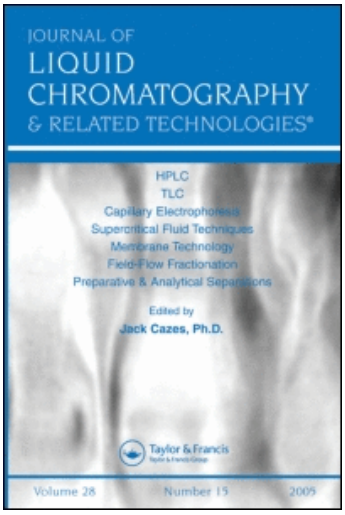

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

HPLC ANALYSIS OF PHENOLIC ACIDS IN *MELISSA OFFICINALIS*

Alicia Caniova[a]; Eva Brandsteterova[a]
[a] Department of Analytical Chemistry, Slovak Technical University, Bratislava, Slovakia

Online publication date: 31 October 2001

To cite this Article Caniova, Alicia and Brandsteterova, Eva(2001) 'HPLC ANALYSIS OF PHENOLIC ACIDS IN *MELISSA OFFICINALIS*', Journal of Liquid Chromatography & Related Technologies, 24: 17, 2647 — 2659
To link to this Article: DOI: 10.1081/JLC-100106092
URL: http://dx.doi.org/10.1081/JLC-100106092

J. LIQ. CHROM. & REL. TECHNOL., 24(17), 2647–2659 (2001)

HPLC ANALYSIS OF PHENOLIC ACIDS IN *MELISSA OFFICINALIS*

Alicia Caniova and Eva Brandsteterova*

Department of Analytical Chemistry, Slovak Technical University, Radlinskeho 9, 812 37, Bratislava, Slovakia

ABSTRACT

A simple and effective HPLC assay for determination of the main phenolic acids (rosmarinic, caffeic, protocatechuic) that were isolated from medicinal plant *Melissa officinalis* has been developed. The main goal of this work was to test and evaluate the liquid extraction procedure for plant samples. As the effective extraction media, mixtures of methanol-water and methanol-water (pH 2.5) were chosen. The following extraction conditions were used: time of extraction step 10 minutes, 3 extraction steps, volume of extraction mixture 10 mL, temperature 25°C.

The extraction recoveries were: rosmarinic acid 100.7%, caffeic acid 99.9%, protocatechuic acid 96.0%. Yields of phenolic acids extracted from *Melissa officinalis* grown in Slovakia were: rosmarinic acid 17.03 mg/g (RSD=1.87%), caffeic acid 1.99 mg/g (RSD=3.65%), and protocatechuic acid 0.041 mg/g (RSD= 5.75%).

* Corresponding author. E-mail: branstet@cvt.stuba.sk

INTRODUCTION

Melissa officinalis belongs to the group of native medicinal plants *Lamiaceae* (together with e.g. *Rosmarinus officinalis*, *Salvia officinalis*, *Origanum vulgare*, *Lavandula angustifolia*,...). It could be used for treatment of several medical conditions such as headaches, gastro-intestinal disorders, rheumatism, and nervousness.(1)

Some studies report that extracts of *Melissa officinalis* have a significant antioxidative activity. The published results confirm the fact that this activity is partly in connection to the content of phenolic acids, mainly rosmarinic acid, that has been found in many medicinal plants in large amounts.(2)

The antioxidant IC_{50} values that have been measured indicate that rosmarinic acid is the only compound that contributes significantly to the antioxidant activities of plant extracts. The reason could be in the presence of four hydroxyl groups in the molecule of rosmarinic acid. Other phenolic acids have only one or two hydroxyl groups.(3). These compounds cannot be responsible for the antioxidant effect, since they are not present in sufficient amounts.(4,5,6)

Rosmarinic acid is present in all species of the *Lamiaceae* family.(1,2,4,7) In spite of this fact, some papers dealing with the HPLC analysis of plant extracts have also described the determination of some other phenolic acids present in low amounts in medicinal plant extracts (e.g. caffeic acid, protocatechuic acid, lithospermic acid, carnosic acid).(1,2,4,7-17)

It has been observed, that some phenolic acids are not stable and start to decompose at temperature above 50°C and heating tends to hydrolyse rosmarinic acid, which produces caffeic acid and results in substantial losses (28,2-56,4%).(8)

The UV light also has a strong influence on phenolic acid stability.(18) In nature, the phenolic acids are present as *trans* isomers, but on exposure to UV radiation or daylight they gradually transform to *cis* isomers.(19)

HPLC analysis of phenolic acids in plant material is relatively simple. The majority of published studies have applied the reversed-phase HPLC system with UV detection and organic-water (pH=2-3) or organic buffer mobile phases. Gradient elution was primarily used.

