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SEPARATION AND IDENTIFICATION OF SOME DERIVATISED DITERPENES AND TRITERPENES IN ROSEMARY (*Rosmarinus officinalis L.*) BY GAS CHROMATOGRAPHY AND MASS SPECTROMETRY[1]

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A rapid analytic method for isolation, separation and determination of some phenolic diterpenes and triterpenes from fresh and dry rosemary leaves (Rosmarinus officinalis L.) has been developed. The components identified in the plant extract were trimethylsilylated derivatives of rosmarinic acid, phenolic diterpenes, carnosol and carnosic acid, pentacyclic triterpenoids, ursolic, oleanolic, betulinic acid and betulin. Experiments were performed to establish the optimum conditions for solvent selection, mode and duration of extraction,

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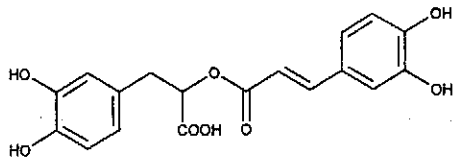
derivatisation, and to set chromatographic conditions, as well. The enumerated compounds have numerous similarities in the chemical and pharmacological character. Publications dealing with separation and determination of diterpenes and triterpenes in plant material report many different analytic methods, but most extended are high performance liquid chromatography and thin layer chromatography. The procedures are complicated, since the separation and the determination of terpenic compounds are difficult because of structure similarity. Therefore, gas chromatography and mass spectrometry were used as methods enabling good separation and parallel determination of phenolic diterpenes and triterpenes.

Introduction

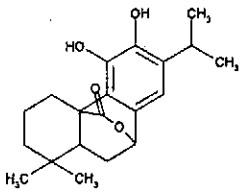
Rosmarinic acid, phenolic diterpenes, carnosol and carnosic acid, pentacyclic tritepenoids, ursolic, oleanolic, betulinic acid and betulin occur widely in the plant realm. These compounds have numerous similarities in chemical and pharmacological character. They are well known for their antioxidative properties [1-6] as well as for their wide range of biological activities and medicinal properties, such as antibacterial, anticarcinomic, antiinflammatory, antitumor, antiviral, fungistatic, and many other effects [4–17]. For this reason, the compounds are very interesting and important for medicine, pharmacy, food-processing and cosmetic industry. Publications dealing with the determination of terpenoids in plant material report many different analytic methods, but most extended are high performance liquid chromatography (HPLC) and thin layer chromatography (TLC) equipped with an UV-Vis or fluorescent detector [4,6,17–21]. Extraction and clean-up procedures described in articles are quite complicated [6,19,24,30–34]. The literature contains relatively little information about the separation and determination of terpenic compounds using gas chromatography and mass spectrometry (GC/MS) [7,22–28]. Total separation of the compounds using HPLC is very difficult because of structure similarity (Fig. 1). Therefore, special efforts were made to devise a procedure that ensures a simple and quantitative extraction, an efficient removal of matrix influences and rapid determination using GC/MS technique.

Samples of *Rosmarinus officinalis L.* were homogenized and extracted with a mixture of tetrahydrofuran and ethanol in an ultrasonic bath. Cleaning of the extract from matrix interferences was performed using size exclusion chromatography (SEC) after adsorption of plant pigments on activated carbon. The isolated terpenoid compounds were silylated. The silyl derivatives were analyzed by gas chromatography and mass spectrometry.

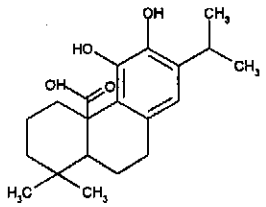
The results of the study confirmed that the method is suitable for separation, identification and determination of triterpenoid compounds in different plant materials.



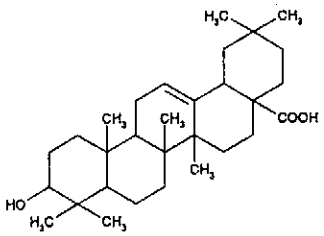
a. ROSMARINIC ACID



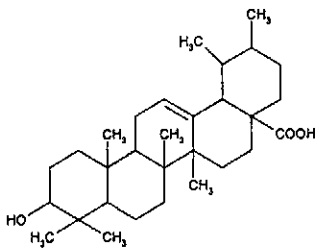
b. CARNOSOL



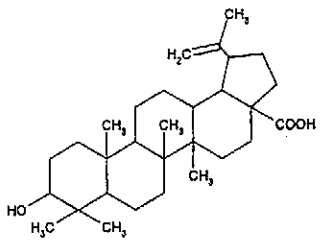
c. CARNOSIC ACID



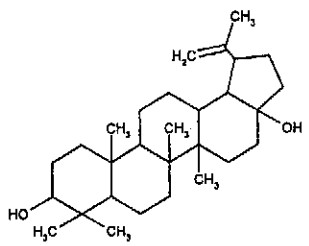
d. ELEANOLIC ACID



e. URSOLIC ACID



f. BETULINIC ACID



g. BETULIN

Fig. 1 Molecular structures of rosmarinic acid (a), phenolic diterpenes, carnosol (b) and carnosic acid (c), pentacyclic triterpenoids oleanolic acid (d), ursolic acid (e), betulinic acid (f) and betulin (g)

Experimental

Chemicals

All the solvents used were at least of analytical grade: methanol, ethanol and hexane (Riedel-de Haen, Germany), tetrahydrofuran (THF), acetone and pyridine (Merck, Germany), dichloromethane and toluene (J. T. Baker, Netherlands), ethyl acetate and tetrabutylmethyl ether (Fluka Chemie, Switzerland).

