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HPTLC-DENSITOMETRY DETERMINATION OF TRITERPENIC ACIDS IN *ORIGANUM VULGARE*, *ROSMARINUS OFFICINALIS* AND *SYZYGIUM AROMATICUM*

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Abstract: Spices play an important role in the chemoprevention and they can be a rich source of biologically active compounds such as triterpenes in the human diet. A method based on high performance thin-layer chromatography combined with densitometry for determination of ursolic and oleanolic acids in some common spices was elaborated. The prechromatographic derivatization with 1% of iodine solution was used to enable simultaneous analysis of these triterpenes. The extracts were separated on HPTLC silica gel 60 F_{254} plates with use of mobile phase consisting of toluene-petroleum ether-ethyl acetate-acetonitrile 5 : 5 : 1 : 0.3 (v/v/v/v). After drying, the plates were sprayed with 10% (v/v) ethanol solution of sulfuric acid (VI) and heated to 120˚C for 3 min. Quantification was performed in fluorescence/reflectance mode at a wavelength of 400 nm using a computer-controlled densitometer Desaga CD 60.

Keywords: oleanolic acid, ursolic acid, spices, *Origanum vulgare*, *Rosmarinus officinalis, Syzygium aromaticum*

In recent years, the increasing interest in antioxidants is observed due to their protective effect on cells, and ability to free radical scavenging. Oxygen free radicals and their reactive derivatives may cause diseases associated with oxidative stress, such as chronic inflammation, cancer, cardiovascular and neurodegenerative disorders (1, 2).

It has been known that secondary metabolites such as flavonoids, anthocyanins and terpenes synthesized by plants have antioxidant properties (3-5). Spice and herbs contain a high amount of natural polyphenols, therefore, they are a valuable source of antioxidant in human diet. They are being used as food additives since ancient times mostly for improving organoleptic properties and as preservatives (6). They are consumed in a variety of combinations depending on taste preferences. Many spices and their extracts can be applied for preservation of lipids and reduce lipid peroxidation in biological systems (7-9). Some of them have also the beneficial digestive stimulant action through an appropriate stimulation of the activities of pancreatic digestive enzymes, enzymes of intestinal mucosa, and stimulation of the liver to produce and secrete bile rich in bile acids. Spices also play an important role in the chemoprevention.

Origanum vulgare L. (Labiatae), commonly known as oregano and *Rosmarinus officinalis* L. (Lamiaceae) are important culinary herbs and they are widely used as a popular spice for food production. Recently, these spices had gained a great importance in medicine and pharmacy owing to their antimicrobial, antifungal and antioxidant activity (10-13).

Cloves are the aromatic dried flower buds of *Syzygium aromaticum* (L.) (Myrtaceae) with anticarcinogenic, antimutagenic, antioxidant, antimicrobial and anti-inflammatory properties (14-16).

Triterpenic acids, such as betulinic, oleanolic and ursolic acids are compounds commonly occurring in the plant kingdom with confirmed antioxidant properties which can strongly influence the biological activity of herbs and plants. These triterpenes possess also wide spectrum of pharmacological activities including anti-inflammatory hepatoprotective, antitumor, anti-HIV, antimicro-

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bial, antifungal, and antihyperlipidemic effects (17-19).

The objective of our work was determination of oleanolic and ursolic acid in commercially available spices, such as *O. vulgare* L., *R. officinalis* L., and *S. aromaticum* L. with use of HPTLC technique combined with densitometry. This method is convenient analytical tool for rapid estimation of plant material because of the possibility of simultaneous determination of several samples in a single chromatographic run.

EXPERIMENTAL

Materials and chemicals

Dried plant materials used in the isolation process: *Origanum vulgare* L., *Rosmarinus officinalis* L. (leaves) and *Syzygium aromaticum* (L.) Merrill (cloves buds) were obtained from the local market. Spices were produced by Kamis, S.A. Stefano.

Oleanolic and ursolic acids were purchased from Chromadex (Santa Ana, CA, USA). All solvents and reagents were at least pro analysis grade from Polish Reagents (POCh, Gliwice, Poland). HPTLC plates were from Merck (Darmstadt, Germany).

Standards and samples preparation

Stock solutions of triterpenes were prepared by dissolving 5 mg of each triterpenic acid in 5 mL of methanol (final concentration: 1 mg/mL).

