Scientific paper

Determination of Major Phenolic Acids, Phenolic Diterpenes and Triterpenes in Rosemary (*Rosmarinus officinalis* L.) by Gas Chromatography and Mass Spectrometry

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Abstract

A gas chromatographic-mass spectrometric (GC-MS) method for the simultaneous identification and quantification of seven major phenolic and terpenic compounds in *Rosmarinus officinalis* L. was developed. The compounds were identified as trimethylsilyl (TMS) derivatives of phenolic acids (caffeic and rosmarinic acid), phenolic diterpene (carnosic acid), and pentacyclic triterpenes (ursolic, oleanolic, betulinic acid and betulin). These compounds have been identified by retention time and comparison of mass spectra. The procedure involves ultrasonic extraction using solvent mixture of tetrahydrofuran and ethanol. Extracts were fractionated by size exclusion chromatography (SEC) after purifying on graphitised carbon. The fraction with phenolic and terpenic compounds was derivatised prior to GC-MS analysis using *N*-methyl-*N*-trimethylsilyl trifluoroacetamide (MSTFA) as a derivatisation reagent. The derivatisation process was optimized regarding temperature and reaction time. The linearity of the method was tested in concentration range 4–25 mg L^{-1}. The correlation coefficients (r^2) were in the range of 0.997 to 0.999. The average recoveries for all compounds ranged from 80 to 82%. The GC-MS technique is specific and sensitive, and can be used for simultaneous identification and determination of a wide range of phenolic and terpenic compounds in different plants even at trace levels.

Keywords: diterpenes, triterpenes, *Rosmarinus officinalis* L., size exclusion chromatography, gas chromatography, mass spectrometry.

1. Introduction

In recent years, natural compounds such as phenolic acids, phenolic diterpenes and triterpenes, present in various plants, have been the subject of intense research due to their potential benefits for human health. It has been demonstrated that the antioxidant and radical scavenging activities are the main properties of these compounds. Therefore, they may contribute to preventing cardiovascular or inflammatory diseases and cancer, which are caused, among others, by harmful effects of free radicals. Besides their primary antioxidant activity, the compounds also display a variety of biological functions such as antibacterial, cytotoxic, antiviral and fungistatic activities.[1–13] Since the addition of synthetic antioxidants to foods is very limited for legislative reasons, natural antioxidants derived from plants, especially phenols, have become of considerable interest from the viewpoint of dietary antioxidant supplementation and food preservation. Many plants and herbs are considered to be excellent and rich sources of phenolic and terpenic compounds.[1,3,5,18,22,25,29,31] Among others, rosemary (*Rosmarinus officinalis* L.) contains high percentages of phenolic acids (e.g. vanillic, caffeic, chlorogenic, rosmarinic acid), phenolic diterpenes (e.g. carnosol, rosmanol, isorosmanol, carnosic acid), and pentacyclic triterpenes (e.g. ursolic, oleanolic, betulinic acid, betulin, alpha-amyrin, beta-amyrin).[14,30,32] Undoubtedly, it is very important to determine the above compounds in aromatic plants. Therefore, reliable and practical methods for separation, identification and quantitative analysis have been proposed. There are many publications dealing with the determination of phenolic and terpenic compounds, but most of the protocols are based on reverse-phase high performance liquid chromatography (HPLC) techniques coupled with spectrophotometric (UV-VIS) or mass spectrometric (MS) detection.[1,3,10–18,31] Electromigration techniques, e.g. iso-

tachophoresis coupled with capillary zone electrophoresis represent another way of analyzing phenolic or terpenic compounds.[19,31] In the literature, we find relatively little information about separation and determination of these compounds using gas chromatography coupled with mass spectrometry (GC-MS).[4,20–27] Although HPLC methods seem to be the preferred choice, GC can also serve as a suitable and reliable way of determination, especially in case of complex natural matrices, such as plant extracts. HPLC methods, in contrast to GC methods, do not require chemical derivatisation prior to analysis for conversion of non-volatile and thermally labile compounds into volatile and thermally stable ones. Since HPLC methods generally use UV detection and because many phenolic or terpenic compounds show UV spectra with λ_{max} in a narrow range (230–320 nm), different and often tedious purification treatments and separation processes of the compounds are required in order to prevent interferences. However, compared to mass spectrometry, UV spectra are not sufficiently diagnostic, and the identification is still difficult because of structure similarity (Figure 2). Capillary gas chromatography, coupled with mass spectrometry (GC-MS) can provide accurate results. It gives a high degree of specificity (with appropriate selection of ions used for quantitation), good sensitivity, and also permits the simultaneous quantitative determination of a wide range of phenolics and terpenes, from monoterpenes and diterpenes to sesquiterpenes and triterpenes, even at trace levels.

Therefore, the purpose of the presented study was to simplify the extraction and to accomplish the purification procedure used to isolate the phenolic and terpenic fraction from *Rosmarinus officinalis* L., to obtain a sensitive, accurate GC-MS method for simultaneous determination of a large number of phenolic and terpenic compounds, and to develop a rapid and efficient method for routine monitoring of individual compounds in the mentioned spice. Finally, the aim of this work was also to verify the applicability of the method on different species of *Lamiaceae* family.

