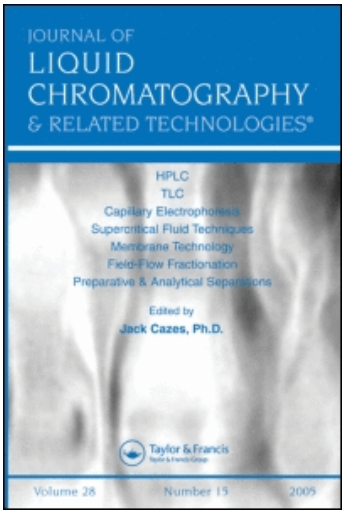

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Validation of HPLC Determination of Phenolic Acids Present in Some Lamiaceae Family Plants

Alica Ziaková[a]; Eva Brandšteterová[a]
[a] Department of Analytical Chemistry, Faculty of Chemical and Food Technology, Slovak Technical University, Bratislava, Slovakia

Online publication date: 02 March 2003

To cite this Article Ziaková, Alica and Brandšteterová, Eva(2003) 'Validation of HPLC Determination of Phenolic Acids Present in Some **Lamiaceae** Family Plants', Journal of Liquid Chromatography & Related Technologies, 26: 3, 443 — 453
To link to this Article: DOI: 10.1081/JLC-120017181
URL: http://dx.doi.org/10.1081/JLC-120017181

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES®
Vol. 26, No. 3, pp. 443–453, 2003

Validation of HPLC Determination of Phenolic Acids Present in Some *Lamiaceae* Family Plants

Alica Ziaková and Eva Brandšteterová*

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

ABSTRACT

HPLC columns with different length, particle size, and chemical properties of sorbent were tested and compared for the application in the development of the universal HPLC assay for determination of phenolic compounds, which could be present in some medicinal plants from the *Lamiaceae* family (*Melissa officinalis*, *Rosmarinus officinalis*, *Salvia officinalis*, *Thymus serpyllum*, and *Origanum vulgare*). More RP-18 columns have been chosen for the simultaneous separation of all phenolic compounds in the study. The basic chromatographic characteristics were evaluated and the HPLC method using one of the suitable columns was validated for all determined analytes. The recommended separation conditions were applied for phenolic compound monitoring in extracts of plant material. Yields of analytes present in all plant samples were evaluated.

*Correspondence: Eva Brandšteterová, Department of Analytical Chemistry, Faculty of Chemical and Food Technology, Slovak Technical University, Radlinskeho 9, 812 37 Bratislava, Slovakia; E-mail: branstet@cvt.stuba.sk.

DOI: 10.1081/JLC-120017181 1082-6076 (Print); 1520-572X (Online)

Key Words: Validation; HPLC; Phenolic acids; Plants.

INTRODUCTION

Phenolic acids constitute a large group of naturally occurring organic compounds with a broad spectrum of pharmacological activities. It was found that they possess not only antioxidant but also antiviral and antibacterial properties. The antioxidant activity of phenolics is generally combined with hydroxyl groups on their molecules. Phenolic acids are widely distributed in the natural plants, e.g., fruits, vegetable, various medicinal, and the 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 derivates in combination with the other groups of phenolic compounds.[1]

The *Lamiaceae* family seems to be a rich source of plant species containing large amounts of phenolic acids, so it is considered to be a promising source of natural antioxidants. Free phenolic acids in 10 species belonging to the *Lamiaceae* family have already been analyzed using the HPLC method, but mobile phases with different composition were used for the determination of less polar rosmarinic acid and the other more polar phenolic acids. Therefore, two independent analyses for each plant sample were needed.[2] Seventy taxa of *Lamiaceae* have also been studied and it was found that many species displayed DPPH radical scavenging activity.[3] DPPH radical scavenging activity in the four *Lamiaceae* plants, including *Melissa officinalis*, was related to the content of rosmarinic acid and its derivatives.[4]

In our previous works[5,6] an HPLC assay was developed for the simultaneous separation and determination of rosmarinic acid, caffeic acid, and protocatechuic acids isolated from *M. officinalis*, as well as different extraction methods (liquid extraction, SPE, PSE, SFE, MSPD), which were tested and evaluated. The aim of this work is to present a universal method for the isolation and determination of phenolic acids from different medicinal plants of the *Lamiaceae* family. For these reasons, different analytical columns and additional phenolic compounds were utilized in this study.