The liquid extraction (LE) is a preferable preseparation technique. Plant samples were extracted with methanol,(6,9,20) methanol followed by ethylacetate,(12) methanol with alkaline and acid hydrolysis,(15) ethanol and hexane,(11) and acetone.(10) Some papers used alcoholic-water extraction media(2,4,7) or acetone-water mixtures.(10) For the isolation of some antioxidants from plant material, the combined LE method with methanol-water, chloroform, and ethyl acetate has been published.(1) Solid phase extraction (SPE) has also been used for cleaning the extracts after LE.(15)

Supercritical fluid extraction (SFE) has also been applied as an extraction procedure for plant material.(17) SFE was compared to the LE.(16) The residue

after the SFE was extracted by LE (acetone) and the combined extracts were fractionated with preparative liquid chromatography.(5)

The aim of the presented work was to develop a simple and effective sample preparation method for the HPLC determination of phenolic acids (rosmarinic, caffeic, protocatechuic) in medicinal plant *Melissa officinalis* grown in Slovakia. Special attention has been given to the optimisation of the liquid extraction of plant material, choice of extraction solvent, number and time of individual extraction steps, and volume of chosen extractant. Suitable columns have also been tested and evaluated for a complete HPLC assay prepared for routine applications.

EXPERIMENTAL

Chemicals and Samples

Methanol (gradient grade) and ethanol (p.a.) were obtained from Merck (Slovakia), acetic acid (p.a.) was from Mikrochem (Slovakia), and formic acid (p.a.) from Lachema (Czech Republic).

Standards of rosmarinic, caffeic, protocatechuic acids, and plant samples grown in Slovakia were obtained from the Research Institute of Food Industry, Biocentrum Modra (Slovakia).

Equipment

A HP 1100 system (Hewlett-Packard, Waldbronn, Germany) consisting of a pump with degasser, a diode-array detector (DAD), and an HP ChemStation was used. Separations of phenolic acids were carried out in the following chromatographic columns: Symmetry®C18 (150x3.9 mm, 5 μm) Waters (Milford, USA), the guard column Symmetry C18 (20x3.9 mm) Waters (USA), and Separon SGX C18 (250x4 mm, 7 μm) Watrex (Bratislava, Slovakia).

The ultrasonic bath, Sonorex (Bandelin electronic, Germany), shaker T-22 Lovena (Prague, Czech Republic), pH meter HI 9321 (Hanna instruments, Portugal), filter paper Filtrak No.368 (Niederschlag, Germany), and nylon microfilter Tessek (Prague, Czech Republic) were used for the sample preparation.

Solutions

Stock solutions of rosmarinic, caffeic, and protocatechuic acids (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.

Chromatographic Conditions

A mobile phase, which consisted of MeOH and water with pH=2.5 (pH adjusted with formic acid), was used for the chromatographic separations. The linear gradients are shown in Tables 1a, b.

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

Dried tops of *Melissa officinalis* were ground to powder and 100 mg of the sample was extracted with 10 mL of extraction agent for 10 minutes in an ultrasonic bath at 25°C. The extracts were filtered and the extraction procedure was repeated twice with the residue. The solutions were filtered through a teflon microfilter prior to injection.

RESULTS AND DISCUSSION

For the development of the HPLC method, the various reversed-phase columns were tested and two of them were chosen after evaluating chromato-

Table 1. The Timetable of the Linear Solvent Gradient System Used in HPLC Analysis of Phenolic Acids

Time [min]	Methanol [%]	Water (pH 2.5) [%]
a) Symmetry®C18 Column		
0	15	85
2	15	85
25	45	55
b) Separon SGX C18 Column		
0	25	75
2	25	75
40	90	10

Table 2. Retention Time (tr), Capacity Factor (k), Resolution (R_{ij}), Asymmetry (A_s), and Number of Theoretical Plate (n) Values for Analytes, Separon SGX C18 Column, MF:HCOOH in Water (pH 2,5):Methanol, Gradient (0-2 min 75:25, 8-40 min 10:90), Flow-rate 0,4 mL/min, t_0 =5.61 min, (n=2)

	tr (min)	k	R_{ij}	A_s	n/m
Protocatechuic acid	12.94	1.31		1.66	7744
Caffeic acid	19.08	2.40	5.50	1.01	17424
Rosmarinic acid	24.95	3.45	5.06	1.20	31120

graphic characteristics (retention times, capacity factors, chromatographic resolutions, assymetry factors, and number of theoretical plates) for the studied analytes (rosmarinic, caffeic, and protocatechuic acid). The gradient elution profiles of the mobile phase for the used columns are described in Experimental. The separation characteristics for chosen columns, Separon SGX C18 and Symmetry®C18, are listed in Tables 2 and 3.

