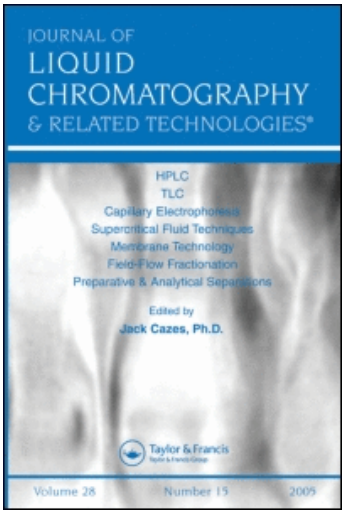

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Abstract: Several extraction techniques, such as Soxhlet extraction, solid phase extraction using molecularly imprinted polymer, matrix solid phase dispersion, and supercritical fluid extraction were evaluated for the isolation and purification of phenolic compounds, e.g., benzoic acids from natural samples of *Melissa officinalis.* The extracts of benzoic acids were analyzed by high performance liquid chromatography (HPLC) in reversed phase modus (C_{18} column) and under gradient elution (acetonitrile/0.074 mol/L formic acid). The results showed that the recovery rates of gallic acid, p-hydroxybenzoic acid, protocatechuic acid, gentisic acid, vanillic acid, and syringic acid from biological materials by MSPD was equivalent with and, in fact, higher than that of conventional extraction methods.

Keywords: Benzoic acid derivatives, *Melissa officinalis*, Soxhlet extraction, Solid phase extraction using molecularly imprinted polymer—MISPE, Matrix solid phase dispersion—MSPD, Supercritical fluid extraction—SFE

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INTRODUCTION

Melissa officinalis (lemon balm) is the herb belonging to the group of native medicinal plants *Lamiaceae*. *Melissa officinalis* contains various constituents like flavonoids, essential oils, bitters, phenolic acids, tannins, triterpenes, and resin. Flavonoids and phenolic acids belong to phenolic compounds, which appear to be responsible for lemon balm's anti-herpes and thyroid-regulating actions.[1] The leaves, stems, and flowers of lemon balm are used medicinally. It could be used for treatment of several medical conditions such as headaches, gastro-intestinal disorders, rheumatism, and nervousness.[2]

The determination of plant phenolic compounds is of great interest. Phenols have natural antioxidant activity. Plants synthesize them as a defense mechanism against microorganisms and strong UV radiation.[3] Plant extracts are natural alternatives to synthetic antioxidants, as they possess similar or even higher antioxidant activity. Plant material also contains a huge variety of different ballast compounds, such as waxes, oils, sterols, chlorophyll, which may interfere with analyzed compounds and, moreover, they could damage the analytical column. Therefore, the sample preparation is very important for the HPLC analysis of phenolic compounds in plant material.

As temperature and pressure play important roles in extraction kinetics, extraction techniques can be classified based on these parameters. Traditional extraction methods for solid samples include Soxhlet extraction that operates under atmospheric pressure with heating. It consumes relatively large volumes of organic solvents and the extraction may take a long time.[4] Moreover Soxhlet extraction is not selective and an additional sample preparation method is often required. Selective extraction can be obtained by a combination of Soxhlet extraction with solid phase extraction (SPE). This approach utilizing C_{18} sorbent was used for the isolation of free phenolic acids in six *Echinacea* species.[5] Solid phase extraction was also applied as a cleanup procedure of plant crude extracts in other works. A selective procedure combining microwave-assisted extraction (MAE) and SPE on polymeric RP-105 SPE sorbent was applied prior to HPLC of phenolic compounds in plant material. The extraction efficiencies were then compared with those obtained by computer controlled, two step Soxhlet like extractions.[6] Off-line and on-line SPE utilizing Oasis HLB polymer was applied for the isolation of phenolic compounds from *Melisa officinalis*, after liquid extraction in an ultrasonic bath.[7]

The technique of molecular imprinting allows specific recognition sites to be formed in synthetic polymers through the use of templates. Molecularly imprinted polymer (MIP) can be used as solid phase in solid phase extraction protocols. A careful selection of the most appropriate solvents to be used in different steps of SPE procedure (sample loading, washing, and elution) is needed in order to extract the target analyte selectively.[8,9] SPE, using MIP prepared with protocatechuic acid as a template, is described in our

previous paper. MIP was applied for cleanup of *Melissa officinalis* extract before determination of benzoic acid derivatives.[10]