The plant material was powdered and accurately weighted. Each sample (2.0 g) was twice extracted with ethanol (2 × 20 mL) within 12 h at room temperature and next, four times in an ultrasonic bath with use of 20 mL of ethanol (4 × 15 min). The obtained extracts were combined and evaporated to dryness. The residue was dissolved in methanol, filtered through a 0.45 μm membrane filter (Millipore, USA) and made up to 20 mL in volumetric flask. Five mL of each extract was purified by SPE technique. The samples were applied on octadecyl microcolumn (J.T. Baker, USA) and eluted with 10 mL portion of methanol. The obtained solutions were finally diluted to 40 mL with methanol.

Chromatography

Chromatography was performed on 20 cm × 10 cm HPTLC silica gel 60 F_{254} plates from Merck (Darmstadt, Germany). The plates were washed with methanol and dried in a stream of hot air before use. Five μL of standard solutions and samples of extract were spotted as 8 mm bands by means of a CAMAG (Muttenz, Switzerland) ATS4 automatic TLC sampler (track distance: 10 mm, distance from the left edge: 15 mm).

Oleanolic and ursolic acid are structural isomers and the similarity of chemical structures make their TLC separation rather difficult. Pre-derivatization with iodine was necessary to separate both analyzed compounds according to the procedure proposed by Wójciak-Kosior (20).

The plates were developed in horizontal Teflon DS chambers (Chromdes, Lublin, Poland) with 1% iodine solution in chloroform on a distance of 15 mm, and next, the start zone was covered by a glass strip and the plates were placed in dark for 10 min. Finally, the plates were dried in a stream of warm air to remove iodine.

The plates were developed with mixture of toluene-petroleum ether-ethyl acetate-acetonitrile 5:5:1:0.3 (v/v/v/v) to a distance of 70 mm in chromatographic chamber, previously saturated with vapors of the mobile phase. After drying, the plates were sprayed with 10% (v/v) H_2SO_4 in ethanol and heated for 3 min at the temperature of 120°C. The quantification was carried out by densitometric scanning (Desaga CD-60, Heidelberg, Germany) in flurescence/reflectance mode at λ = 400 nm (slit dimension: 4 × 1 mm). The source of radiation was a mercury lamp. To measure the fluorescent light only, a cut-off filter at λ = 420 nm was used to block the excitation wavelength shorter than specified. The plates were scanned within 30 min; afterwards a progressive degradation was observed. Documentation was obtained with use of a digital camera at 365 nm.

RESULTS AND DISCUSSION

Spices are a rich source of biologically active compounds in the human diet. Triterpenic acids are common constituents of many medicinal herbs and plants and can strongly influence their therapeutic properties. In the present paper, oleanolic and ursolic acid in commonly used spices, such as *O. vulgare* L., *R. officinalis* L. and *S. aromaticum* was determined.

In preliminary investigations the mobile phase composition was optimized. To find the most suitable eluent, numerous tests using organic solvents such as: toluene, ethyl acetate, petroleum ether, diethyl ether, acetonitrile and acetone in various ratios were made. The best results were obtained for the mixture of toluene : petroleum ether : ethyl acetate : acetonitrile 5:5:1:0.3 (v/v/v/v). The analyzed bands were dense, compact and well separat-

ed from the accompanying components of the extracts (Figs. 1-3). The identification of triterpenic acids in investigated extracts was done on the basis of R_f values. The purity of the peaks in the sample was ascertained by comparison of absorption spectra with those obtained from the standards.

For densitometric determination the measurement of fluorescence is recommended owing to greater selectivity (the matrix, which is not fluorescent, is not measured) and better linearity. Pentacyclic triterpenes have no native fluorescence; therefore, derivatization with use of 10% sulfuric acid in ethanol was necessary. The formation of fluorescent zones and their intensities was dependent on the duration of heating and temperature. The conditions of derivatization were chosen experimentally. The example of chromatographic plate is presented in Figure 4.