Oleanolic, ursolic, rosmarinic and betulinic acid, cholesterol and cholesteryl acetate were supplied by Sigma-Aldrich (Germany). Sodium sulphate anhydrous was purhased from J. T. Baker (Netherlands). Carnosic acid and betulin were products of VITIVA (Slovenia) and *N*-methyl-*N*-trimethylsilyl trifluoroacetamide-MSTFA of Fluka Chemie (Switzerland).

Instrumentation

The derivatised acid extracts were analyzed with Finnigan MAT gas chromatograph (San Jose, CA, USA) equipped with an ion trap mass spectrometer detector Finnigan GCQ and CTC autosampler A200S. Chromatographic separation was performed on chemically bonded fused silica capillary column HP-5MS (J&W Scientific, Folsom, CA, USA), stationary phase 5 % phenyl-95 % methylpolysiloxane, 0.25 mm internal diameter, 0.25 μm film thickness, 30 m length. The control of instruments and data processing were preformed with the programme package Xcalibur 1.2 (Windows operating system). The gas-chromatographic conditions were as follows:

- electron ionization: EI+ (positive electron mode),
- transfer line: 290 °C,
- injector temperature: 265 °C,
- ion source temperature: 230 °C,
- carrier gas: helium, constant flow 1.5 ml min^{-1}, constant velocity 40 cm s^{-1},
- electron impact: 70 eV,
- calibration: PFTBA (perfluorotributylamine),
- scan mode: SCAN (full scan, entire mass spectrum), SIM (selective ion monitoring, chosen mass fragments),
- mass range (full scan): 55 – 800 amu,
- injection mode: splitless,
- injection volume: 2 μl,
- temperature program : initial temperature 105 °C, 0.8 min isothermal, 15 °C min^{-1} up to 220 °C, 40 °C min^{-1} up to 300 °C, 20 min isothermal.

In addition, the following equipment was used: grinder, centrifuge vials, centrifuge LC 321 (Tehnica Železniki, Slovenia), rotary evaporator (Bűchi

Rotavapor R-205, Switzerland), ultrasonic bath (Sonis 4), SPE mini columns filled with activated carbon, Carbopack B (Superclean™ Envi™ Carb SPE tubes 6 ml, Supelco, Bellefonte), glass column (15 mm internal diameter and 30 mm length), Bio-BeadsÒ S-X3 Beads gel (200 to 400 mesh, Bio-Rad Laboratories, Richmond), Pasteur's pipette, automatic pipette (10 – 100 µl, 100 – 1000 µl), conical glass flasks, sand bath, vials for GC/MS analysis.

Selection of Extraction Solvent

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

Extraction of Plant Material

The plant material (*Rosmarinus officinalis L.*) was gathered during July and August 2004 from different regions of Slovenia and Croatia (Maribor, Koper, Lastovo, Korčula). 5 g of fresh or air dried rosemary leaves were ground. 1 g of homogenized leaves was transferred into 50 ml centrifuge tube and extracted twice with 10 ml of mixture of THF and ethanol (v/v, 1:1) on an ultrasonic bath for 30 min. Then the extract was centrifugated and supernatants were combined and dried with 3 g of anhydrous sodium sulphate. Dried solution was transferred into 50 ml conical glass flask and concentrated by rotary evaporation to dryness. The residue was redissolved in 3 ml of ethanol.

Cleanup Procedure using Activated Carbon

The cleaning procedure was performed in a vertically aligned mini column filled up with pulverized activated carbon, Carbopack B. The column was moistened with ethanol. An aliquot (300 µl) of extract was transferred onto the top of the column and rinsed at least twice using 1 ml of ethanol, respectively. The eluate was collected into new 50 ml conical flask and concentrated by rotary evaporation to dryness. The dry yellow extract was redissolved in 1 ml THF and in this way ready for SEC.

SEC Cleanup Procedure

THF was used as solvent for beds swelling and as mobile phase. The cleaning procedure was performed in glass SEC column. Twenty-four hours before use for size exclusion chromatography, Bio-Beads gel was slurred in pure THF and stored for swelling. After 24 hours approximately 50 ml of the gel was filled into a column. The flow rate was 1 ml min^{-1}.

1 ml THF plant extract was transferred into the column. The extract was rinsed with 3 × 1 ml THF. After the sample sunk into the column bed, 50 ml of solvent was added by careful flow. Attention had to be paid that the column has not become dry at any moment. Three different fractions (0 – 10 ml, 10 – 25 ml, 25 – 50 ml) were collected and concentrated to dryness.