Supercritical fluid extraction (SFE), which utilizes the unique properties of supercritical fluids, is also one of the modern extraction methods. It is performed under elevated pressure and/or temperature. The extraction is faster, more efficient, and sample throughput is high. With relatively less consumption of organic solvents, it is a more environmentally friendly way of extraction.[4] SFE has been applied successfully to the extraction of a variety of organic compounds from herbs and other plants.[11] Total phenol was successfully quantified in the SFE and liquid-solvent extracts of olive leaves.[12] Polyphenolic compounds (gallic acid, catechin, epicatechin) were extracted from white grape seeds with the extraction recovery of 79%.[13] Low extraction yields were achieved in SFE of phenolic acids (rosmarinic, caffeic, protocatechuic) and protocatechuic aldehyde from *Melissa officinalis*.[7]

Matrix solid phase dispersion (MSPD) is a simple approach to the disruption of biological material that allows for the rapid fractionation and isolation of sample's components. This process combines the use of mechanical forces generated from the grinding of samples with silica or polymer based solid supports, to produce a sample/column material from which dispersed sample matrix components can be selectively isolated. It simplifies the extraction and cleanup steps, reduces sample manipulation, and is faster than conventional techniques.[14] MSPD has been almost exclusively applied to the analysis of drugs and pollutants in foods.[15,16] There are not many papers dealing with the application of MSPD as a preseparation technique for analysis of natural compounds present in plant samples. It was successfully applied to the isolation of carotenoids lutenin and zeaxanthin from spinach samples,[17] phenolic acids from the herb *Melissa officinalis*,[18] isoflavonoids from the herb *Radix astragali*,[19] and some phenolic compounds (gallic acid, syringic acid, catechin, epigallocatechin gallate, and rutin) from fruit green tea.[20]

The aim of this work was to test the applicability of some selected extraction methods (SFE and MSPD) for the isolation of benzoic acid derivatives from *Melissa officinalis* before HPLC analysis. SFE was compared to a classical Soxhlet extraction, which has already been optimized in our previous paper,[21] and MSPD extraction was compared to the cleanup procedure using MIP that was also described in our previous work.[10]

EXPERIMENTAL

Apparatus

An SE-1 apparatus (SEKO-K, Ltd. Brno, Czech Republic) was used for all supercritical fluid extractions. Analysis of each extract was carried out with

an HPLC series 1050 from Hewlett Packard (Waldbronn, Germany) equipped with a quaternary gradient pump and a UV-Vis detector with a measuring cell with a volume of 8 μL.

Soxhlet apparatus and a vacuum manifold processor (Alltech, Lokeren, Belgium) were also used for the extractions of plant material.

Chemicals and Solutions

Benzoic acids derivatives, e.g., gallic acid (GA), p-hydroxybenzoic acid (pHBA) were purchased from Merck (Damstadt, Germany), protocatechuic acid (PA) was obtained from the Research Institute of Food Industry (Biocentrum Modra, Trnava, Slovakia), gentisic acid (GeA), vanillic acid (VA) were supplied by MGP (Czech Republic), and syringic acid (SyrA) by Fluka Chemie (Buchs, Switzerland). Methanol, acetonitrile, hexane, and dichloromethane (all HPLC grade) were obtained from Scherlau Chemie S.A. (Barcelona, Spain). Formic acid (p.a.) was supplied from Lachema (Brno, Czech Republic). Deionized water from Milli-Q system (Millipore, El Passo, TX, USA) was used. Bakerbond octadecyl (C_{18}) solid phase, 40 μm, 60 Å (J.T. Baker, Deventer, Netherlands) was used in MSPD.

Stock standard solutions of each of the acids (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 mobile phase.

