gallic acid	2.70	17.02	0.9991	0.010	1.2
Protocatechuic acid	7.94	41.65	0.9997	0.010	1.2
p-Hydroxybenzoic acid	2.92	16.70	0.9995	0.015	1.2
Gentisic acid	0.06	83.03	0.9935	0.040	1.2
Vanillic acid	21.67	40.57	0.9998	0.020	1.1
Syringic acid	9.53	16.52	0.9884	0.020	1.0

[a]RSD from ten measurements.

Table 5. Yields (μg/g of plant material) of benzoic acids using 80 mL of methanol for Soxhlet extraction and various time of extraction (1 h, 4 h, 8 h)

	1 h	4 h	8 h
Gallic acid	56.3	—	—
Protocatechuic acid	31.7	21.2	28.5
p-Hydroxybenzoic acid	11.6	5.7	10.8
Gentisic acid	—	—	—
Vanillic acid	3.6	5.4	3.9
Syringic acid	235.7	101.3	134.7

RSD were 3.8–5.2% (n = 3)

from 1 h to 8 h did not considerably change the content of a majority of isolated benzoic acids. Even the loss of gallic acid during a longer time of extraction was observed. Therefore, 1 hour of the duration of the Soxhlet extraction was used in the next experiments. The composition of extraction solutions was also studied in the following experiments. The extraction of plant material with mixtures of methanol–water (60 : 40, v/v and 80 : 20, v/v) were carried out. A powder, 0.7 g, was extracted with 80 mL of the extraction agent for 1 h by Soxhlet extraction. The extraction yields (μg/g of plant material) are listed in Table 6. Higher amounts of analytes were isolated using additional water in the extraction agent. The results were better using methanol–water (80 : 20, v/v) than in the case of methanol–water (60 : 40, v/v).

The mixture of methanol–water (80 : 20, v/v) was used for the isolation of benzoic acids before HPLC determination. The extraction recoveries of all analytes were determined for this extraction procedure and the recoveries of

Table 6. Yields (μg/g of plant material) of benzoic acids using 80 mL of different extraction solution (methanol, methanol with 40% of water and methanol with 20% of water) for Soxhlet extraction, duration of extraction 1 h

	Methanol (μg/g)	Methanol/water 60 : 40, v/v (μg/g)	Methanol/water 80 : 20, v/v (μg/g)	Recovery[a] (%)
Gallic acid	56.3	11.7	16.4	101.8
Protocatechuic acid	31.7	65.9	75.3	100.8
p-Hydroxybenzoic acid	11.6	11.7	10.5	91.3
Gentisic acid	—	—	—	—
Vanillic acid	3.6	23.0	14.5	88.9
Syringic acid	235.7	451.3	540.8	45.3

RSD were 4.3–5.3% (n = 3).
[a]RSD were 4.5–5.5% (n = 3).

all benzoic acids exceeded 88%. In the case of syringic acid, the recovery was only 45.3%. This can be caused by interferance of the peaks of syringic acid and some other unknown compound with absorption maximum at 290 nm and 325 nm. Syringic acid has only one absorption maximum at 280 nm. Therefore, different wavelengths for the quantification of syringic acid can not be used.

The chromatogram of the extract of *Melissa officinalis* is shown in Figure 3.

Lemon balm (*Melissa officinalis*) is consumed in the form of aqueous extracts (200 mL of boiling water poured over the tea spoon of lemon balm is brewed for 10 minutes). Therefore, the amounts of benzoic acids released into water were determined. The extraction yields are shown in Table 7. They are given in μg/g of plant material in order to compare them with the amounts of benzoic acids in lemon balm determined by analysis of water–methanol (80 : 20, v/v) extracts. It was found out that 73–76% of gallic

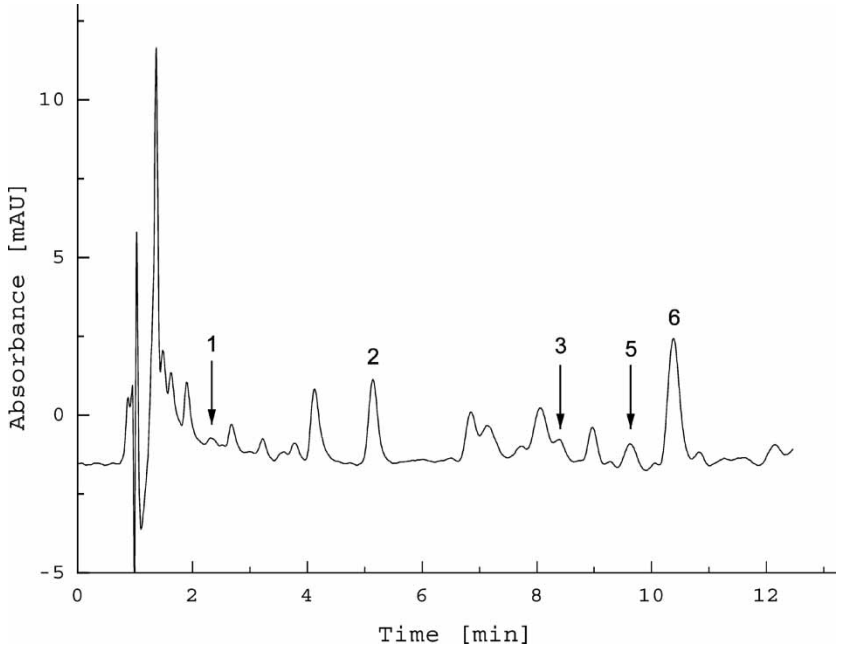

Figure 3. HPLC chromatogram of the extract of *Melissa officinalis* after Soxhlet extraction with 80 mL of methanol–water (80 : 20, v/v), duration of extraction 1 h. HPLC conditions: Column: Alltima C18-Rocket (53 × 7 mm, 3 μm) with precolumn Separon SGX C18 (10 × 4 mm, 7 μm), mobile phase: acetonitrile-formic acid (0.074 mol/L) with the linear gradient (from 5% to 15% of acetonitrile in 12 min), flow-rate 1.5 mL/min, DAD 254 nm, injection volume 20 mL. 1-gallic acid, 2-protocatechuic acid, 3-p-hydroxybenzoic acid, 5-vanillic acid, 6-syringic acid.

Table 7. Yields (μg/g of plant material) of benzoic acids using 200 mL of water (100°C) for the extraction, duration of the extraction 10 min. (according the instructions for the preparation)

	Gallic acid	Protocatechuic acid	p-Hydroxybenzoic acid	Gentisic acid	Vanillic acid	Syringic Acid
(μg/g)	12.5	55.3	7.7	—	9.3	109.3

RSD were 2.5–3.7% (n = 3).

acid, protocatechuic acid, and p-hydroxybenzoic acid present in plant material are released into water. In the case of vanillic acid it is about 64%. Only about 20% of syringic acid is released into water.

In conclusion, the presented extraction procedure of analytes into methanol–water (80 : 20, v/v) and HPLC analysis on the Alltima C18-Rocket analytical column is applicable for the isolation and determination of gallic acid, protocatechuic acid, p-hydroxybenzoic acid, gentisic, and vanillic acid in *Melissa officinalis.* But, no gentisic acid was detected in the methanolic extracts, or extracts of aqueous methanol.

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