All HPLC studies were realised with a DAD detector, so quantitative analyses could be performed at absorption maxima of the analysed compounds (RA 330 nm, CA 280 nm, PA 260 nm). For the comparative analyses, HPLC chromatograms were detected at 280 nm. The detection limits at signal/noice ratio 3 were determined at this wavelenght as follows: rosmarinic acid 20 ng/mL, caffeic acid 5 ng/mL, and protocatechuic acid 10 ng/mL. Linear responses of DAD were determined for the concentration ranges RA: 1-50 μg/mL, CA: 0.6-17 μg/mL, PA: 5-200 ng/mL; and then calibration curves were calculated:

RA	y=-1.996+1.016x	r=0.9981
CA	y=-0.7159+0.9414x	r=0.9988
PA	y=-0.0004+0.5819x	r=0.9986

From the data in Tables 2 and 3, it is obvious that both columns are suitable for the HPLC separation of all analytes. The separation time of this mixture is

Table 3. Retention Time (tr), Capacity Factor (k), Resolution (R_{ij}), Asymmetry (A_s), and Number of Theoretical Plates (n) Values for Analytes, Symmetry®C18 Column, MF:HCOOH in Water (pH 2,5):Methanol, Gradient (0-2 min 85:15, 2-25 min 55:45), Flow-rate 0,4 mL/min, t_0 =2.78 min, (n=2)

	tr (min)	k	R_{ij}	A_s	n/m
Protocatechuic acid	8.61	2.09		2.01	11638
Caffeic acid	11.42	3.11	4.74	1.11	76891
Rosmarinic acid	16.56	4.96	8.47	1.33	41410

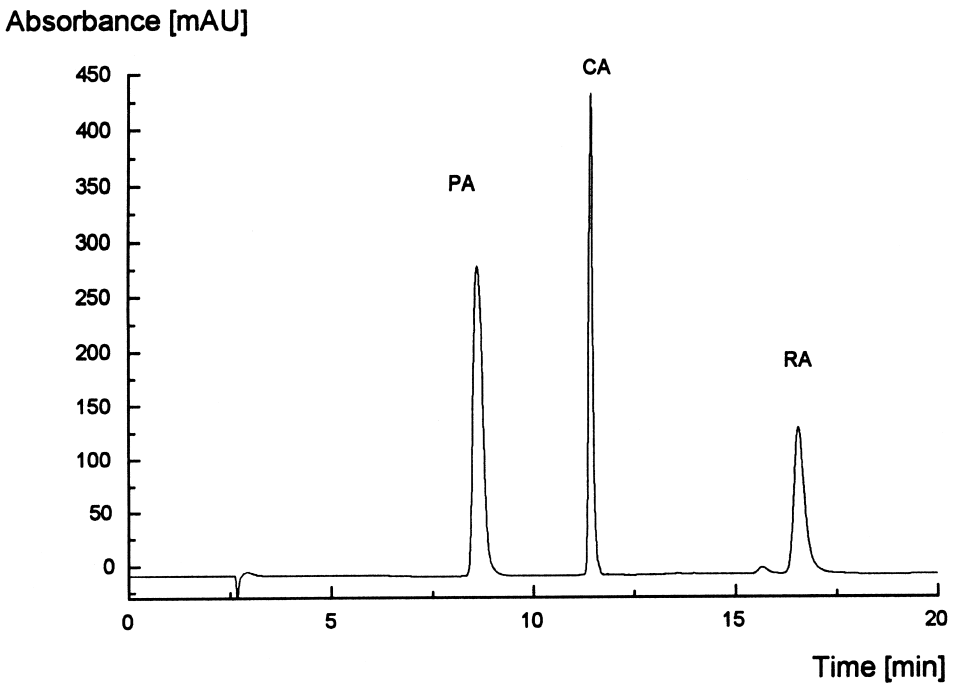

Figure 1. HPLC chromatogram of phenolic acids standards : PA- protocatechuic acid (55.1 μg/mL), CA- caffeic acid (19.0 μg/mL), RA- rosmarinic acid (35.5 μg/mL). Chromatographic conditions: chromatographic column, Symmetry C18 (150x3.9 mm 5 μm); mobile phase MeOH-water (pH 2.5) gradient elution; flow-rate, 0.4 mL/min; detection, DAD 280 nm; injection volume of 20 μL.