EXPERIMENTAL

Chemicals, Samples, and Solutions

Standards of rosmarinic (RA), caffeic (CA), protocatechuic (PA), gallic (GA), and synapic acids (SA), 3,4-dihydroxybenzaldehyde (protocatechualdehyde) (DBA), and catechin (CAT), were obtained from Research Institute of

Food Industry, Biocentrum Modra (Slovakia). Plant samples of lemon balm (*M. officinalis*), rosemary (*Rosmarinus officinalis*), sage (*Salvia officinalis*), thyme (*Thymus serpyllum*), and oregano (*Origanum vulgare*) were bought in the local market.

Stock solutions of standards (ca. 1 mg/mL) were prepared in methanol and stored in a 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 water or mobile phase. HPLC grade methanol was purchased from Merck (Slovakia) and formic acid (p.a.) from Lachema (Czech Republic).

HPLC Equipment

The HPLC analysis of phenolics was performed using an HP 1100 system (Hewlett-Packard, Waldbronn, Germany) consisting of a pump with degasser, a diode-array detector (DAD), and an HP ChemStation.

Chromatographic Conditions

The following analytical columns were tested: Symmetry® C18 (150 × 3.9 mm, 5 μm) Waters (Milford, USA), Symmetry Shield™ C18 (150 × 3.9 mm, 5 μm) Waters (Milford, USA); Separon SGX C18 (250 × 4 mm, 7 μm) Watrex (Bratislava, Slovakia); Develosil ODS (100 × 4.6 mm, 3 μm) Watrex (Bratislava, Slovakia); GROM SIL 100 ODS-0 AB (50 × 4 mm, 3 μm) Grom (Germany); GROM SIL 120 ODS-3 CP (50 × 4 mm, 3 μm) Grom (Germany); GROM SIL 120 ODS-4 HE (50 × 4 mm, 3 μm) Grom (Germany); GROM SIL 120 CYANO-3 CP (50 × 4 mm, 5 μm) Grom (Germany); GROM SIL 100 OCTYL-4 FE (50 × 4 mm, 3 μm) Grom (Germany).

A mobile phase consisting 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 0.5 mL/min and injection volume 20 μL. All analyses were carried out at laboratory temperature. Diode-array detector was working in the range of 200–400 nm and chromatograms were acquired at different wavelengths according to absorption maxima of analyzed compounds.

Sample Preparation

The extraction procedure of phenolic compounds from plant material was realized according to our previously published method.[3] Dried plants 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 extraction procedure was repeated twice with the residue. The solutions were filtered through a nylon microfilter Tessek (Czech Republic) prior to injection into the HPLC column.

RESULTS AND DISCUSSION

For the HPLC separation of phenolic acids various reversed-phase C18 columns (GROM-SIL 100 ODS-0 AB, 120 ODS-3 CP, 120 ODS-4 HE, SEPARON SGX C18, Symmetry Shield C18, Symmetry C18, Develosil ODS), one C8 (GROM-SIL 100 OCTYL-4 FE) and one CN (GROM-SIL 120 CYAN-3 CP) columns filled with different sorbents, of various lengths and particle sizes have been tested. Chromatographic characteristics (retention time, capacity factor, chromatographic resolution, asymmetry factor, and number of theoretical plates) for the studied phenolic compounds were evaluated and compared. Assymetry factor values were calculated in 10% of peak height. It was found out, that the cyanic stationary phase is not suitable for the separation of analytes, because of insufficient resolution between gallic and protocatechuic acids ($R_{ij} = 1.00$) and between (+)-catechin and caffeic acid ($R_{ij} = 0.90$). There were also high asymmetry values evaluated for this column, especially for rosmarinic acid.