2. Experimental

2.1. Chemicals

All reagents and solvents used were at least of analytical grade. Methanol, ethanol and hexane were purchased from Riedel-de Haen (Germany), tetrahydrofuran (THF), acetone, dimethyldichlorosilane (DMDCS) and pyridine from Merck (Germany), dichloromethane, anhydrous sodium sulphate and toluene from J.T. Baker (Netherlands), *N*-methyl-*N*-trimethylsilyl trifluoroacetamide (MSTFA) and ethyl acetate from Fluka Chemie (Switzerland). Rosmarinic acid (97%), oleanolic acid (97%), ursolic acid (90%), betulinic acid (90%), betulin (98%), cholesterol (99%) and cholesteryl acetate (95%) were supplied by Sigma-Aldrich (Germany). Carnosic acid was acquired from Alexis corporation (Switzerland). Bio-Beads S-X3 gel (200 to 400 mesh) was from Bio-Rad Laboratories (Richmond).

2.2. Calibration

Internal stock solutions were prepared by dissolving internal standard cholesterol (ISTD) and injection standard cholesteryl acetate (InjSTD) with THF in 100-mL volumetric flasks to obtain concentrations of 102.5 mg L^{-1} and 96.6 mg L^{-1}, respectively (Table 1). Standard stock solutions of caffeic, rosmarinic, carnosic, oleanolic, betulinic, ursolic acid and betulin were prepared by dissolving each of the components in THF. From all stock solutions, one working calibration solution (A) was prepared containing investigated compounds in concentrations listed in Table 1. Five calibration solutions were prepared by combining, separately, 50, 100, 150, 200 and 250 μL of solution A with 200 μL of ISTD, 100 μL of MSTFA and 50 μL of pyridine. Each solution was derivatised by heating for 2 h at 70 °C (see section 2.7.). Then 200 μL of InjSTD was added and the solution was dilluted to 1 mL with THF. The concentrations of ISTD and InjSTD in all five cali-

Table 1: GC-MS and calibration parameters for TMS derivatives of investigated compounds.

Investigated compound (purity %)	t_R (min)	Molecular ion- *m/z* (relative intensity %)	Two major fragment ions- *m/z* (relative intensity %)		Conc. (mg L^{-1})
cis-Caffeic acid (99.4%)	22.28	396 (40)	219 (100)	381 (30)	
trans-Caffeic acid (99.4%)	25.89	396 (40)	219 (100)	381 (30)	103.4
Carnosic acid (96.4%)	34.50	548 (15)	335 (100)	431 (90)	48.7
cis-Rosmarinic acid	45.79	720 (5)	219 (95)	396 (100)	
trans-Rosmarinic acid (97%)	48.45	720 (5)	219 (95)	396 (100)	100.8
Betulin (98%)	50.07	586 (5)	189 (100)	393 (35)	99.9
Oleanolic acid (97%)	50.14	600 (5)	203 (100)	320 (20)	97.8
Betulinic acid (90%)	50.36	600 (2)	189 (100)	320 (15)	91.1
Ursolic acid (90%)	50.70	600 (5)	203 (100)	320 (35)	90.9
Cholesterol – ISTD (99%)	43.98	458 (40)	368 (100)	329 (80)	102.5
Cholesteryl acetate – InjSTD (95%)	45.01	428 (2)	368 (100)	326 (25)	96.6

bration solutions were 20.5 mg L^{-1} and 19.32 mg L^{-1}, respectively. Five calibration solutions were injected in triplicate; also six replicate analyses of the calibration solutions were performed. Curves were constructed by linear regression of the peak-area ratio (y) of individual phenolic and terpenic compound to the ISTD, versus the concentration (x) in mg L^{-1}.

2.3. Plant Material

Different plant samples of *Rosmarinus officinalis* L. (rosemary), *Salvia officinalis* L. (sage), *Satureja montana* (winter savory), *Salvia sclarea* (clary sage), and *Salvia glutinosa* (sticky sage), were used for analyses. Samples were obtained from spontaneous plants grown in their natural habitat in different regions of Slovenia and Croatia. The plant material was air dried and stored in the darkness at room temperature prior to sample preparation step and analysis.

2.4. Selection of Extraction Solvent

To choose the optimum extraction solvent, the solubility of investigated compounds in different solvents and in different mixtures of solvents was examined. Tetrahydrofuran (THF), dichloromethane, ethyl acetate, acetone, methanol, ethanol, toluene, hexane, and pyridine were compared. Based on results, the organic mixture of tetrahydrofuran and ethanol (v/v, 1:1) was used for further work.

2.5. Extraction From Plant Material

5 g of air dried plant sample was ground. 1 g of homogenized sample was transferred into a 50 mL centrifuge tube. The corresponding amount of ISTD was added, prior to the extraction. The compounds were then extracted, using optimal extraction conditions, three times with 20 mL of mixture of THF and ethanol (v/v, 1:1) in an ultrasonic bath for 30 min. The extracts were centrifuged and supernatants were combined and dried with 3 g of anhydrous sodium sulphate. The dried extract was transferred into a 50-mL conical glass flask and concentrated by rotary evaporation to dryness. The residue was redissolved in 3 mL of the same solvent mixture.