from 17-27 minutes; the R_{ij} values are more than sufficient for the quantitative analysis. The A_s factors for both columns are comparable, but the Symmetry column has significantly higher numbers of theoretical plates. The HPLC chromatograms of phenolic acid mixture with Separon SGX C18 and Symmetry®C18 columns are demonstrated in Fig. 1 and Fig. 2. From both chromatograms, it is possible to observe that a small peak is eluting before the rosmarinic acid peak. As it was mentioned in the Introduction, a small amount of *cis* isomer could be detected and separated from *trans* isomer in the operation at daylight. The HPLC separation of *cis* and *trans* isomers of rosmarinic acid is illustrated in the chromatogram. (Fig. 3) This model solution was obtained after the irradiation at $\lambda_{cut\ off}$ > 315 nm for 6 hours.

It is very interesting that the R_{ij} and A_s values for both columns are similar, though it is known that Separon SGX C18 column is packed with 7 μm particles

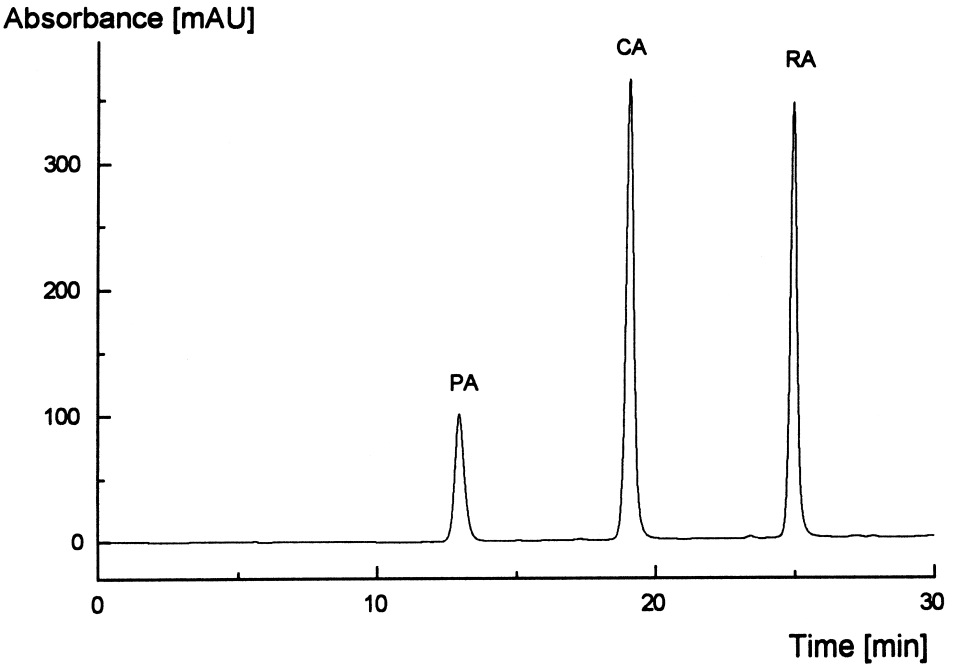

Figure 2. HPLC chromatogram of phenolic acids standards: PA- protocatechuic acid (28.6 μg/mL), CA- caffeic acid (42.5 μg/mL), RA- rosmarinic acid (84.8 μg/mL). Chromatographic conditions: chromatographic column, Separon SGX C18 (250x4 mm; 7 μm); mobile phase MeOH-water (pH 2.5) gradient elution; flow-rate, 0.4 mL/min; detection, DAD 280 nm; injection volume of 20 μL.

and only partially end-capped. The acidic properties of the analysed compounds and the low pH of the applied mobile phase could explain this fact. At this pH value, the residual silanol groups in C-18 sorbent do not react very strongly with the analytes and it could be the reason for the high symetries of phenolic acid peaks in both columns.

The main part of this work was devoted to the optimisation of plant sample preparation with the application of the liquid extraction technique. After the preliminary experiments, it has been found out that the application of ultrasonic bath with the controlled temperature is more efficient in comparison to classical extraction assay, which requires shaking of the extracted material with the solvent mixture. If the ultrasonic bath in the same time interval was used, the extraction yields for all analytes were higher. Therefore, the method of liquid extraction in the ultrasonic bath at temperature 25°C has been chosen for optimisation of the extraction assay.