The method was validated for linearity, precision and accuracy. A calibration plot was established by analysis of standard solution at five different concentrations in the ranges 230-2300 ng/spot for oleanolic acid and 45-450 ng/spot for ursolic acid. For each point, three measurements were made

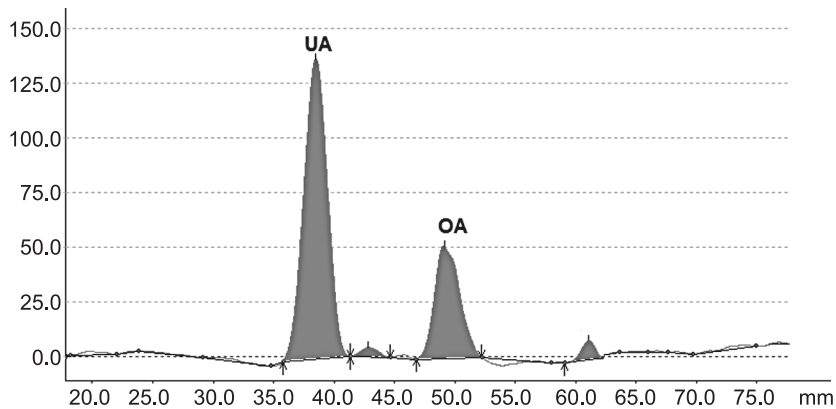

Figure 1. Densitogram of extract from leaves of *Rosmarinus officinalis* obtained at λ = 400 nm after derivatization with 10% (v/v) H_2SO_4 in ethanol

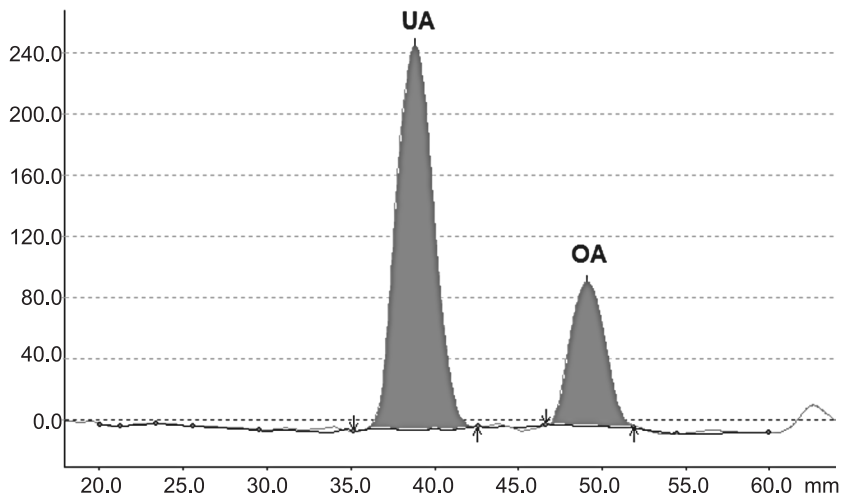

Figure 2. Densitogram of extract from leaves of *Origanum vulgare* L. obtained at λ = 400 nm after derivatization with 10% (v/v) H_2SO_4 in ethanol

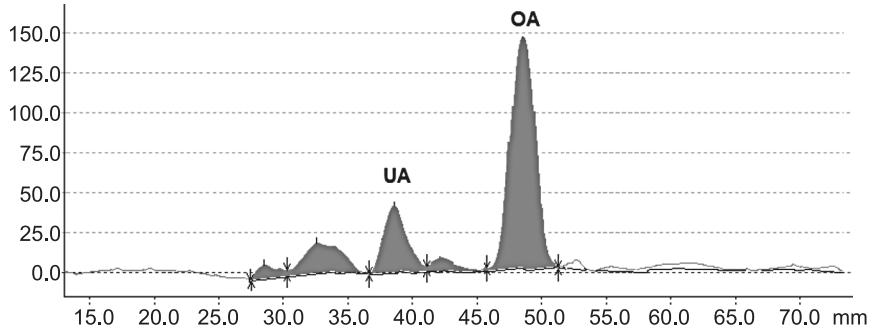

Figure 3. Densitogram of extract from cloves buds of *Syzygium aromaticum* (L.) Merrill. Obtained at λ = 400 nm after derivatization with 10% (v/v) H_2SO_4 in ethanol