2.6. Purification of Plant Extract

2.6.1. Cleanup Procedure Using Graphitized Carbon

The cleaning procedure using solid phase extraction (SPE) was performed in mini columns filled with graphitized carbon, Carbopack B (Superclean Envi Carb SPE tubes 6 mL, Supelco, Bellefonte). Columns were preconditioned with ethanol (2 × 6 mL).

An aliquot of 200 µL of plant extract was quantitatively transferred into the column. Elution was carried out with 30 mL of ethanol. The eluate was collected into conical flasks and concentrated by rotary evaporation to dryness. The dry residue was redissolved in 1 mL of THF and thus prepared for separation using size exclusion chromatography (SEC).

2.6.2. SEC Cleanup Procedure

THF was used as a solvent for beds swelling and as a mobile phase. The cleaning procedure using SEC was performed in a glass column (15 mm I. D. and 30 cm long). 24 h before use, Bio-Beads S-X3 gel was suspended in pure THF and stored for swelling. After 24 h, 50 mL of the slurry was filled into the column.

The residue, which was previously dissolved in 1 mL of THF, was quantitatively transferred into the column. After the extract sunk into the column bed, 50 mL of THF was added at flow rate 5 mL min^{-1}. Attention had to be paid not to let the column fall dry at any moment. Three different fractions (0–15 mL, 15–30 mL, 30–50 mL) were collected. The fraction of terpenic compounds (15–30 mL) was concentrated to dryness and redissolved in 1 mL of THF. The aliquot of 100 µL from terpenic fraction was taken for derivatisation and analysis.

2.7. Derivatisation Procedure

The trimethylsilyl (TMS) derivatives of investigated compounds in calibration solutions and in plant extracts were prepared in the same way. The silylation procedure was performed in glass tubes, previously deactivated with 5% DMDCS in toluene, and rinsed twice with ethanol, twice with toluene and twice with THF.

Prior to derivatisation the solvent from calibration solutions or from purified extract was evaporated and residues were dried using a gentle steam of nitrogen. Residues were silylated by adding 100 µL of the silylation reagent MSTFA and 50 µL of pyridine. To optimize derivatisation, solutions were exposed to different temperatures (room temperature, 50 °C 60 °C, 70 °C, 80 °C) for different periods of time (10 min, 15 min, 30 min, 60 min, 120 min, 24 h). After cooling down to room temperature 200 µL of InjSTD was added and the solution was diluted to 1 mL with THF. Finally an aliquot of 200 µL was transferred into the vials for GC-MS analysis.

2.8. Instrumentation and GC-MS Conditions

Analyses were performed using a Finnigan GCQ ion trap mass spectrometer coupled to a Finnigan MAT gas-chromatograph (Thermoquest, Germany), equipped with a split injection port. Chromatographic separation was performed on DB-5MS capillary column (J&W Scientific, Folsom, CA, USA); the dimensions of the column were 30 m × 0.25 mm I.D., 0.25 µm film thickness. Helium was used as the carrier gas with a constant linear velocity of 40 cm s^{-1}. The oven temperature program was: 0.8 min

at 105 °C, from 105 °C to 220 °C at 15 °C min^{-1}, from 220 °C to 300 °C at 40 °C min^{-1} and 20 min at 300 °C. Injector temperature was set at 290 °C. Samples were injected in split mode (split ratio 1 : 75). The injection volume was 2 μL. The transfer line temperature was held at 290 °C. The mass spectrometer was operated in the electron positive-mode ionisation (EI), with electron energy at 70 eV. Ion source temperature was 235 °C. The MS data were obtained in full scan mode (total ion current-TIC, mass range 50–750 amu). Identification of phenolic and terpenic compounds in derivatised plant extracts was established by comparing their retention times and mass spectra to the derivatised investigated compounds or by comparison of their spectral properties with literature data. For quantification reconstructed ion chromatograms were used, where usually two fragment ions with greater intensities were selected. Molecular ions and two specific fragment ions with relative intensities of TMS derivatives for the investigated compounds are presented in Table 1.

3. Results and Discussion

Calibration curves were prepared by analysis of calibration solutions of investigated compounds in the concentration range from 4 to 25 mg L^{-1}. Five calibration solutions were injected in triplicate; six replicate analyses of the calibration solutions were performed within two weeks. Curves were constructed by linear regression of the peak-area ratios (y) of each analyte to the ISTD, versus concentrations (x). Equations of calibration curves and their correlation coefficients are presented in Table 2. The r^2 values were in the range from 0.997 to 0.999 which confirmed the linearity of the method. The reproducibility of chromatographic analyses was evaluated by the relative standard deviation (RSD) of six replicate analyses of five calibration solutions. RSD was between 4.1 and 8.4%.

Table 2: Regression equations and correlation coefficients for silylated investigated compounds.

