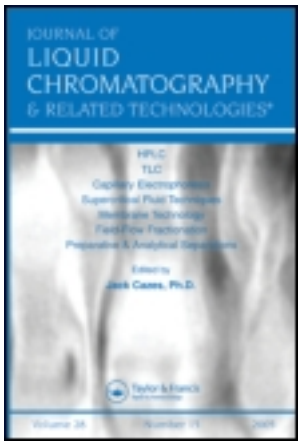


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# APPLICATION OF DIFFERENT PREPARATION TECHNIQUES FOR EXTRACTION OF PHENOLIC ANTIOXIDANTS FROM LEMON BALM (*MELISSA OFFICINALIS*) BEFORE HPLC ANALYSIS

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## ABSTRACT

Four different preparation techniques [SPE (Solid phase extraction) in off-line and on-line modes, PSE (Pressurized solvent extraction) and SFE (Supercritical fluid extraction)] were tested for the isolation of some phenolic compounds from *Mellissa officinalis.* Solid phase extraction methods using the hydrophilic–lipophilic sorbent (OASIS HLB) was used in off-line mode for clean-up of the water extract of medical plants. A generic SPE method was slightly modified, both washing and elution steps were optimized. The contents of methanol for both steps were recommended. Extraction recoveries for all analyzed compounds were about 100% with RSD values in the range 1.1–2.7%. On-line SPE has also been applied for phenolic compounds isolation.

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Pressurized solvent extraction and SFE are not recommended as preparation techniques suitable for polar phenolics extraction from plant material. Yields of all analytes were low in comparison to liquid extraction and SPE, and the cleanness of chromatograms has also been worse than after SPE with OASIS HLB sorbent.



## INTRODUCTION

Phenolic acids are widely distributed in the plant kingdom and are present, among others, in medicinal plants. They are very often responsible for the antioxidant activity of the plant extracts.[1–3]

Plant material 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 a very important part of the method development for HPLC analysis of phenolic compounds in plant material.

### Liquid Extraction (LE)

With this technique, the most important steps of the method development are: the choice of solvent, the pH, the temperature, the sample-to-solvent volume ratio, and the number and the time intervals of individual extraction steps.[4]

Alcohols (methanol, ethanol) and aqueous alcohols are the most used extraction agents for extracting phenolic compounds from the plant material.[1,2,5–13] Different extraction solvents, such as petroleum ether, chloroform, ethyl ether, ethyl acetate, and acetone have also been tested for extraction steps for removing interfering compounds. Boiling water has also been recommended as an extraction agent for the extraction of antioxidants from some medical plants and tea samples.[3,14,15]. In some cases, acidified alcohol could also be applied for the isolation of phenolic acids from plant material.[16,17]

### Off-Line Solid Phase Extraction (SPE)

Solid phase extraction is a good choice for the clean-up procedure of plant crude extracts. The advantages of SPE compared to LLE are that SPE is faster and more reproducible, cleaner extracts are obtainable, emulsion creation is avoided, and smaller sizes are needed. From an environmental point of view, a lower consumption of toxic solvents is used in most SPE procedures.[18]

Solid phase extraction combining two cartridges with different sorbents ($C_{18}$ and quaternary amine) was used for the isolation and purification of phenolic acids from *Echinacea* extracts, *Eleutherococcus senticocus* root extracts, and extracts from different *Lamiaceae* plants.[2,9,19]

Liquid extraction and SPE procedures have been combined to identify polyphenols in *Phillyrea angustifolia* L. leaves.[12] After three-fold extraction with 80% ethanol with 2% sodium disulfide and extraction with hexane, samples were purified by LLE using ethyl acetate or by SPE using activated serial Bond-Elut CH and Bond-Elut SAX cartridges.

A SPE procedure for extraction of phenolics from grape was described.[20] Samples were extracted with 80% MeOH, extracts were filtered, methanol was removed from the combined extracts, and residue was redissolved in 0.01 N HCl. Then, it was passed through a $C_{18}$ isolute endcapped SPE cartridge previously conditioned with ethyl acetate, methanol, and 0.01 N HCl. The loaded cartridge was washed with 0.01 N HCl, dried, and phenolic compounds other than anthocyanins were eluted with ethyl acetate. The solvent was evaporated and the residue was redissolved in methanol. Two kinds of $C_{18}$ SPE columns were tried, end-capped and non-endcapped. The end-capped columns gave higher recoveries of colorless phenolic compounds.