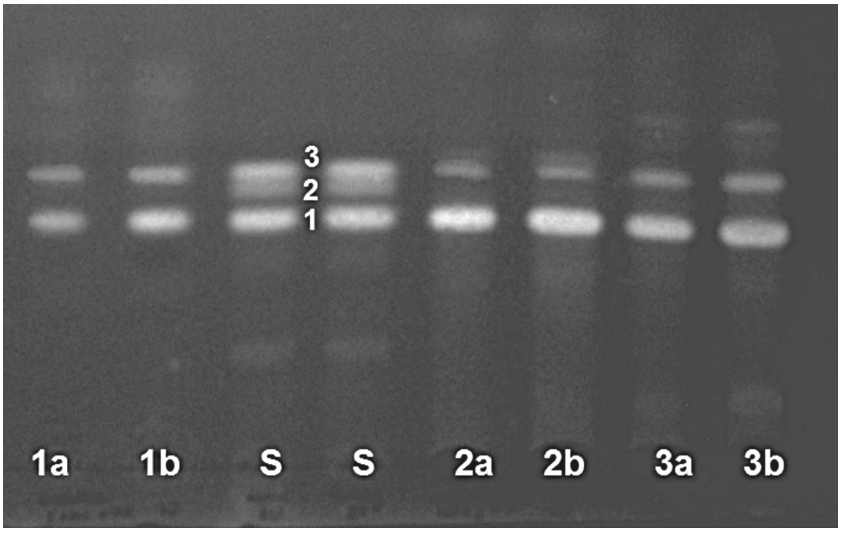

Figure 4 The photograph of HPTLC plate: 1a, 1b – *Syzygium aromaticum* (L.) Merrill; S – mixture of standards (1-UA, 2-BA, 3-OA); 2a, 2b – *Rosmarinus officinalis* L.; 3a, 3b – *Origanum vulgare* L. Documentation was obtained after derivatization with 10% (v/v) H_2SO_4 at λ = 365 nm

to improve repeatability. The average percentage relative standard deviation (% RDS) for oleanolic acid ranged from 1.5 to 2.5% and for ursolic acid ranged from 1.1 to 2.7%. The mean peak areas (n = 5) were taken for the construction of the calibration curve. The data were analyzed by linear regression least squares model and showed a good linear relationship over the tested range (r = 0.9973 for oleanolic acid and r = 0.9984 for ursolic acid). The linear regression equations for oleanolic and ursolic acids were y = 0.2420x - 8.6582 and y = 1.3834x – 9.0036, respectively. The accuracy of the method was established by performing recovery experiments at two different levels. Known amounts of each compound were spotted and analyzed as described above. The average recovery was 100.7% for oleanolic acid and 102.3% for ursolic acid. Limit of detection and limit of quantification were calculated by use of the equations: LOD = 3 × N/b and LOQ = 10 × N/b, where N is the standard error of the estimate, and b is the slope of the calibration curve. The limits of detection were 31 ng/spot for oleanolic acid and 13 ng/spot for ursolic acid. The limits of quantification were 103 ng/spot and 43 ng/spot, respectively. The validation data are summarized in Table 1.

Table 1. Summary of validation data for determination of ursolic and oleanolic acid.

Validation parameter	Oleanolic acid	Ursolic acid
Linearity range	230–2300 ng/spot	45–450 ng/spot
Regression equation	0.2420x – 8.6582	1.3834x – 9.0036
Correlation coefficient (R^2)	0.9973	0.9984
Limit of detection (LOD)	31 ng/spot	13 ng/spot
Limit of quantification (LOQ)	103 ng/spot	43 ng/spot
Precision (% RSD)	1.5 – 2.5	1.1 – 2.7
Recovery (%)	100.7	102.3

Table 2. The results of quantification of triterpenic acids in spices.

Sample of extract	Oleanolic acid (mg/g of dry plant material)	Ursolic acid (mg/g of dry plant material)
Origanum vulgare L.	6.51	10.04
Rosmarinus officinalis L.	18.10	7.51
Syzygium aromaticum (L.) Merrill	20.02	1.10

The presence of oleanolic and ursolic acids in various amounts was observed in all investigated spices. The highest concentration of oleanolic acid was noted in *Syzygium aromaticum* and amounted to 20.02 mg/g of plant material. The content of ursolic acid ranged from 1.10 mg/g (*Syzygium aromaticum*) to 10.04 mg/g (*Origanum vulgare*). Total content of both acids was the highest in *Rosmarinus officinalis* (25.61 mg/g), however, the lowest amount was determined in *Origanum vulgare* (16.55 mg/g).

The obtained results of quantitative analysis of the extracts are presented in Table 2.

CONCLUSION

HPTLC combined with densitometry is a rapid and cost-effective tool for investigation of plant extracts. It enables measurement of many samples in a short time and can be applied to routine analysis of spices, e.g., for their quality control.

This method was successfully applied to simultaneous quantification of oleanolic and ursolic acid in *Origanum vulgare* L., *Rosmarinus officinalis* L. and *Syzygium aromaticum* after prechromatographic iodine derivatization. Both triterpenes have confirmed biological activity and can influence the medicinal properties of spices.

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