Investigated compound	Correlation coefficient (r_2)	Regression equation
cis-Caffeic acid	0.9968	y = 0.3379 x + 0.586
trans-Caffeic acid	0.9988	y = 0.9127 x + 0.5539
Carnosic acid	0.9993	y = 0.2071 x + 0.094
cis-Rosmarinic acid	0.9981	y = 0.1316 x – 0.092
trans-Rosmarinic acid	0.9994	y = 0.2506 x – 0.315
Betulin	0.9990	y = 0.022 x – 0.016
Oleanolic acid	0.9966	y = 0.007 x – 0.002
Betulinic acid	0.9984	y = 0.0576 x + 0.1393
Ursolic acid	0.9994	y = 0.136 x + 0.0894

The extraction procedure was optimized regarding extraction solvent and recovery. By examining the solubility of phenolic and terpenic compounds in different solvents, it was established that the highest solubility was achieved with a mixture of tetrahydrofuran and ethanol (v/v, 1:1). It was also found that the best recoveries (over 82%) were obtained by using three equal volumes of solvent mixture (3 × 20 mL), while using only two equal volumes gave lower recoveries (about 68%). The recovery was evaluated using cholesterol which was added to the homogenized sample before the extraction, clean-up and silylation procedures. Cholesteryl acetate was used for volume correction. Both compounds are structurally related, on the capillary column they behave similarly as derivatised analytes and they are not found in the investigated plants. Therefore they are suitable for quantitative analysis of investigated compounds. It is known that phenolic and terpenic compounds are stable in aprotic solvents (e.g. dimethyl sulfoxide), but less stable in protic ones (e.g. methanol).[16] This statement was also confirmed in our investigations, since investigated compounds dissolved in THF remained stable for at least one week in darkness at the temperature 0–4 °C. The stability of derivatised compounds was also investigated and it was confirmed that they remained stable for at least 5 days in darkness at the temperature 0–4 °C.

Plant extracts were additionally cleaned. Method using ENVI-Carb SPE tubes, filled with Carbopack B, and the SEC method, using a column, filled with Bio-Beads S-X3 gel, were suitable for removing interferences like chlorophylls, carotenoides and paraffin waxes and for isolating the phenolic and terpenic fraction. Chlorophylls and other pigments were eliminated using graphitised carbon, while all other interferences were eliminated using SEC. Three different fractions (0–15 mL, 15–30 mL, 30–50 mL) were collected by SEC. The first fraction contained large amounts of high molecular compounds and therefore it was rejected. The second fraction contained phenolic and terpenic compounds. The third fraction did not contain any other compounds.

The investigated compounds were not suitable for direct GC analysis, and therefore derivatisation was performed. MSTFA was used as trimethylsilyl (TMS) donor for the silylation of hydroxy and carboxylic groups. For silylation we had to ensure both extracts and solvents to be dry and all protic solvents to be removed to achieve optimal results. Water was eliminated by adding the anhydrous sodium sulphate and ethanol by concentrating to dryness on the rotary evaporator. The silylation procedure was improved by adding pyridine as a catalyst and as an acid scavenger. The results of experiments indicated that heating at 70 °C for 120 min was sufficient for quantitative silylation. Lower temperature and shorter time were not suitable for quantitative derivatisation. A further increase of reaction time did not improve the measured data. At higher temperatures the investigated compounds could be decomposed.

In Figure 1 TIC chromatograms of TMS derivatives of investigated compounds, present in the calibration solution and in the different plant extracts are presented. By

GC-MS analysis different active biological components of *Rosmarinus officinalis* L., including phenolic acids (*cis*-caffeic acid, *trans*-caffeic acid, *cis*-rosmarinic and *trans*-rosmarinic acid), phenolic diterpene (carnosic acid) and pentacyclic triterpenes (betulin, oleanolic, betulinic and ursolic acid) were separated, identified and quantified (Figures 1, 2). *Trans*-isomers of phenolic acids, phenolic diterpenes and triterpenes have been confirmed by retention time and mass spectral comparison to the derivatised investigated compounds, while *cis*-isomers of phenolic acids were identified by comparing their mass spectra with literature data.[27,28] The compounds in plant extracts were quantified from the corresponding calibration curves. *Cis*-isomers were quantified using calibration curve of *trans*-isomers, as the *cis* forms were not available. The reproducibility of analytical method was determined by analysis