## On-Line Solid Phase Extraction

On-line SPE combines the selectivity of off-line SPE with complete automation. The key to on-line SPE is direct elution of the extract from the SPE cartridge into the LC system by the LC mobile phase. Several laborious handling steps are thus omitted, making on-line SPE much more efficient and providing superior analytical results.

The sample is injected into a small trapping column, analyte is trapped on a sorbent, and contaminants are washed off the cartridge to waste. Subsequently, the content of SPE cartridge is eluted onto the HPLC column.[21]

## Pressurized Solvent Extraction (PSE)

Pressurized solvent extraction (PSE) also known as accelerated solvent extraction, is a technique for extracting solid and semisolid samples with liquid solvents. Pressurized solvent extraction uses higher temperatures (50–200°C) and pressures (1500–3000 psi) to accelerate the extraction process. The higher temperature increases the extraction kinetics, while the increase in pressure keeps the solvent from boiling. This greatly improves solvating efficiency and also the efficiency of the extraction process.

The stability of phenolic compounds (e.g. caffeic, *p*-coumaric, gentisic acids, protocatechuic aldehyde, catechin) during PSE using methanol at different temperatures (40, 50, 100, and 150°C), has been studied.[22] The average recovery was over 90%, with the exception of catechin and epicatechin at 150°C. The same solutions of phenolic compounds were kept at the boiling point of methanol (65°C) as long a time as that used by PSE (45 min). Using this method, yields of some compounds were significantly lower.



## Supercritical Fluid Extraction (SFE)

Supercritical fluid extraction is a technique, which applies the unique properties of a supercritical fluid to selectively extract and fractionate valuable non-polar components from different samples. The main advantages of using supercritical fluids for extractions is that they are inexpensive, contaminant free, and less costly to dispose safely than organic solvents. Extraction yield and selectivity can be controlled by adjusting the pressure, temperature, and flow rate within the supercritical extraction system, or by adding modifiers to the supercritical fluid. A common modifier is methanol (typically 1–10%), which increases the polarity of supercritical $CO_2$.

Supercritical fluid extraction was used to extract polyphenolic compounds (gallic acid, catechin, epicatechin) from grape seeds. Results from SFE were compared with those obtained by liquid-solvent extraction and sonicated assisted liquid solvent extraction (SALSE).[23]

Supercritical fluid extraction was compared with the Soxhlet extraction, steam distillation, and maceration, for the isolation of phenolic compounds from chamomile flowerheads. The yields of essential oils were four times higher than that produced by steam distillation, and recovery of flavonoid apigenin was also better compared to Soxhlet extraction and maceration. However, highly polar flavonoid was not extracted by pure $CO_2$ (recovery values $<1.1\%$). Its extraction efficiency improved by the addition of methanol (5% v/v) to the supercritical fluid, but the obtained recoveries were unsatisfactory. Supercritical fluid extraction is not suitable for the extraction of highly polar compounds.[24]

In our previous work,[3] a simple and effective HPLC assay has been described for the determination of phenolic acids isolated from lemon balm, as well as an optimized liquid extraction procedure for these compounds.

The aim of this work was to test different extraction and clean-up methods, in order to find the most suitable simple method for the extraction and purification of phenolic compounds (rosmarinic, caffeic, protocatechuic acids, and protocatechuic aldehyde) from *M. officinalis.*

## EXPERIMENTAL

### Chemicals, Samples, and Solutions

Standards of rosmarinic, caffeic, protocatechuic acids, 3,4-dihydroxybenzaldehyde (protocatechuic aldehyde), and plant samples of *M. officinalis* L., grown in Slovakia were obtained from Research Institute of Food Industry, Biocentrum Modra (Slovakia). Stock solutions of standards (ca. 1 mg/mL) were prepared in methanol and stored in the freezer at −20°C. The stability of stock solutions was controlled and no change in concentrations was observed. Working solutions were prepared by diluting the stock solutions with mobile phase.