The tested C8 column showed worse peak shapes and higher asymmetry values compared to C18 columns, so this phase has also not been recommended for the separation of studied phenolic compounds.

All tested C18 phases were suitable for the separation of investigated analytes; the chromatographic resolutions were sufficient for the quantitative analysis. There were only differences in peak symmetries and retention times caused by variety in particle size, column length, and different sorbent endcapping. The best symmetries of all analytes were obtained for Symmetry Shield, Symmetry and Grom Sil 120 ODS-4 HE columns. But, also, the other tested C 18 columns could be applied for this kind of analysis and the separation parameters for all analytes are very good. Chromatographic characteristics for five tested C-18 columns are illustrated in Table 1 (a)–(e).

The main validation parameters [listed in Table 2 (a)–(d)] were evaluated to check the suitability of the chromatographic assay developed for the determination of rosmarinic acid, caffeic acid, protocatechuic acid and its aldehyde using Symmetry C18 column. The repeatabilities of the retention times were determined from 10 injections of standards at different concentrations. The repeatabilities of the injection were calculated at two concentration levels. The repeatabilities of retention times for all standards were under 1% and repeatabilities of the injection were all under 2%. The

Table 1. Capacity factor (k), chromatographic resolution (R_{ij}), assymetry factor (A_s), values and number of theoretical plates (n) for all analytes using different analytical columns.

	k	R_{ij}	A_s	n/m
(a) Separon SGX C18 250 × 4.6 mm, 7 μm				
GA	1.11		0.87	5,476
PA	2.00	4.28	0.93	16,700
CAT	2.51	2.88	1.10	26,896
CA	3.56	5.21	1.00	25,440
SA	4.81	5.87	0.83	54,089
RA	5.11	1.45	1.11	52,105
(b) Develosil 100 × 4.6 mm, 3 μm ODS				
GA	1.32		1.00	8,600
PA	2.89	5.71	1.00	43,270
CAT	3.89	4.47	1.00	86,490
CA	5.68	7.56	1.33	103,230
SA	7.89	9.33	1.14	285,610
RA	10.16	4.78	1.14	287,640
(c) Grom SIL 120 ODS-4 HE 50 × 4 mm, 3 μm				
GA	1.68		1.00	3,080
PA	3.68	2.92	1.11	17,460
CAT	6.00	4.63	1.00	73,700
CA	8.74	4.95	1.00	54,000
SA	13.63	8.09	0.98	206,090
RA	18.95	7.77	1.00	149,630
(d) Symmetry C18 150 × 3.9 mm, 5 μm				
GA	1.29		1.00	4,500
PA	3.06	5.45	1.10	20,310
CAT	4.03	3.67	1.10	48,740
CA	6.09	7.37	1.00	71,200
SA	8.44	8.42	1.13	171,740
RA	11.53	10.50	1.30	134,430
(e) Symmetry Shield C18 150 × 3.9 mm, 5 μm				
GA	2.00		1.08	8,170
PA	3.63	5.18	1.00	27,990,
CAT	5.09	5.67	1.00	75,610
CA	7.29	8.56	1.00	89,700
SA	8.57	5.00	1.00	187,040,
RA	11.37	10.89	1.13	199,990

Note: Mobile phase: HCOOH in water (pH 2.5) : methanol, gradient elution, flow-rate 0.5 mL/min, ($n = 3$).

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

Table 2. Validation parameters for the chromatographic system using symmetry C18 column.