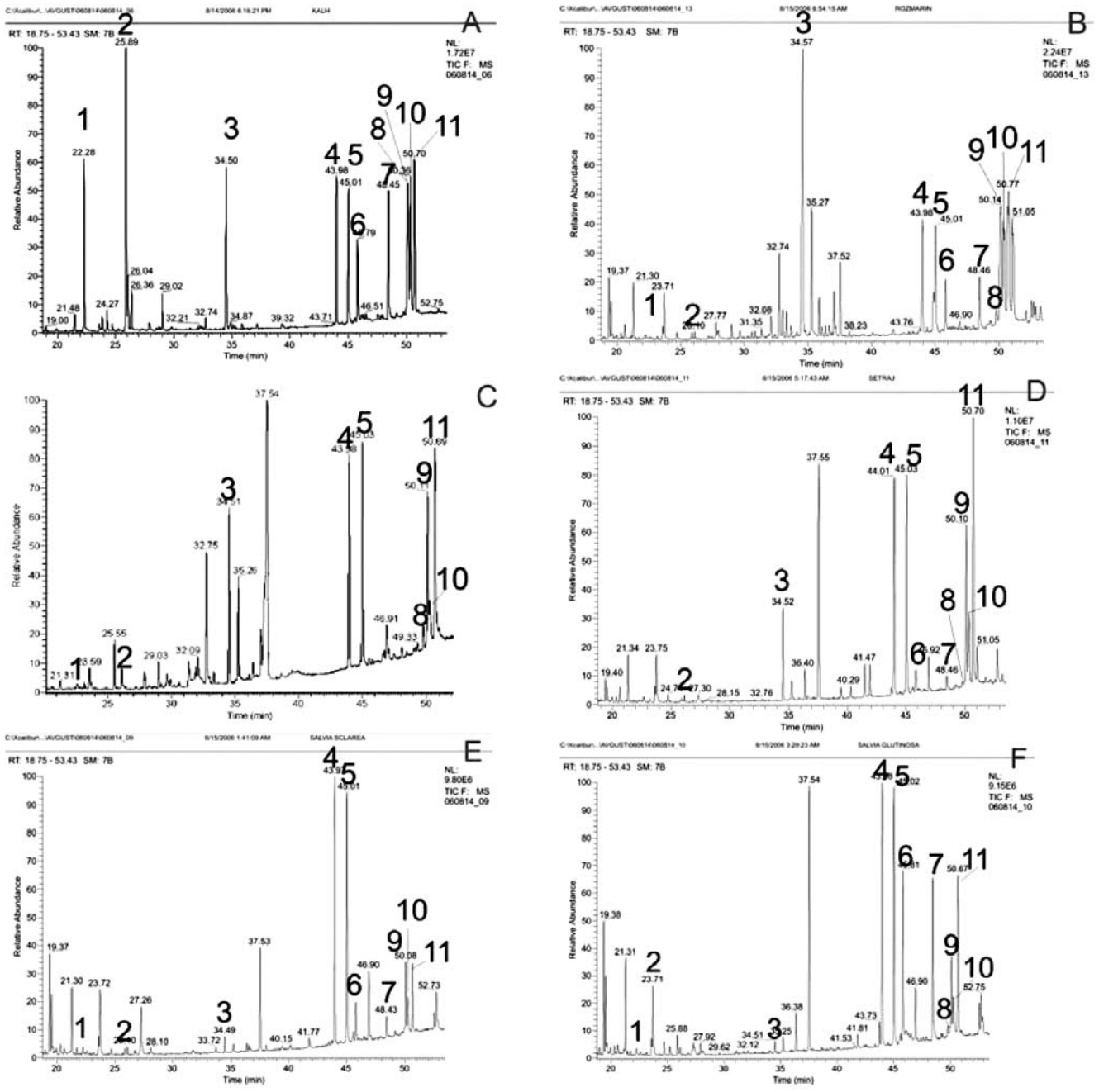

Figure 1. TIC chromatograms: silylated investigated compounds present in calibration solution (A); and silylated compounds present in extracts of rosemary-*Rosmarinus officinalis* L. (B), sage-*Salvia officinalis* L. (C), winter savory-*Satureja montana* (D), clary sage-*Salvia sclarea* (E) and sticky sage-*Salvia* glutinosa (F). Peak numbers refer to the TMS derivatives of compounds enumerated in Figure 2.