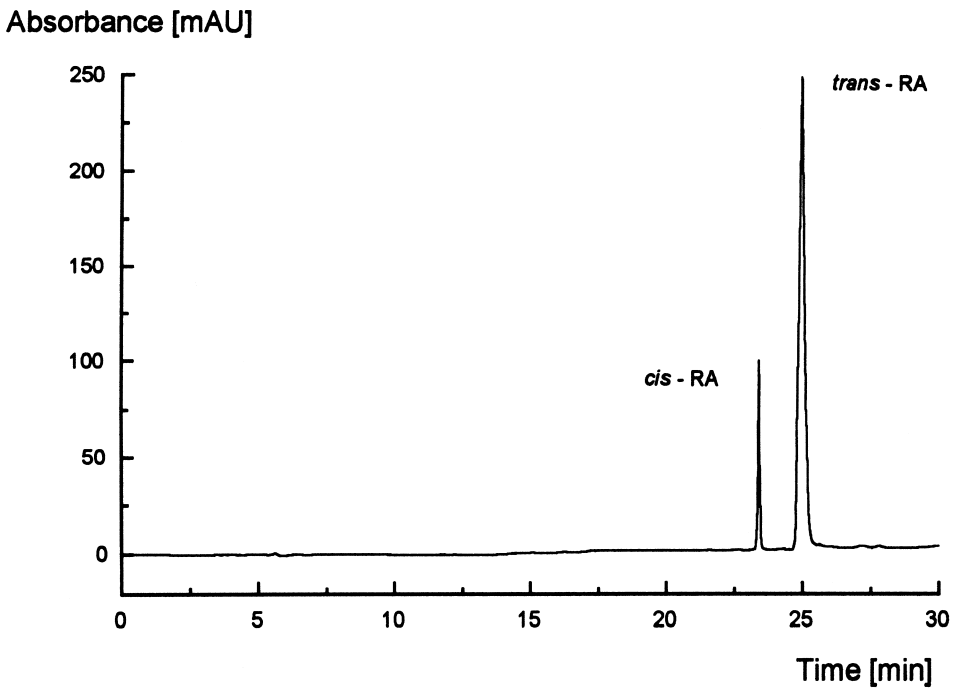

Figure 3. HPLC chromatogram of *cis* and *trans* isomers of rosmarinic acid. Chromatographic conditions: chromatographic column, Separon SGX C18 (250x4 mm; 7 μm); mobile phase MeOH-water (pH 2.5) gradient elution; flow-rate, 0.4 mL/min; detection, DAD 280 nm; injection volume of 20 μL.

As it is known from the literature, nearly all papers recommend alcoholic or water-alcoholic extraction media for the isolation of phenolic acids from plant materials. However, comparisons of extraction recoveries for different extraction media and aspects of optimisation of extraction conditions have not recently been published.

Methanol-water, ethanol-water, with or without pH regulation (pH 2.5), and water (pH 2.5) have been tested for RA, CA, and PA in two extraction steps. Various volumes of extraction solvent (5, 10, 15 mL) and different times for the individual extraction step (5, 10, 20 min) were used. It was found out, that yields of all analytes were not higher with 15 mL of extraction solvents used and the yields for 10 mL of extractants were only about 6% higher for RA, 8% for CA, and 13% for PA. This means that when only 5 mL of extraction mixture was used, nearly 90% of all analytes could become isolated from the plant material. The same situation was observed with the extraction time. The time of 10 min-

Table 4. Yields of Rosmarinic, Caffeic, and Protocatechuic Acids Extracted with Different Extraction Agents from *Melissa officinalis*

	RA		CA		PA	
	1* [mg/g]	2* [mg/g]	1 [mg/g]	2 [mg/g]	1 [mg/g]	2 [mg/g]
MeOH:Water	14.70	16.94	1.45	1.91	0.0345	0.0410
MeOH:Water (pH 2.5)	15.30	17.21	1.48	1.72	0.0276	0.0383
Water (pH 2.5)	11.86	15.53	0.84	0.99	0.0156	0.0183
EtOH:Water	13.52	15.54	1.14	1.36	0.0016	0.0016
EtOH:Water (pH 2.5)	15.30	17.16	1.64	1.81	0.0051	0.0059

*Number of extraction steps.
Extraction conditions: two extraction steps, volume of extraction agent, 10 mL; time of extraction step 10 minutes (n=2).

utes was completely sufficient to gain the maximum yields of analysed compounds. The extraction yields for the two-step procedure, when different extraction media have been used, are illustrated in Table 4.