HPLC grade methanol was obtained from Merck (Slovakia), formic acid (p.a.) was from Lachema (Czech Republic).

### HPLC Analysis

The separation of phenolics was achieved with an HP 1100 system (Hewlett-Packard, Waldbronn, Germany) consisting of a pump with degasser, a diode-array detector (DAD), and an HP ChemStation. A Symmetry® C18 (150 × 3.9 mm, 5 μm) analytical column (Waters, USA) with the Symmetry C18 (20 × 3.9 mm) guard column (Waters, USA) were used. A mobile phase, which consisted of MeOH and water (pH = 2.5, adjusted with formic acid) with linear gradient (from 15% to 75% of methanol in 40 min), was used for the chromatographic separations. The flow rate was 0.4 mL/min and injection volume 20 μL. All analyses were carried out at ambient temperature.

UV spectra were recorded in the range of 200–400 nm. Chromatograms were acquired at 260, 280, and 330 nm.

### Sample Preparation

#### Liquid Extraction

Extraction of phenolic compounds from *M. officinalis* was carried out according to the previously published method.[3] Dried tops of *M. officinalis* were ground to powder and 100 mg of the sample was extracted with 10 mL of water, pH 2.5, for 10 min in an ultrasonic bath Sonorex (Bandelin electronic, Germany) at 25°C. The extracts were filtered and the extraction procedure was repeated twice with the residue. The solutions were filtered through a nylon microfilter Tessek (Czech Republic) prior to injection.

### Solid Phase Extraction

A 1 mL sample of acidic water extract (pH = 2.5) or diluted methanol–water extract of *M. officinalis* was loaded onto the preliminary conditioned (1 mL MeOH, 1 mL water (pH 2.5)) OASIS™ HLB SPE cartridge 1 cc/30 mg Waters (USA). After washing with 1 mL of MeOH–water, pH 2.5 (20 : 80), phenolic compounds were eluted with 2 mL of MeOH–water, pH 2.5 (80 : 20). The eluent was filtered through a nylon microfilter and injected into the HPLC system. The SPE scheme is illustrated in Fig. 1. For on-line SPE, Novapak cartridges, Waters (USA) were applied as a precolumn in the column-switching system.

### Pressurized Solvent Extraction

Pressurized solvent extraction was performed at the Department of Analytical Chemistry, University of Pardubice (Czech Republic) using a Pressurized Solvent Extractor *one*PSE made by Applied separations (USA).

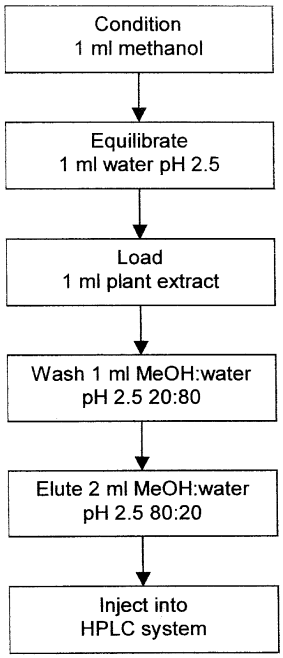


***Figure 1.*** Solid phase extraction method for phenolic compounds in lemon balm (*M. officinalis*) samples.

An extraction vessel was filled with 0.5 g of ground dried *M. officinalis* and washed glass balls and then sealed in the extractor. Samples were extracted in two 5 min long static cycles with 1 min $N_2$ flushing at the end at pressure 0.3 MPa. Extraction temperature was 80°C and extraction pressure was 10 MPa.

### Supercritical Fluid Extraction

Supercritical fluid extraction was performed at the Department of Analytical Chemistry, University of Pardubice (Czech Republic) using a supercritical fluid extractor SE-1 (SEKO-K, Brno, Czech republic) with a 50 μm restrictor. For each experiment, the extraction cell was filled with 0.1 g of ground-dried lemon balm and washed glass balls and the cell was placed into the extractor. Dynamic extractions were performed, extraction temperature was 60°C, extraction pressure 40 MPa. Restrictor temperature was set to 100°C and extraction time was 60 min.