Plant Material

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

Sample Preparation

Supercritical Fluid Extraction

Static extractions were conducted in stainless steel vessels and all experiments were performed in duplicate. Plant material (200 mg) previously ground to a powder, were used in each extraction. Extraction conditions were studied by varying the parameters (extraction temperature, extraction pressure, trap temperature, modifier type and percent, trap solution). The various parameters are shown in Table 1. The time of an extraction also varied (20, 40, 60 min). The restrictor temperature (150°C) and the volume of trap solvent (2.5 mL) were common in all experiments. The modifier was added just before sealing the extraction chamber.

Table 1. Conditions of SFE experiments

Experiment	p (MPa)	T (°C)	T_{trap} (°C)	Modifier	Trap solvent	Time (min)
1	10	40	25	250 μL MeOH	2.5 mL water/HCOOH	40
2	10	60	25	250 μL MeOH	2.5 mL water/HCOOH	40
3	10	80	25	250 μL MeOH	2.5 mL water/HCOOH	40
4	20	60	25	250 μL MeOH	2.5 mL water/HCOOH	40
5	30	60	25	250 μL MeOH	2.5 mL water/HCOOH	40
6	20	60	40	250 μL MeOH	2.5 mL water/ HCOOH	40
7	20	60	60	250 μL MeOH	2.5 mL water/HCOOH	40
8	20	60	25	250 μL EtOH	2.5 mL water/HCOOH	40
9	20	60	25	250 μL acetone	2.5 mL water/HCOOH	40
10	20	60	25	500 μL MeOH	2.5 mL water/HCOOH	40
11	20	60	25	250 μL MeOH	2.5 mLMeOH/ water/HCOOH	40

p—extraction pressure, T—extraction temperature, T_{trap}—trap temperature.

Matrix Solid Phase Dispersion

Dry plant material was ground to a powder and a 0.5 g aliquot was placed into a mortar. It was mixed with 2 g of sorbent (Bakerbond C_{18}) and 1 mL of n-hexane until a homogenous mixture was obtained. The blend was transferred into a glass syringe with a frit placed on the bottom. The sample was covered with another frit and pressed using a syringe plunger. The prepared column was washed with 10 mL of n-hexane, 10 mL of dichloromethane, and benzoic acid derivatives were eluted with 10 mL of a mixture methanol–formic acid in water (pH of water 2.5); 80/20 v/v. The eluent was filtered through a nylon microfilter and injected into the HPLC system.

Soxhlet Extraction and MISPE Procedure

The conditions of Soxhlet extraction of derivatives of benzoic acid from *Melissa officinalis* were optimized in our previous work.[19] The extraction was performed with a mixture of methanol–water, 80/20 v/v for 1 hour.

The purification of the extract was achieved by the MISPE procedure described in ref.[10] MIP was prepared in porogen acetonitrile for protocatechuic acid as a template. Acrylamide was used as a functional monomer and ethylene glycol dimethacrylamide as a crosslinker. The amount of 200 mg of MIP was packed into a cartridge and conditioned with methanol, water, and acetonitrile. Of the extract, 1.5 mL was reconstituted in 10 mL of acetonitrile. The deluted extract, 5 mL, was applied onto the conditioned MIP. The cartridge was washed with 2 mL of water and 3 mL of acetonitrile. The benzoic acid derivatives were eluted with 5 mL of a mixture of methanol-acetic acid (9:1 v/v). The effluent was evaporated to dryness and redissolved in the mobile phase and injected into the HPLC system.

HPLC Analysis

An Alltima C18-Rocket (53 × 7 mm, 3 m) (Alltech, Lokeren, Belgium) was used for all extracts assays. The UV wavelength was fixed at 254 nm. A binary mobile phase consisting of acetonitrile and formic acid in water (0.074 mol/L), at a flow rate of 1.6 mL/min was used. The linear gradient was applied: 0–12 min: 5–15% acetonitrile. The data analyses were carried out using an HP ChemStation.

RESULTS AND DISCUSSION

In our previous work, derivatives of benzoic acid were extracted by Soxhlet extraction.[21]As it can be seen in Table 2, the classical method of extraction provided recoveries of 88.9% (VA), 91.3% (pHBA), 100.8% (PA), 101.8% (GA), and 45.3% (SyrA). The recoveries of a cleanup method, MISPE, are also presented in Table 2.