It can be seen, that the highest yields for all analytes have been achieved when methanol-water mixture was used. Only the RA extraction yields are slightly higher for acidified alcoholic-water mixtures. Thus, methanol-water (60:40) was chosen for the optimisation of the required number of extraction steps. The results presented in Table 5 confirm that three extraction steps are sufficient for complete isolation of all phenolic acids from Melissa plant samples. The precision of this assay was evaluated by RSD values (Table 5), and the accuracy was evaluated by extraction recoveries after adding known concentrations of

Table 5. Yields and RSD Values of Rosmarinic, Caffeic, and Protocatechuic Acids Extracted from *Melissa officinalis*

	1*		2		3		4		5	
	[mg/g]	RSD [%]	[mg/g]	RSD [%]	[mg/g]	RSD [%]	[mg/g]	RSD [%]	[mg/g]	RSD [%]
RA	14.70	1.50	16.94	1.64	17.03	1.87	17.03	1.92	17.04	1.96
CA	1.45	2.84	1.91	3.05	1.99	3.65	1.99	3.65	1.99	3.65
PA	0.0345	4.98	0.0410	5.01	0.0410	5.75	0.0413	5.75	0.0413	5.75

* Numbers 1 through 5 refer to number of extraction steps.
Extraction conditions: extraction agent, MeOH:voda (60:40); volume of extraction agent, 10 mL; time of extraction step 10 minutes (n=3).

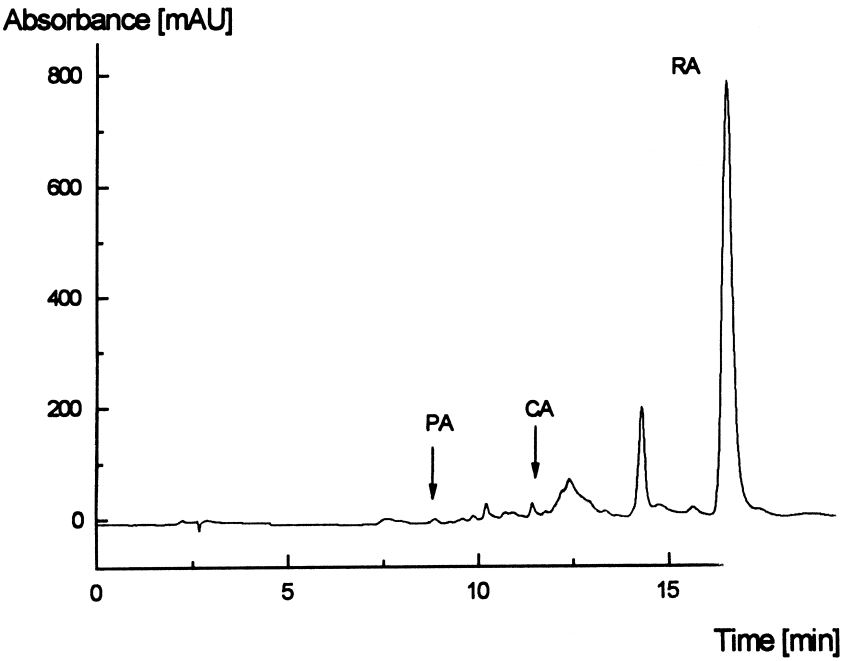

Figure 4. HPLC chromatogram of *Melissa officinalis* extract. Chromatographic conditions: chromatographic column, Symmetry C18 (150x3.9 mm 5 μm); mobile phase MeOH-water (pH 2.5) gradient elution; flow-rate, 0.4 mL/min; detection, DAD 280 nm; injection volume of 20 μL.

all analytes to the plant sample before the extraction procedure. The extraction recoveries were high: RA 100.7% (c=17.2 μg/mL), CA 99.9% (c=7.1 μg/mL), PA 96.0% (c=0.08 μg/mL).