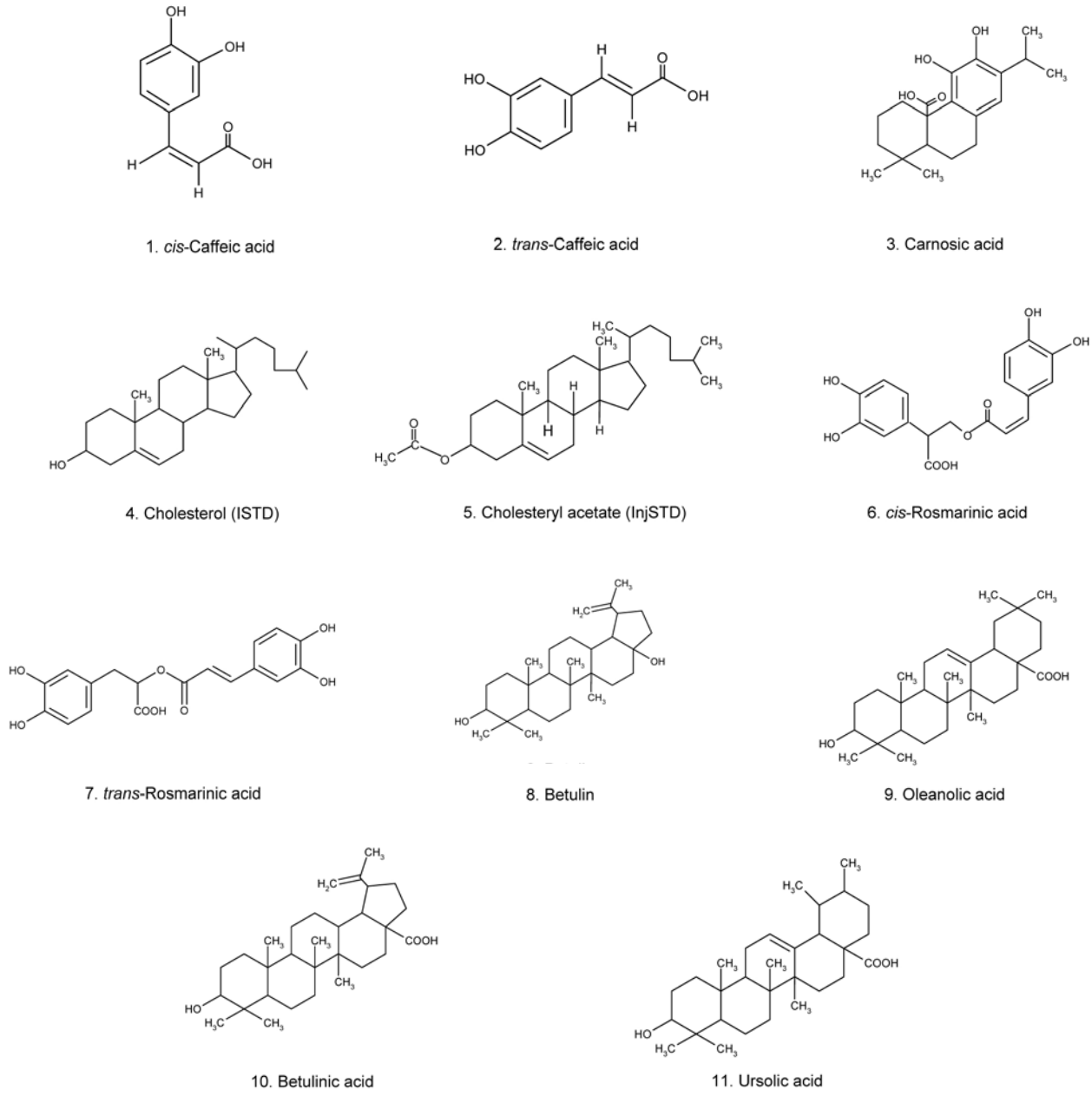

Figure 2. The chemical structures and names of the identified phenolic and terpenic compounds **(1–3, 6–11)**, internal standard **(4)** and injection standard **(5)**. Compound number refers to the peak number in chromatograms (Figure 1).

of homogenized samples of *Rosmarinus officinalis* L. The derivatised extracts of rosemary were injected in triplicate; five replicate analyses were performed within two weeks. RSD was lower than 10%. Some other compounds such as carnosol, methylcarnosic acid, beta-sitosterol were also identified, but results were not quantitatively evaluated, because we did not have authentic standard compounds.

Although the method was validated using *Rosmarinus officinalis* L., it was also applied to five different *Lamiaceae* species (*Salvia officinalis* L. – sage, *Satureja montana* – winter savory, *Salvia sclarea* – clary sage and *Salvia glutinosa* – sticky sage, Figure 1). Two replicate analyses were performed for each plant and the contents of investigated compounds were calculated. The average contents of phenolic and terpenic compounds in plant extracts (mg g^{-1} dry weight) are presented in Table 3. Rosmarinic acid is the main component in *Rosmarinus officinalis* L. and *Salvia glutinosa.* The content of ursolic acid and its position isomer oleanolic acid are also relatively

Table 3: Contents of phenolic and terpenic compounds in extracts of rosemary-*Rosmarinus officinalis* L., sage-*Salvia officinalis* L., winter savory-*Satureja montana,* clary sage-*Salvia sclarea* and sticky sage-*Salvia glutinosa* (mg g^{-1} dry weight). Each content value is the mean of five or two replications.

Compound	*Rosmarinus officinalis* L. (n = 5)	*Salvia officinalis* L. (n = 2)	*Satureja montana* (n = 2)	*Salvia sclarea* (n = 2)	*Salvia glutinosa* (n = 2)
cis-Caffeic acid	0.15	0.02	*	0.07	0.10
trans-Caffeic acid	0.12	*	*	*	0.17
Carnosic acid	4.95	2.56	1.13	0.10	0.07
cis-rosmarinic acid	7.07	*	1.47	2.01	8.01
trans-Rosmarinic acid	4.49	0.19	0.71	0.93	5.70
Betulin	1.73	3.47	4.01	1.55	1.58
Oleanolic acid	2.42	10.91	10.68	4.31	3.71
Betulinic acid	2.58	0.53	1.52	0.04	0.03
Ursolic acid	1.89	4.15	6.67	1.01	2.39

*In trace amounts.

high in all investigated plants. Caffeic acid is present in all of investigated plants in the lowest concentrations.