## RESULTS AND DISCUSSION

### Off-Line Solid Phase Extraction

As was mentioned in our previous work,[3] phenolic acids in crude plant extract could also be quantified without any additional clean-up step. But analyzed extracts have a yellow color. So, it was obvious that they contained some colored ballast compounds, which could damage the column. Therefore, the further purification of crude plant extracts using the SPE method was developed. It could also be used for the preconcentration of protocatechuic acid and its aldehyde, which are present in low concentrations.

For the sample clean-up, Oasis HLB cartridges were employed. The Oasis HLB sorbent is a macroporous copolymer made from a balanced ratio of two monomers, the lipophylic divinylbenzene and the hydrophilic *N*-vinylpyrrolidone. The sorbent is stable from pH 1 to 14. Unlike traditional $C_{18}$ bonded silica sorbents, the Oasis HLB copolymer reversed-phase adsorption mechanism is uncomplicated by the often irreproducible population of surface silanols or metal impurities. This means that acidic, basic, and neutral compounds, whether polar or non-polar, can be isolated reproducibly with high recoveries.[25]

A modified generic method recommended for an Oasis HLB cartridge was used. After conditioning with methanol, cartridges were equilibrated with water, pH 2.5, to wash out methanol and prepare an acidic environment for the extract. After loading the plant extract onto the cartridge, solutions with different methanol concentrations were tested for washing the ballast compounds and elution of analyzed phenolic compounds (Fig. 1).

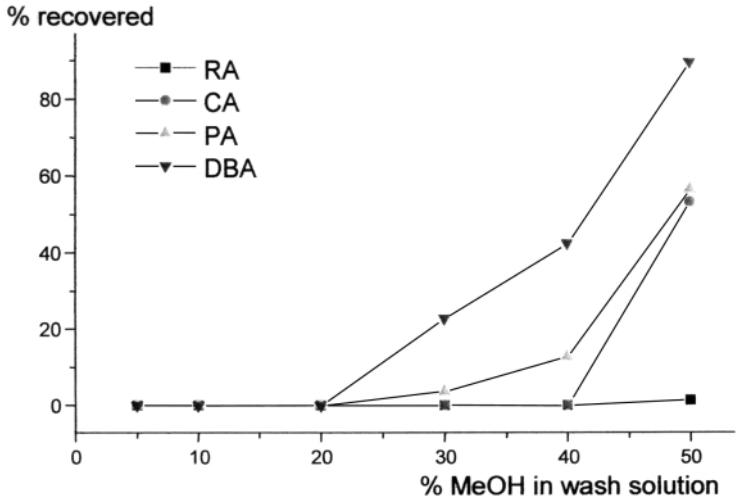


***Figure 2.*** Relationship between content of methanol in washing solution and recoveries of phenolic compounds from Oasis HLB SPE cartridge. Washed with 1 mL of each solution.

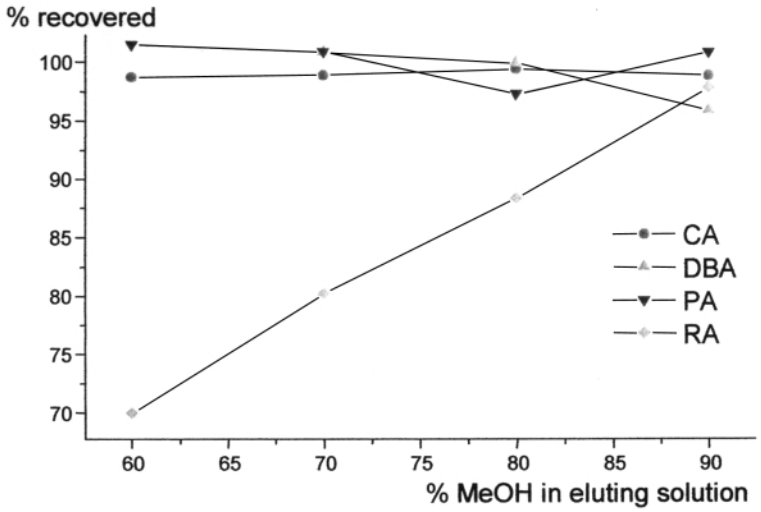


***Figure 3.*** Relationship between content of methanol in eluting solution and recoveries of phenolic compounds from Oasis HLB SPE cartridge. Eluted with 2 mL of each solution. Extraction recoveries for SPE assay were the following: PA: 99.2%, RSD 2.2%; DBA: 95.3%, RSD 1.7%; CA: 98.0%, RSD 2.7%; RA: 92.1%, RSD 1.1%.