Parameter	Value
(a) Rosmarinic acid	
Repeatability	
of retention times[a]	0.27%
of the injection[b]	
9.09 μg/mL	1.26%
93.44 μg/mL	1.54%
Calibration curve[c]	
intercept	$-0.829 \times 10^{-2} \pm 3.692 \times 10^{-2}$
slope	0.161 ± 0.001
r^2	0.9997
LOQ	30 ng/mL
Recovery	
17.20 μg/mL	100.7%
62.29 μg/mL	101.1%
(b) Caffeic acid	
Repeatability	
of retention times[a]	0.66%
of the injection[b]	
0.497 μg/mL	1.10%
4.97 μg/mL	1.78%
Calibration curve[c]	
intercept	$0.506 \times 10^{-2} \pm 1.592 \times 10^{-2}$
slope	0.280 ± 0.004
r^2	0.9988
LOQ	10 ng/mL
Recovery	
3.97 μg/mL	88.0%
0.70 μg/mL	91.1%
(c) Protocatechuic acid	
Repeatability	
of retention times[a]	0.87%
of the injection[b]	
0.59 μg/mL	1.76%
7.37 μg/mL	1.61%
Calibration curve[c]	
intercept	$0.130 \times 10^{-2} \pm 0.179 \times 10^{-2}$
slope	0.191 ± 0.001
r^2	0.9999

(continued)

Table 2. Continued

Parameter	Value
LOQ	15 ng/mL
Recovery	
0.59 μg/mL	95.2%
2.46 μg/mL	99.1%
(d) DBA	
Repeatability	
of retention times[a]	0.82%
of the injection[b]	
5.74 μg/mL	1.76%
0.574 μg/mL	1.46%
Calibration curve[c]	
intercept	$1.972 \times 10^{-2} \pm 2.967 \times 10^{-2}$
slope	0.192 ± 0.001
r^2	0.9999
LOQ	15 ng/mL
Recovery	
0.574 μg/mL	96.3%
2.296 μg/mL	98.7%

[a]RSD from ten measurements.
[b]RSD from six measurements.
[c]Duplicate injection of six calibration standards.

parameters of the calibration curves and their correlation coefficients show very good linearity selected in concentration ranges. The limit of quantification (LOQ) was calculated from the peak height based on signal-to-noise ratio of 10.

The extraction recoveries of all analytes in *M. officinalis* samples were determined at two concentration levels by adding pure standards to the plant samples prior to the extraction procedure. The recoveries were very high (more than 95%) for all analytes, except caffeic acid, where the recoveries were 88.0 and 91.1% for tested concentration levels.

After column testing and method evaluation, different Lamiaceae plants were extracted and analysed using the method described in Experimental. As is possible to see from Table 3 and Fig. 1(a)–(c), rosmarinic acid is the most predominant phenolic acid in all plant samples, which is in accordance with previously published results.[2–4] Also, caffeic acid was present in all samples.

Table 3. Yields and RSD values of RA, CA, PA, and DBA and extracted from different medicinal plants.

	RA		CA		PA		DBA	
	(mg/g)	RSD (%)	(mg/g)	RSD (%)	(mg/g)	RSD (%)	(mg/g)	RSD (%)
O. vulgare	14.752	0.81	0.216	4.08	0.305	2.82	0.0675	2.21
R. officinalis	23.578	2.31	0.203	3.69	0.202	2.79	—	—
S. officinalis	7.546	2.77	0.171	1.85	—	—	—	—
T. serpyllum	13.173	2.78	0.262	1.76	0.201	2.93	0.0465	2.54
M. officinalis	21.002	2.29	0.192	3.65	0.044	1.56	0.0549	4.99

Note: The extraction conditions are extraction agent, MeOH : water (60 : 40); volume of extraction agent, 10 mL; time of extraction step 10 min ($n = 3$).

No protocatechuic acid was detected in *S. officinalis* and no protocatechualdehyde was found in sage and rosemary samples.