In future work we will try to identify and quantify more unknown compounds, presented in phenolic and terpenic fraction (Figure 1). We are interested in compound eluting immediately after the ursolic acid. We suppose that it is one of the oleanane or ursane type of pentacyclic triterpene.[23] In further investigations we will isolate the compound and determine its structure with different spectroscopic methods.

4. Conclusions

High performance liquid chromatography has been especially widely used for separation and determination of bioactive phenolic and terpenic compounds in a variety of plants, while gas chromatography is rarely used. The main reason is that some compounds are not really suited for GC analysis due to their non-volatility or stability. However, it is possible to analyse investigated compounds with GC-MS analysis but prior to analysis the compounds must be extracted, additionally cleaned and derivatised. Derivatisation with the trimethylsilyl group makes terpenoid compounds apolar, thermostable and volatile enough for GC. This method has good separation power when the extract is cleaned from matrix compounds. Although the proposed procedure is more time consuming in comparison with HPLC, it offers a complete separation and simultaneous determination of phenolic acids, phenolic diterpenes and triterpenes in different plant extracts. Our qualitative and quantitative results proved that GC-MS method offers very good alternative for identification, separation and quantification of investigated compounds in comparison to other conventional methods. Contents of phenolic and terpenic compounds in plant samples are comparable to those reported in literature, but it has to be emphasized that results can differ because there are several factors that can impact significantly on the phenolic and terpenic content in plants.[33–35] Namely, phenolics are uneven distributed in plant tissue (leaves, stems, sepals, petals, seeds, roots). Therefore it is important to state which part of plant was used for analysis.[36] It was also reported, that there is strong seasonal variation in concentrations of investigated compounds in rosemary. Usually solar radiation during the summer, resulting in water and light stress, decreases concentrations of some phenolics, while they are increased during the winter.[13] Finally the extraction and clean-up methods have strong influence on the determination of investigated compounds in plants. The ultimate goal of an efficient extraction and purification is the preparation of sample extract enriched in all components of interest and free from interfering matrix components. The correct extraction (time of extraction, temperature, pH, solvent, …) has to be ensured to avoid chemical modification, degradation and other biochemical changes of the components in the sample.[16,30,31,37] Our results indicated that phenolic and terpenic compounds were successfully isolated from rosemary leaves and from some other plants. The advantages of the cleanup procedures (SPE and SEC) used in this study over previous conventional methods are better removal of matrix interferences and better separation of investigated compounds. Although, only few GC-MS methods have been developed to characterize and quantify phenolics and terpenics in *Rosmarinus officinalis* L., they are viable alternatives for such analysis due to the excellent resolving power and detection capabilities. GC-MS method gives good specificity and sensitivity and therefore it can be used for determination of the compounds in different plants even at trace levels.