In this work, we tried to use several extraction techniques for the isolation of benzoic acid derivatives from *Melissa officinalis*. SFE and MSPD were tested for this purpose. The SFE extraction was compared to Soxhlet extraction in terms of extraction yields of the analytes. The use of solid

Table 2. Extraction yields and percent recoveries of benzoic acid derivatives in μg/g of dry material obtained by Soxhlet extraction[21] and MISPE procedure[10]

		GA	PA	pHBA	VA	SyrA
Soxhlet extraction	Yield (μg/g)	16.4	75.3	10.5	14.5	540.8
	Recovery (%)	101.8	100.8	91.3	88.9	45.3
MISPE	Recovery (%)	56.4	77.1	70.0	82.1	16.5
	RSD (%)	7.0	4.2	3.8	3.3	7.5

phase for the extraction of benzoic acid derivatives in MSPD extraction was compared to the results from Soxhlet extraction and MISPE procedure.

The SFE has already been applied for the extraction of rosmarinic, caffeic, protocatechuic acids, and protocatechuic aldehyde from *Melissa officinalis*.[7] The extraction was performed at the following conditions: 100 mg of ground, dried lemon balm and washed glass balls were extracted at extraction temperature of 60°C, extraction pressure 40 MPa, restrictor temperature of 100°C, and extraction time 60 min. Although the yields of phenolic acids were very low, we tested the possibility of isolation of benzoic acid derivatives from *Melissa officinalis* by varying the parameters of SFE in order to find conditions for higher extraction yields. The restrictor temperature was constant for all experiments. In order to overcome problems of plugging the restrictor the restrictor temperature was set to 150°C. The addition of modifier into the extraction cell was performed in all experiments, because according to preliminary experiments it was mandatory for the extraction. The effect of the extraction temperature on the amount of acids extracted was studied at constant pressure (10 MPa). Three different temperatures (40, 60, 80°C) corresponding to CO_2 densities of 0.62, 0.28, and 0.22 g/mL for pressure 10 MPa, respectively, were tested. The effect of extraction pressure, trap solvent and temperature, type and amount of modifier, were studied in the same way. Conditions of all experiments are listed in Table 2. The results from all experiments are presented in Table 3, and the chromatogram of SFE extract of *Melissa officinalis* can be seen in Figure 1. The extraction yields of analytes were in the ranges: GA 0.3–1.5 μg/g, PA 0.2–1.6 μg/g, pHBA 0.3–1.7 μg/g, VA 0.4–1.7 μg/g, SyrA 1.6–10.2 μg/g. It is obvious there are only small differences between results of different experiments. The variation of the parameters of SFE didn't affect the efficiency of the extraction. Surprisingly, the effect of extraction temperature

Table 3. Extraction yields of benzoic acid derivatives in μg/g of dry material obtained by SFE. (the average values of two measurements)

Experiment	GA	PA	pHBA	VA	SyrA
1	0.4	0.6	0.6	0.7	10.2
2	0.4	0.7	1.0	0.5	7.0
3	0.5	0.7	1.0	0.5	4.4
4	0.7	1.0	1.6	1.0	6.5
5	0.7	0.2	0.3	0.6	2.3
6	0.6	0.7	0.6	1.7	4.5
7	0.3	0.3	0.4	0.4	3.6
8	0.5	0.3	0.4	0.6	1.6
9	—	—	—	—	—
10	0.4	0.4	0.6	0.5	6.9
11	1.5	1.6	1.7	1.4	9.5