In conclusion, the presented HPLC assay could be used as a generic method for the simultaneous determination of phenolic acids present in medicinal plants of the *Lamiaceae* family. It was found, that rosmarinic acid is the main phenolic compound detected in all studied plant samples. The other phenolics are present in more than 100 times lower concentrations, and for this reason, could not contribute to a great deal to the antioxidant activity of plant extracts. The demands on the choice of columns for HPLC determination of phenolics in this kind of plant samples are not very strict, most of the higher quality of sorbents are suitable and the column length or

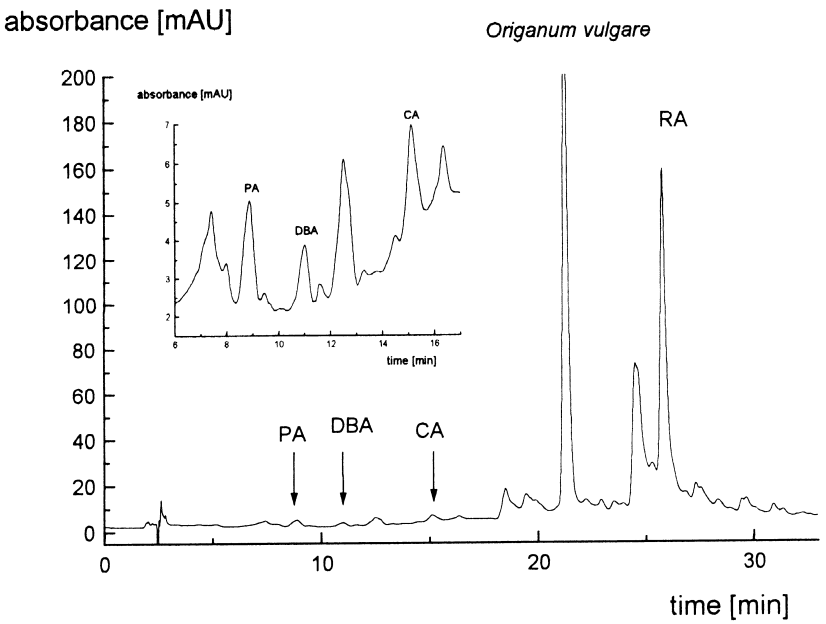

Figure 1. HPLC chromatograms of different Lamiaceae plant extracts. (a) oregano (*O. vulgare*), (b) thyme (*T. serpyllum*), (c) rosemary (*R. officinalis*). Chromatographic conditions: column: Symmetry C18 (150 × 3.9 mm, 5 μm) with Symmetry C18 precolumn (20 × 3.9 mm), mobile phase, MeOH–water (pH 2.5), gradient elution, flow-rate 0.5 mL/min, detection, DAD 280 nm, injection volume 20 μL.

(continued)