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6. References

1. J. Tóth, M. Mrlianová, D. Tekel´ová, M. Korenová, *Acta Fac. Pharm. Univ. Comen.* **2003***,* 139–146.
2. M. Petersen, M. S. J. Simmonds, *Phytochemistry* **2003**, *62(2)*, 121–125.
3. D. Baricevic, S. Sosa, R. Della Loggia, A. Tubaro, B. Simonovska, A. Krasna, A. Zupancic, *J. Ethno. Pharmacol.* **2001**, *75*, 125–132.
4. G. Janicsák, K. Veres, M. Kállai, I. Máthé, *Chromatographia* **2003**, *58*, 295–299.
5. J. Liu, *J. Ethno. Pharmacol.* **1995**, *49*, 57–68.
6. N. Osakabe, H. Takano, C. Sanbongi, A. Yasuda, R. Yanagisawa, K. I. Inoue, T. Yoshikawa, *Biofactors* **2004**, *21*, 127–131.
7. C. Renzulli, F. Galvano, L. Pierdomenico, E. Speroni, M. C. Guerra, *J. Appl. Toxicol.* **2004**, *24*, 289–296.
8. C. Sanbongi, H. Takano, N. Osakabe, N. Sasa, M. Natsume, R. Yanagisawa, K. I. Inoue, K. Sadakane, T. Ichinose, T. Yoshikawa, *Clin. Exp. Allergy* **2004**, *34*, 971–977.
9. N. Osakabe, A. Yasuda, M. Natsume, T. Yoshikawa, *Carcinogenesis* **2004**, *25*, 549–557.
10. S. Albu, E. Joyce, L. Paniwnyk, J. P. Lorimer, T. J. Mason, *Ultrason. Sonochem.* **2004**, *11*, 261–265.
11. M. Backleh, G. Leupold, H. Parlar, *J. Agric. Food Chem.* **2003**, *51*, 1297–1301.
12. E. Ibañez, A. Kubátová, F. J. Señoráns, S. Cavero, G. Reglero, S. B. Hawthorne, *J. Agric. Food Chem.* **2003**, *51*, 375–382.
13. S. Munné-Bosch, L. Alegre, K. Schwarz, *Eur. Food Res. Technol.* **2000**, *210*, 263–267.
14. M. J. Del Bano, J. Lorente, J. Castillo, O. Benavente-García, J. A. Del Río, A. Ortuño, K.-W. Quirin, D. Gerard, *J. Agr. Food Chem.* **2003**, *51*, 4247–4253.
15. B. H. De Oliveira, C. A. M. Santos, A. P. D. M. Espindola, *Phytochem. Anal.* **2002**, *13*, 95–98.
16. M. A. Thorsen, K. S. Hildebrandt, *J. Chromatogr. A* **2003**, *995*, 119–125.
17. B. Claude, Ph. Morin, M. Lafosse, P. Andre, *J. Chromatogr. A* **2004**, *1049*, 37–42.
18. M. Burnouf-Radosevich, N. E. Delfel, *J. Chromatogr. A* **1984**, *292(2)*, 403–409.
19. R. Hamoudová, M. Urbánek, M. Pospíšilová, M. Polášek, *J. Chromatogr. A* **2004**, *1032*, 119–125.
20. V. P. Papageorgiou, M. N. Bakola-Christianopoulou, K. K. Apazidou, E. E. Psarros, *J. Chromatogr. A* **1997**, *769*, 263–273.
21. C. Mathe, G. Culioli, P. Archier, C. Vieillescazes, *J. Chromatogr. A* **2004**, *1023*, 277–285.
22. P. J. M. Cordeiro, J. H. Y. Vilegas, F. M. Laças, *J. Braz. Chem. Soc.* **1999**, *10*, 523–526.
23. M. Burnouf-Radosevich, N. E. Delfel, *Phytochemistry* **1985**, *24 (9)*, 2063–2066.
24. M. Saitta, S. L. Curto, F. Salvo, G. D. Bella, G. Dugo, *Anal. Chim. Acta* **2002**, *466*, 335–344.
25. T. Galgon, D. Höke, B. Dräger, *Phytochem. Anal.* **1999**, *10*, 187–190.
26. A. Ghosh, S. Misra, A. K. Dutta, A. Choudhury, *Phytochemistry* **1985**, *24 (8)*, 1725–1727.
27. A. Reschke, *Z. Lebensm. Unters. Forsch.* **1983**, *176*, 116–119.
28. A. Caniova, E. Brandsteterova, *J. Liq. Chrom. & Rel. Technol.* **2001**, 24 *(17)*, 2647–2659.
29. P. M. Kris-Etherton, K. D. Hecker, A. Bonanome, S. M. Coval, A. E. Binkoski, K. F. Hilpert, A. E. Griel, T. D. Etherton, *Am. J. Med.* **2002**, *113*, 71–88.
30. M. Suhaj, *J. Food Compos. Anal.* **2006**, *19*, 531–537.
31. M. Antolovich, P. Prenzler, K. Robards, D. Ryan, *Analyst* **2000**, *125*, 989–1009.
32. T. Brglez, Master Thesis, University of Ljubljana, 2002.
33. V. Čuláková, M. Máriássyová, L. Heilerová, *Chem. Listy* **2005**, *99*, s277–s278.
34. G. Janicsák, K. Veres, A. Z. Kakasy, I. Máthé, *Biochem. Syst. Ecol.* **2006**, *34*, 392–396.
35. G. Janicsák, I. Máthé, V. Miklóssy-Vári, G. Blunden, *Biochem. Syst. Ecol.* **1999**, *27*, 733–738.
36. S. Munné-Bosch, L. Alegre, *Plant. Physiol.* **2001**, *125*, 1094–1102.
37. A. Cert, W. Moreda, M. C. Pérez-Camino, *J. Chromatogr. A* **2000**, *881*, 131–148.

Povzetek

Razvili smo metodo za sočasno identifikacijo in kvantitativno določitev sedmih fenolnih in terpenskih spojin v rožmarinu (*Rosmarinus officinalis* L) s plinsko kromatografijo in masno spektrometrijo (GC-MS). Spojine smo identificirali kot trimetilsilil (TMS) derivate fenolnih kislin (kofeinske in rožmarinske kisline), fenolnega diterpena (karnozolne kisline) in pentacikličnih triterpenov (ursolne, oleanolne, betulinske kisline in betulina). Naštete spojine so bile potrjene s primerjavo retencijskih časov in masnih spektrov derivatov preiskovanih spojin. Metoda vključuje ultrazvočno ekstrakcijo preiskovanih spojin z mešanico topil tetrahidrofuran in etanol. Ekstrakte smo po čiščenju na aktivnem oglju, frakcionirali z velikostno izkjučitveno kromatografijo. Frakcijo, ki je vsebovala fenolne in terpenske spojine, smo pred GC-MS analizo derivatizirali z *N*-metil-*N*-trimetilsilil trifluoroacetamidom (MSTFA). Postopek derivatizacije smo optimirali glede na čas reakcije in temperaturo. Linearnost metode smo preverili v koncentracijskem območju 4–25 mg L^{-1}. Povprečni izkoristki za preiskovane spojine so bili med 80 to 82 %, korelacijski koeficienti (r^2) so bili med 0,997 in 0,999. GC-MS tehnika je specifična in občutljiva, omogoča dobro ločevanje spojin in je zato primerna za sočasno identifikacijo in določevanje širokega spektra fenolnih in terpenskih spojin v različnih rastlinah, tudi ko so spojine prisotne v sledovih.