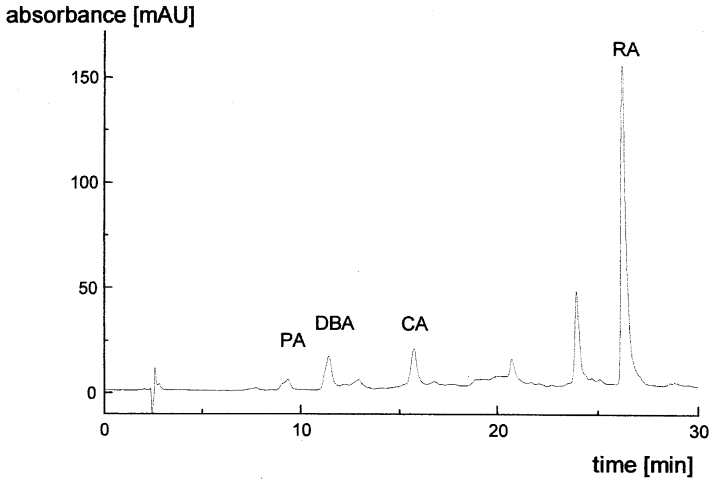


***Figure 4.*** HPLC chromatogram of *M. officinalis* extract after SPE. Chromatographic conditions: as mentioned in experimental.

Relationships between concentration of methanol in washing (2) or eluting (3) solutions, and the recoveries of phenolic compounds from the Oasis HLB cartridge are illustrated in Figs. 2 and 3. According to these graphs, it is obvious that 20% methanol in washing solution could be sufficient, as no amount of studied compounds was washed out from the cartridge. Using elution mixtures containing 30% methanol, more than 22% of protocatechuic acid and 3% of protocatechuic aldehyde were washed out (Table 1).

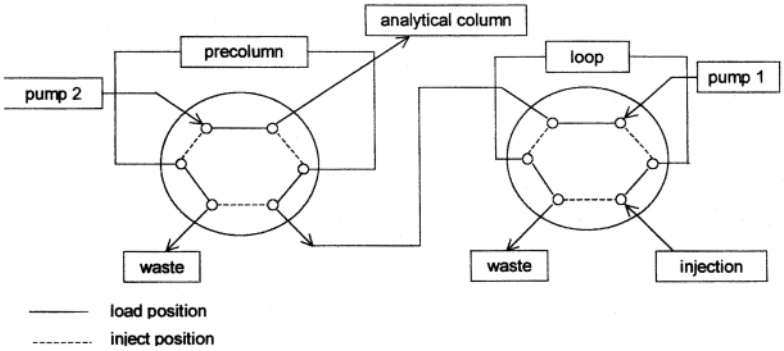


***Figure 5.*** Scheme of the column-switching system used in the present study.

***Table 1.*** % of Phenolic Compounds Washed Out in Washing Step Using Washing Solutions with Different Concentration of Methanol

| % MeOH in Washing Solution | % of Washed Phenolic Acids | | | |
|---|---|---|---|---|
| | RA | CA | DBA | PA |
| 5 | 0 | 0 | 0 | 0 |
| 10 | 0 | 0 | 0 | 0 |
| 20 | 0 | 0 | 0 | 0 |
| 30 | 0 | 0 | 3.63 | 22.8 |
| 40 | 0 | 0 | 12.85 | 42.47 |
| 50 | 1.4 | 53.38 | 56.68 | 89.77 |

As is possible to see from Table 2 and Fig. 3, the best recoveries of studied analytes were obtained when the eluting solution with 90% of methanol was used. But the high methanol content had a bad influence on peak shapes. Since the peak shapes were much better when 80% methanol was used, and the recoveries of studied compounds were also satisfactory (88–99%), eluting solutions containing 80% methanol was chosen as the optimal.