After the optimisation of the complete extraction process, the following extraction conditions have been recommended:

Extraction solvent: methanol-water (60:40)
Volume of extraction solvent: 10 mL
Extraction time: 10 minutes
Number of extraction steps: 3
Extraction temperature: 25°C

Robustness of the HPLC assay was confirmed by using two different HPLC columns for the plant extracts analyses. The R_{ij} values were evaluated for

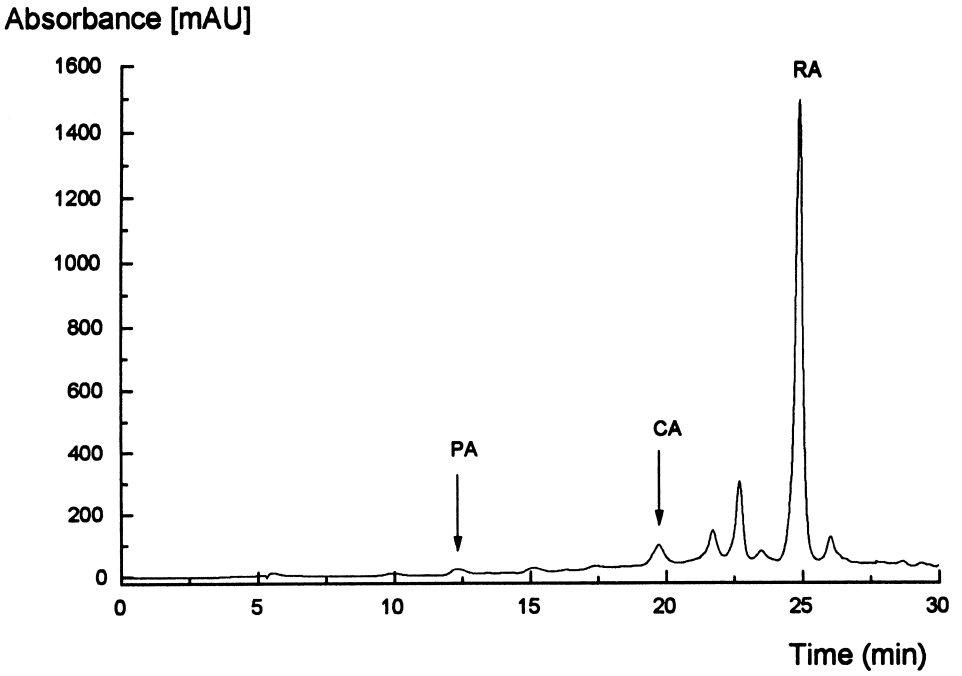

Figure 5. HPLC chromatogram of *Melissa officinalis* extract. Chromatographic conditions: chromatographic column, Separon SGX C18 (250x4 mm; 7 μm); mobile phase MeOH-water (pH 2.5) gradient elution; flow-rate, 0.4 mL/min; detection, DAD 280 nm; injection volume of 20 μL.

the peak eluting in extract before and after the analytes for both tested columns. The R_{ij} values were in the range 1.43 - 2.66 for the Symmetry®C18 column and 1.31 - 2.17 for the Separon SGX C18 column. It is clear that the R_{ij} values are comparable and sufficient for the quantitative analysis.

The HPLC chromatograms of extracts from *Melissa officinalis* using both recommended columns, are demonstrated in Figs. 4 and 5. It may be seen, that no additional clean-up step to purify the extracts is necessary and all three phenolic acids could be quantified.

In conclusion, this optimisation of the extraction procedure could be applied for HPLC monitoring of rosmarinic, caffeic, and protocatechuic acids in *Melissa officinalis* grown in Slovakia, as well as for stability studies of all analysed phenolic acids, mainly rosmarinic acid.

ACKNOWLEDGMENTS

This research was supported by the Slovak Grant Agency for Science, VEGA, Grant No.1/6102/99. The authors would like to thank Waters Corp. Milford, USA for a Symmetry C-18 column and a guard column.