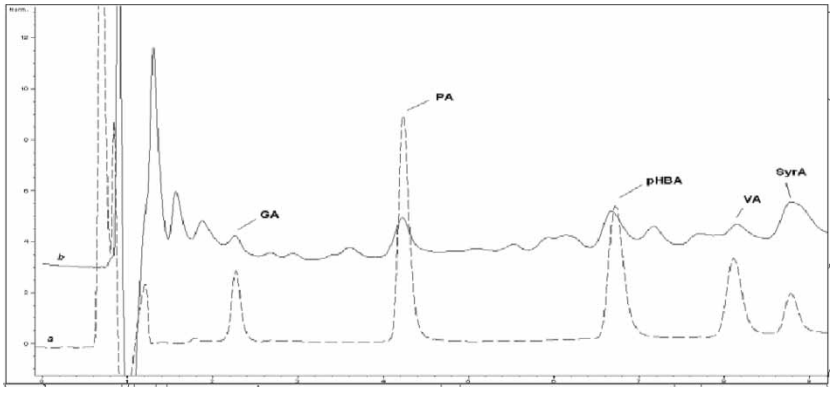

Figure 1. Separation of a mixture of benzoic acid derivatives (*a*) and the chromatogram of SFE extract of *Melissa officinalis* (*b*). For HPLC conditions see Experimental. SFE conditions: modificator 250 μL MeOH, pressure 20 MPa, extraction temperature 60°C, restrictor temperature 150°C, trap temperature 25°C, trap 2.5 mL of MeOH/water/HCOOH; extraction time: 40 min. GA—gallic acid, PA—protocatechuic acid, pHBA—p-hydroxybenzoic acid, VA—vanillic acid, SyrA—syringic acid.

and pressure on the amounts of acids extracted is negligible. This means the density of CO_2 is not crucial for the extraction.

Even the addition of organic solvent as a CO_2 modifier didn't increase the yields of analytes significantly. Although the addition of methanol was essential for SFE and the extraction of benzoic acid derivatives from *Melissa officinalis* was unsuccessful without the modifier, the use of methanol enables obtaining only low amounts of analytes. Other solvents were tested but very similar results were achieved with ethanol as a modifier. To the contrary, when acetone was applied as a CO_2 modifier no analytes were detected in the extracts. Acetone was quite unsuitable for this purpose.

Since phenolic acids are soluble in methanol and water the extracts were trapped in methanol in preliminary experiments. It was found that methanol was not a suitable trap solvent. There were no analytes detected in the extracts (data not shown). It was probably caused by the evaporation of methanol during the extraction. Therefore, better results were observed when water acidified with formic acid (it was used also in the mobile phase in HPLC analysis) was applied as a trap solvent. Approximately two times higher extraction yields were achieved by trapping the extract into the mixture of methanol and acidified water (50/50, v/v). But, the extraction yields are still several times lower than those obtained by Soxhlet extraction. The extraction yields of acids obtained by SFE correspond to 0.7–16.2% of the extraction yields obtained using the Soxhlet apparatus. According to these results, it can be concluded that SFE is not a suitable method of isolation of benzoic acid derivatives from *Melissa officinalis.*

Also tested was another extraction technique for this purpose. The MSPD method was studied as an alternative of Soxhlet extraction of the benzoic acid derivatives from plant material. The generic MSPD utilizing non-polar C_{18} sorbent has already been applied for the extraction of some phenolic compounds from *Melissa officinalis*[18] or fruit green tea.[20] MSPD was successfully used for the isolation of benzoic acid derivatives from *Melissa officinalis.* The obtained results of MSPD application are shown in Figure 2.

The extraction yields of MSPD were graphically compared to those obtained by Soxhlet extraction and the combination of Soxhlet extraction with MISPE presented in our previous works[10,21] (Figure 3). It is obvious, that in the cases of GA, pHBA, and VA the results of MSPD were comparable to the results of Soxhlet extraction and MISPE. Taking into consideration the recoveries of Soxhlet extraction 91% (pHBA) and 88% (VA), it seems MSPD is more effective for the extraction of pHBA and VA than Soxhlet extraction, because it provides higher extraction yields for these compounds. On the other hand, MSPD was not so efficient for the isolation of PA and SyrA compared to Soxhlet extraction. The amount of PA extracted by MSPD represents only 34.9% of the amount extracted by Soxhlet extraction. In the case of SyrA it is even less (2.9%). The isolation of these benzoic acid derivatives probably requires some modification of the generic MSPD procedure used in this work. Still MSPD has an advantage of a simple and fast extraction that combines sample homogenization, extraction of analytes, and sample cleanup in a single process. Although, the MSPD extraction it is not as selective as the MISPE extraction procedure utilizing molecularly imprinted polymer, it can be an attractive technique used for the isolation of some