## On-Line Solid Phase Extraction-HPLC (Column Switching)

Column switching in the back-flush mode was used for the on-line SPE of phenolic acids. The mobile phase II (pumped by pump II.) was the mixture of methanol : water pH 2.5 (linear gradient, from 20 to 75% of methanol in 40 min); mobile phase I contained the same components as the main mobile phase. The methanol content of 20% was chosen to achieve a satisfactory washing effect. A time of 2 min (flow-rate 0.5 mL/min) was sufficient for removing

***Table 2.*** Recoveries of Phenolic Compounds Using Eluting Solutions with Different Concentration of Methanol

| % MeOH in Elution Solution | % of Eluted Phenolic Acids | | | |
|---|---|---|---|---|
| | RA | CA | DBA | PA |
| 90 | 97.88 | 98.93 | 95.94 | 100.89 |
| 80 | 88.41 | 99.46 | 99.94 | 97.32 |
| 70 | 80.24 | 98.93 | 100.86 | 100.89 |
| 60 | 69.98 | 98.74 | 98.16 | 101.49 |

weakly retained compounds from the precolumn. After that, the valve was switched to the inject position to transfer the analytes from the precolumn onto the analytical column. A time of 1 min was adequate for a complete elution of phenolic acids. Subsequently, the valve was switched back and the precolumn was washed with mobile phase I to prepare for the next analysis.

HPLC chromatograms of PA, CA, and RA standard mixtures after on-line SPE-HPLC, at two different wavelengths, are demonstrated in Fig. 6.



## Pressurized Solvent Extraction

Dried lemon balm tops were extracted with methanol at 70 and 80°C and also with methanol containing 0.2% of formic acid at 60, 70, and 80°C. All the extractions were run in triplicate. Table 3 shows the yields and RSD values of extracted compounds. It can be seen, that the use of acidified methanol significantly improved yields of all analyzed compounds. The temperature rise also has a positive effect on yields of phenolics. The best results were obtained using acidified methanol at 80°C. Still, the recoveries were quite low compared to liquid extraction using methanol : water, pH 2.5 (60 : 40) as extraction agent. Recoveries were: 38.8% for rosmarinic acid, 28.3% for caffeic acid, 23.3% for protocatechuic acid, and 35.9% for protocatechuic aldehyde.

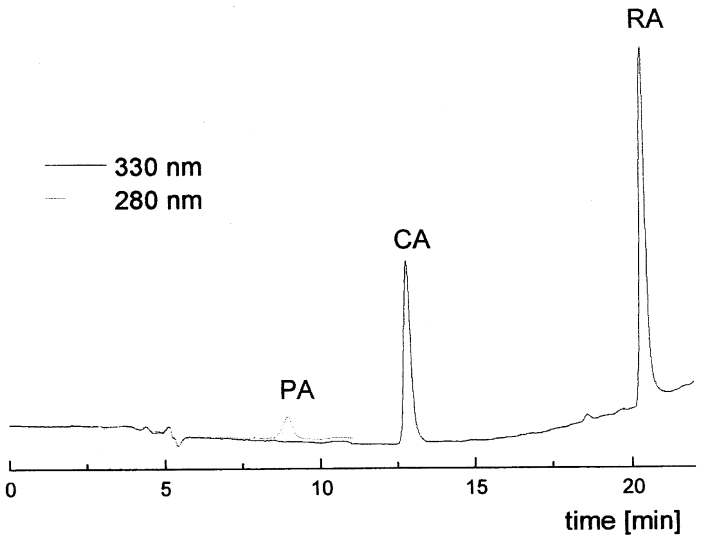


***Figure 6.*** HPLC chromatogram of PA, CA and RA standard mixture after on-line SPE-HPLC at two different wavelengths. Chromatographic conditions: as mentioned in experimental.