Derivatisation/Silylation

All the dry fractions were dissolved in 100 µl of dry pyridine and 200 µl of the silylation reagent MSTFA was added. To find the optimum conditions for silylation, the derivatisation was performed with heating for different time intervals at different temperatures. After cooling down to room temperature, the trimethylsilyl (TMS) derivatives were diluted with THF and transferred into vials for GC/MS analyses.

Results and Discussion

In examination of the solubility of terpenic compounds in different solvents, we established that the best dissolution was achieved with a mixture of tetrahydrofuran and ethanol (v/v, 1:1). Apolar part of investigated compounds is dissolved in tetrahydrofuran, while the polar part is dissolved in ethanol.

Before the silylation procedure all protic solvents had to be removed. Therefore, water was eliminated by adding anhydrous sodium sulphate and ethanol by concentrating to dryness on a rotary evaporator. Silylation/derivatisation procedure was improved by adding pyridine. The results of our experiments indicated that heating at 80 °C for 120 min was sufficient for complete silylation. Lower temperatures and shorter time were not suitable for quantitative derivatisation. Further increasing of reaction time did not affect the data measured. Maximum silylation temperature was 80 °C, because at higher temperatures natural components could be decomposed. The derivatised samples remained stable for 1 week at temperature 0 to 4 °C.

Both methods, namely, the cleaning method using Carbopack B and SEC method, using a column, filled with Bio-Beads gel, were highly suitable cleanup

procedures for removing interferences like chlorophylls, carotenoides, paraffin waxes etc. Chlorophylls were eliminated using activated carbon, while all other interference substances were eliminated using SEC.

By SEC, the following fractions were collected:

- first fraction: 0 – 10 ml (high molecular weight compounds of matrix interferences),
- second fraction: 10 – 25 ml (diterpene and triterpene fraction),
- third, last fraction: 25 – 50 ml (rinsing the column).

Because of large amounts of matrix components, the first fraction was rejected. The second fraction was the purified fraction and contained terpenoids rosmarinic, ursolic, oleanolic, betulinic, carnosic acid, betulin and carnosol. The third fraction was used for rinsing the column and its preparing for next analysis.

GC/MS analysis of silylated derivatives gives very well separated peaks of phenolic diterpenes (carnosol and carnosic acid), pentacyclic triterpenoids (ursolic, oleanolic, betulinic acid and betulin) and rosmarinic acid (Fig. 2). Working in SIM mode, the method is sensitive enough to determine traces of these compounds. The identity of compound 8 has to be determined. We suppose, that it is dehydro form of one of the searched compounds. In further investigations we will try to isolate the compound and to determine its structure by means of various spectroscopic methods.

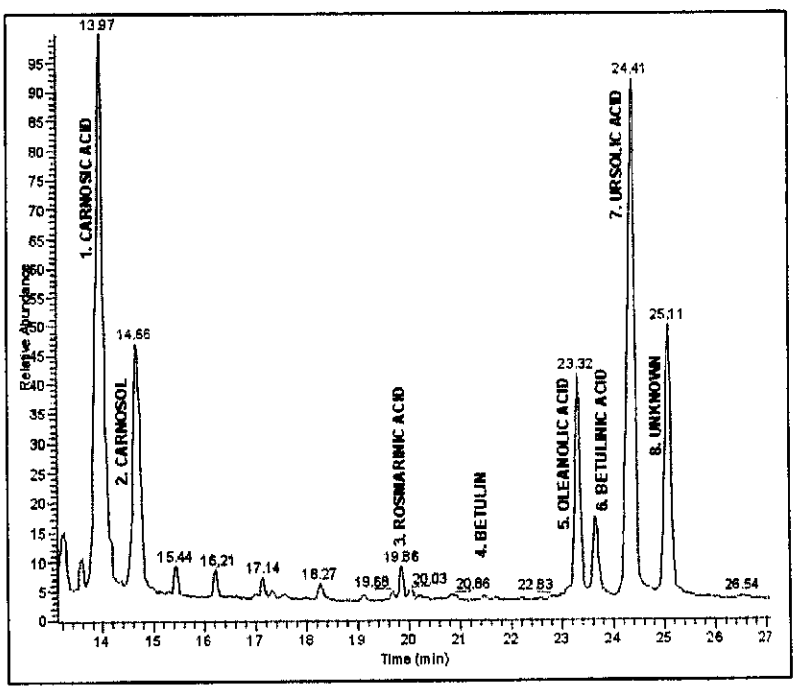


Fig. 2 TIC chromatogram of TMS derivatives of terpenic compounds after carbon and SEC clean-up procedures

Conclusion

Diterpenes and pentacyclic triterpenoids were successfully isolated from both fresh and dry rosemary leaves.

The advantage of our two cleanup procedures (carbon and SEC) over previous conventional methods is better removal of matrix interferences, better separation and better sharpness of chromatogram peaks of terpenic compounds using GC/MS technique. GC/MS also enables simultaneous separation and determination of hydrophilic and hydrophobic compounds without matrix interferences.

The results of the study have confirmed that the method is suitable for separation and determination of diterpenes and triterpenes in various plant materials.

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