REFERENCES

1. Tagashira, M.; Ohtake, Y. A New Antioxidative 1,3-Benzodioxole from *Melissa Officinalis*. Planta Med. **1998**, *64*, 555-558.
2. Lamaison, J.L.; Petitjean-Freytet, C.; Carnat, A. Lamiacées Médicinales à Propriétés Antioxydantes, Sources Potentielles d`Acide Rosmrinique. Pharm. Acta Helv. **1991**, *66* (7), 185-188.
3. Chen, J.H.; Ho, C.T. Antioxidant Activities of Caffeic Acid and its Related Hydroxycinnamic Acid Compounds. J. Agric. Food Chem. **1997**, *45*, 2374-2378.
4. Hohmann, J.; Zupkó, I.; Rédei, D.; Csányi, M.; Falkay, G.; Máthé, I.; Janicsák, G. Protective Effects of the Aerial Parts of *Salvia Officinalis*, *Melissa Officinalis* and *Lavandula Angustifolia* and Their Constituents Against Enzyme-Dependent and Enzyme-Independent Lipid Peroxidation. Planta Med. **1999**, *65*, 576-578.
5. Lu, Y.; Foo, Y. Rosmarinic Acid Derivatives from *Salvia Officinalis*. Phytochemistry **1999**, *51*, 91-94.
6. Guédon, D.J.; Pasquier, B.P. Analysis and Distribution of Flavonoid Glycosides and Rosmarinic Acid in 40 *Mentha x Piperita* Clones. J. Agric. Food Chem. **1994**, *42*, 679-683.
7. Janicsák, G.; Máthé, I.; Miklóssy-Vári, V.; Blunden, G. Comparative Studies of the Rosmarinic and Caffeic Acid Contents of Lamiaceae Species. Biochem. Syst. Ecol. **1999**, *27*, 733-738.
8. Rawn, H.; Pedersen, M.F.; Borum, J.; Andary, C.; Anthoni, U.; Christophersen, C.; Nielsen, P.H. Seasonal Variation and Distribution of Two Phenolic Compounds, Rosmarinic Acid and Caffeic Acid, in Leaves and Roots-Rhizomes of Eelgrass (*Zostera marina* L.). Ophelia **1994**, *40* (1), 51-61.
9. Yuan, J.P.; Chen, H.; Chen, F. Simultaneous Determination of Rosmarinic Acid, Lithospermic Acid B and Related Phenolics in *Salvia Miltiorrhiza* by HPLC. J. Agric. Food Chem. **1998**, *46*, 2651-2654.
10. Morimoto, S.; Goto, Y.; Shoyama, Y. Production of Lithospermic Acid B and Rosmarinic Acid in Callus Tissue and Regenerated Plantlets of *Salvia Miltiorrhiza*. J. Nat. Prod. **1994**, *57* (6), 817-823.

11. Cuvelier, M.E.; Berset, C.; Richard, H. Separation of Major Antioxidants in Sage by High Performance Liquid chromatography. Sci. Aliments **1994**, *14*, 811-815.
12. Kovatcheva, E.; Pavlov, A.; Koleva, I.; Ileva, M.; Mihneva, M. Rosmarinic Acid from *Lavandula Vera* MM Cell Culture. Phytochemistry **1996**, *43* (6), 1243-1244.
13. Tada, H.; Murakami, Y.; Omoto, T.; Shimomura, K.; Ishimaru, K. Rosmarinic Acid and Related Phenolics in Hairy Root Cultures of *Ocimum Basilicum*. Phytochemistry **1996**, *42*, 431-434.
14. Okamura, N.; Fujimoto, Y.; Kuwabara, S.; Yagi, A. High-Performance Liquid Chromatographic Determination of Carnosic Acid and Carnosol in *Rosmarinus Officinalis* and *Salvia Officinalis*. J. Chromatogr. **1994**, *679*, 381-386.
15. Andrade, P.B.; Seabra, R.M.; Valentao, P.; Areias, F. Simultaneous Determination of Flavonoids, Phenolic Acids, and Coumarins in Seven Medicinal Species by HPLC/Diode-Array Detector. J. Liq. Chrom. & Rel. Technol. **1998**, *21* (18), 2813-2820.
16. Tena, M.T.; Valcarel, M.; Hidalgo, P.J.; Ubera, J.L. Supercritical Fluid Extraction of Natural Antioxidants from Rosemary: Comparison with Liquid Solvent Sonication. Anal. Chem. **1997**, *69*, 521-526.
17. Señoras, F.J.; Ibañez, E.; Cavero, S.; Tabera, J.; Reglero, G. Liquid Chromatographic-Mass Spectrometric Analysis of Supercritical-Fluid Extracts of Rosemary Plants. J. Chromatogr. **2000**, *870*, 491-499.
18. Rodriguez de Sotillo, D.; Hadley, M.; Holm, E.T. Phenolics in Aqueous Potato Peel Extract: Extraction, Identification and Degradation. J. Food Sci. **1994**, *59* (2), 649-651.
19. Buiarelli, F.; Cartoni, G.; Coccioli, F.; Levetsovitou, Z. Determination of Phenolic Acids in Wine by High-Performance Liquid Chromatography with Microbore Column. J. Chromatogr. **1995**, *695*, 229-235.
20. Donovan, J.L.; Meyer, A.S.; Waterhouse, A.L. Phenolic Composition and Antioxidant Activity of Prunes and Prune Juice (*Prunus domestica*). J. Agric. Food Chem. **1998**, *46*, 1247-1252.

Received December 22, 2000 Manuscript 5467
Accepted March 20, 2001