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***Table 3.*** Yields and RSD Values of Rosmarinic, Caffeic, Protocatechuic Acids and Protocatechuicaldehyde Extracted from *M. officinalis* Using Pressurized Solvent Extraction. Extraction Conditions: Pressure: 10 MPa, 2 Extraction Cycles, Time of Extraction Cycle: 5 min (n = 3)

| Extraction Agent | RA | | CA | | PA | | DBA | |
|---|---|---|---|---|---|---|---|---|
| | [mg/g] | RSD [%] | [μg/g] | RSD [%] | [μg/g] | RSD [%] | [μg/g] | RSD [%] |
| MeOH 70°C | 2.358 | 4.70 | 19.989 | 3.46 | 3.183 | 4.42 | 7.703 | 4.83 |
| MeOH 80°C | 3.808 | 3.55 | 31.873 | 4.11 | 4.654 | 3.94 | 8.020 | 2.16 |
| MeOH + 0.2% HCOOH 60°C | 4.593 | 3.04 | 34.846 | 6.03 | 5.404 | 5.32 | 13.552 | 3.85 |
| MeOH + 0.2% HCOOH 70°C | 7.277 | 2.38 | 46.488 | 4.52 | 8.899 | 4.57 | 17.993 | 4.05 |
| MeOH + 0.2% HCOOH 80°C | 8.147 | 2.27 | 54.305 | 4.72 | 10.097 | 2.53 | 19.589 | 4.18 |

An HPLC chromatogram of PSE extract of *M. officinalis* using methanol with 0.2% HCCOH, 80°C is illustrated in Fig. 7.

## Supercritical Fluid Extraction

The application of SFE has also been studied for the isolation of phenolic compounds from plant material. But since the SFE is more suitable for the extraction of less polar compounds, yields of polar phenolic acids were very low. No amounts of caffeic and protocatechuic acids were detected in SFE extracts. Using only $CO_2$ without modifier, the recovery of rosmarinic acid was very low, and its extraction efficiency improved when methanol was used as a modifier. After addition of methanol containing 0.2% of formic acid to the supercritical fluid, the recovery for rosmarinic acid was a little higher, but it was not comparable with extraction recoveries of liquid extraction or SPE. For this reason, this technique was not recommended for sample preparation of *M. officinalis* plant.

In conclusion, the optimized generic off-line SPE method using hydrophilic–lipophilic sorbent is the most effective and simple isolation and clean-up

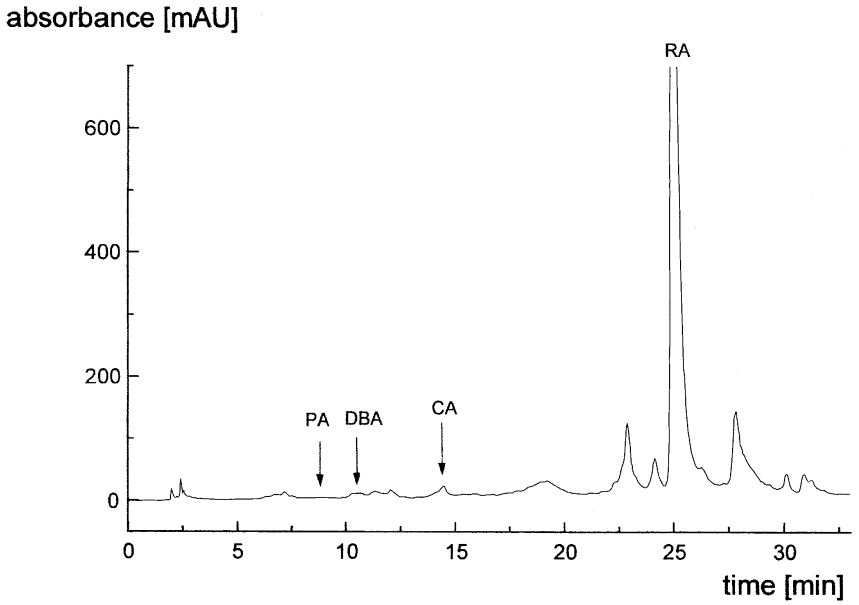


***Figure 7.*** HPLC chromatogram of *M. officinalis* PSE extract. Extracted with MeOH + 0.2% formic acid at 80°C. Chromatographic conditions: as mentioned in experimental.

technique for the preparation of liquid extracts, before the HPLC monitoring of phenolic compounds in *M. officinalis.* Solid phase extraction in on-line mode is now also being tested for the analysis of natural plant samples, and results will be prepared for publishing in a short time. Pressurized solvent extraction and SFE are not the best choice for the simultaneous isolation of phenolic compounds with so different polarities and chemical properties; so, the yields are significantly lower in comparison to the SPE assay.

## ACKNOWLEDGMENT

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