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

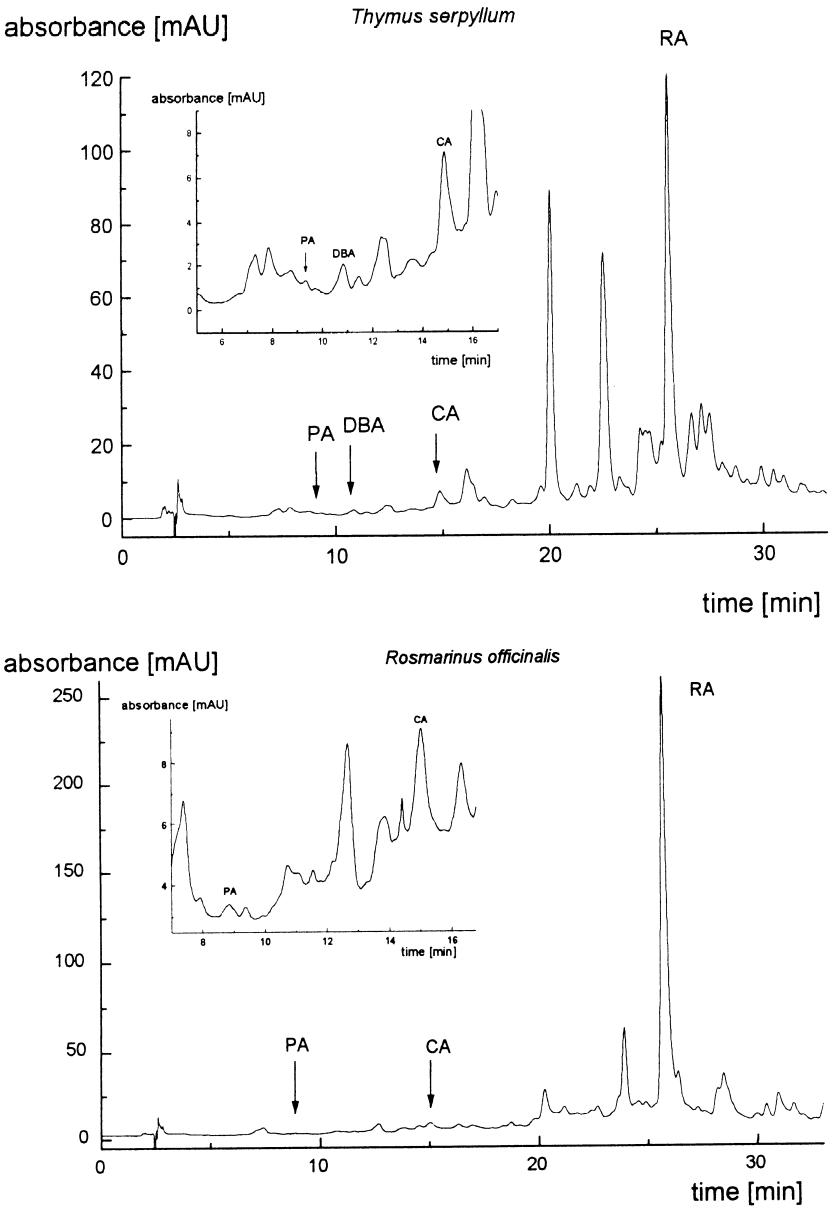

Figure 1. Continued.

particle size have influenced only the column efficiency. Symmetries of peaks or the other chromatographic parameters have been comparable for all tested columns.

ACKNOWLEDGMENTS

This work was supported by the Slovak Grant Agency for Science, VEGA, No. 1/6102/99 and 1/9128/02. The authors would like to thank Waters Corp. Milford, USA, for Symmetry C18 and SymmetryShield C18 columns and Grom Analytik, Germany for HPLC columns.

REFERENCES

1. Brandšteterová, E.; Ziaková-Caniová, A. Phenolic acids in natural plants, analysis by HPLC. *Encyclopedia of Chromatography*; Cazes, J., Ed.; Marcel Dekker Inc.: 2002; *in press*.
2. Zgorka, G.; Glowniak, K.; Variation of free phenolic acids in medicinal plants belonging to the *Lamiaceae* family. J. Pharm. Biomed. Anal. **2001**, *26*, 79–87.
3. 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.
4. Tagashira, M.; Ohtake, Y. A new antioxidative 1,3-benzodioxole from *Melissa officinalis.* Planta Med. **1998**, *64*, 555–558.
5. Caniová, A.; Brandšteterová, E. HPLC analysis of phenolic acids in *Melissa officinalis.* J. Liq. Chromatogr. & Rel. Technol. **2001**, *24* (17), 2647–2659.
6. Ziaková, A.; Brandšteterová, E. Application of different preparation techniques for extraction of phenolic antioxidants from lemon balm (*Melissa officinalis*) before HPLC analysis. J. Liq. Chromatogr. & Rel. Technol. **2002**, *in press.*

Received August 27, 2002
Accepted September 24, 2002
Manuscript 5953