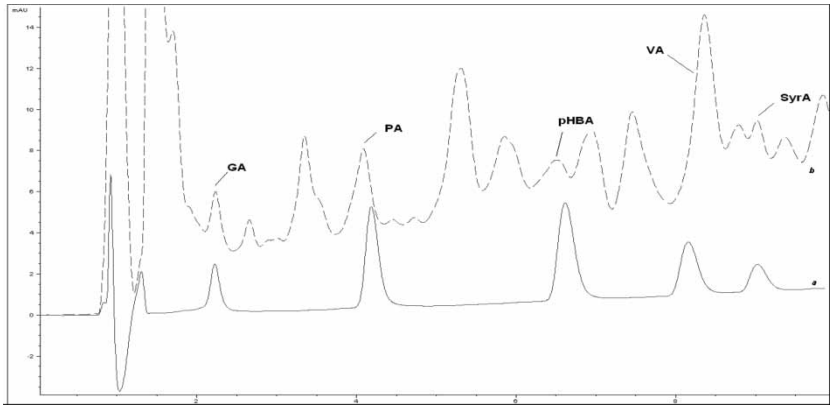

Figure 2. Separation of a mixture of benzoic acid derivatives (*a*) and the chromatogram of MSPD of *Melissa officinalis* extract (*b*). For HPLC conditions and MSPD procedure see Experimental. GA—gallic acid, PA—protocatechuic acid, pHBA—p-hydroxybenzoic acid, VA—vanillic acid, SyrA—syringic acid.

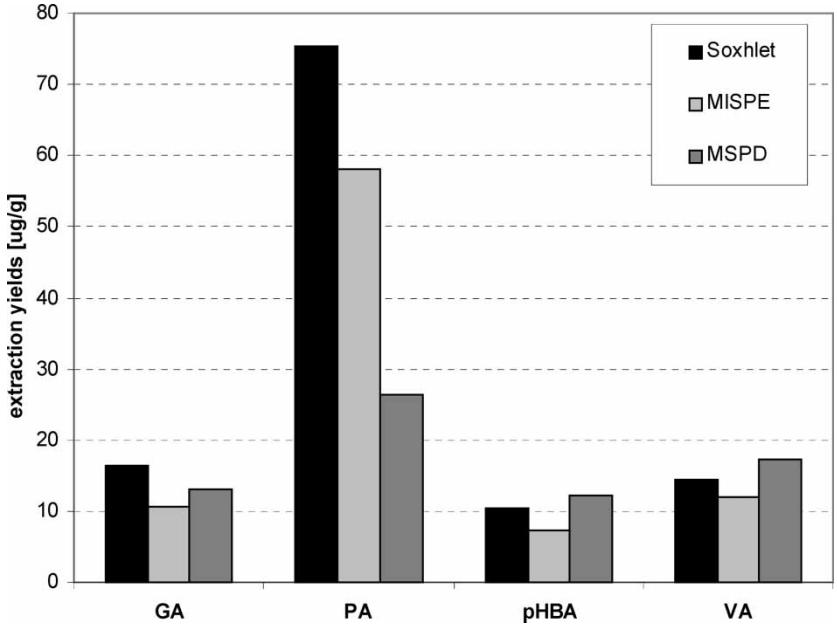

Figure 3. Comparison of extraction yields of p-hydroxybenzoic acid derivatives obtained from *Melissa officinalis* by different extraction methods (Soxhlet extraction, MISPE—combination of Soxhlet extraction and MISPE, MSPD).

studied analytes (gallic, p-hydroxybenzoic, and vanillic acid) from *Melissa officinalis*. For the selective extraction, the combination of MSPD with MISPE could be applied.

CONCLUSIONS

Both tested methods, MSPD and SFE, are strongly matrix dependent. Generally, SFE extraction of polar compounds from plant material is problematic. From the results presented here, it can be concluded that SFE is not a suitable method for the isolation of derivatives of benzoic acid from plant material of *Melissa officinalis*.

In the case of MSPD, the sample matrix is dispersed in the entire volume of the MSPD column so it is the most affecting factor of the extraction. If the MSPD procedure using C_{18} sorbent is applied for the extraction of gallic, p-hydroxybenzoic acid, and vanillic acid from *Melissa officinalis*, yields comparable to Soxhlet extraction were achieved. MSPD can be used as a simple, fast, and environmentally friendly alternative of Soxhlet extraction for these compounds.

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