SOLID-PHASE EXTRACTION FOR PHOTOMETRIC DETERMINATION OF ROSMARINIC ACID IN LEMON BALM (*MELISSA OFFICINALIS*) EXTRACTS

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Abstract: The aim of this study was evaluation of the solid-phase extraction for elimination of interference compounds from lemon balm extracts aimed for photometric determination of rosmarinic acid. In experiments, evaluated conditions were as follows: composition and volume of mobile phase, ratio between volume of sample and mass of stationary phase and flow rate of mobile phase during separation. The results indicated that interfered compounds were eliminated. The lemon balm extracts should be pretreated by adsorption on normal stationary phase (silica gel) in ratio sample volume to silica gel weight 1:1 (v/w), elution by mobile phase – diethyl ether: acetic acid (9:1; v/v) – volume - 40 times of crude extract volume – with flow rate 5 ml/min. After selection of SPE conditions, the method was validated with comparison to HPLC analysis. The results suggest that this method may be useable for determination of rosmarinic acid by photometric measurement based on the complexation of Fe^{2+} ions with rosmarinic acid.

Key words: rosmarinic acid, determination, lemon balm, HPLC, SPE, photometry

1. Introduction

Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid (PEREIRA *et al*., 2005; PETERSEN, 1997). It is commonly found in species of the *Boraginaceae* and *Lamiaceae*. One of these plants is lemon balm (*Melissa officinalis*) that express broad spectrum of biological activities. *M. officinalis* was used in traditional medicine dating back as far back as ancient Greek and Roman times for treating disorders of the nervous system and melancholy (ADINEE *et al*., 2008). Today, balm is very useful for nervous agitation, and for promoting sleep, and ameliorates functional gastrointestinal dysfunction (BOUSBIA *et al*., 2009). It is recommended as a plant juice, cream or tea infusion for nervous complaints, lower abdominal disorders, gastric complaints, hysteria and melancholia, chronic bronchial catarrh, migraine, nervous debility, toothache, earache, headache and high blood pressure and externally, for rheumatism, nerve pains and stiff necks (compress) (DASTMALCHI *et al*., 2007). Rosmarinic acid is responsible for many published activities, especially antimicrobial (BAIS *et al*., 2002), antiallergic (ITO *et al*., 1998; MATSUNO *et al*., 2002), antiviral and anti-inflammatory activity (GEORGIEV *et al*., 2006; HUANG *et al*., 2006; KAMATOU *et al*., 2005).

For analysis of its content during the extraction process in technological scale, the photometric methods were developed (LOPEZ-ARNALDOS *et al*., 1995; ÖZTÜRK *et*

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al., 2010). These methods are based on the increasing of UV-VIS absorbance by the forming of complex between rosmarinic acid with Fe^{2+} (LOPEZ-ARNALDOS *et al*., 1995) and Zr^{4+} ions (ÖZTÜRK *et al*., 2010). Photometric methods in comparison with high performance liquid chromatography (HPLC) or capillary electrophoresis are rapid, cheep and sufficiently accurate. Disadvantage of these methods is small selectivity of complexation reactions. Increasing of absorbance at studied wavelengths by the reaction of Fe^{2+} and Zr^{4+} with another organic acid can introduce mistakes to the analysis.

In present work, the application of solid-phase extraction aimed for removal of interference compounds by the determination of rosmarinic acid by photometric analysis was studied.

2. Material and methods

2.1 Chemicals

Rosmarinic acid 95% (Sigma), methanol, ethanol, propan-2-ol, ethyl acetate, dichloromethane, chloroform, diethyl ether and acetic acid all p.a. grade (Mikrochem), ferrous sulphate p.a. (Chemapol), tris-(hydroxymethyl)-aminomethane p.a. (Sigma) and silica gel (Macherey-Nagel).

2.2 Plant material

Dry leaves of lemon balm (*Melissa officinalis*) were obtained from local area Diviaky, district Banska Bystrica, Slovak Republic.

2.3 Preparation of crude extracts from lemon balm

Dried leaves of lemon balm (1 g) was extracted by 20 mL of following solvents: distilled water, methanol, ethanol, propan-2-ol and by their aqueous solutions (25, 50 and 75 %) at 40 °C for 1 h. Prepared extracts were decanted into the test tubes. The final volume was measured and then the concentration of rosmarinic acid was determined by HPLC and photometric method.

2.4 Analytical determination

2.4.1 Photometric determination of rosmarinic acid

For photometric analysis of rosmarinic acid in the crude extracts from lemon balm, method based on the complex reaction with Fe^{2+} ions was used. 40 µL sample and 4 mL of Tris/acetate buffer (pH 6.0) was mixed. The buffer contained $FeSO_4$ in the concentration of 0.5 g/L. The mixture was incubated for 1 h at room temperature in the darkness. The absorbance of the reaction medium was read at 572 nm. Calibration was carried out with standard of rosmarinic acid and control without rosmarinic acid (LOPEZ-ARNALDOS *et al*., 1995).

2.4.2 HPLC analysis of rosmarinic acid

The rosmarinic acid content in prepared extracts was determined by HPLC method using Purospher Star RP18e, 250 × 4 mm, 5 μm, gradient elution of 0.1 % trifluoracetic acid in water (A) and acetonitrile (B), at flow rate 1 mL/min: 0 min 15 % B, 18 min 30 % B, 25 min 80 % B, 27 min 80 % B with detection at 325 nm (ONDREJOVIČ *et al.*, 2009).

2.4.3 TLC analysis of crude extracts from lemon balm

The plant leaf extracts were additionally analyzed on silica gel plates by TLC. 10 μL of each extracts were applied and the plates were developed in different organic solvents and their mixtures (acetic acid: methanol: dichlormethane 4:15:35; acetic acid: methanol: dichloromethane 4:2:36; acetic acid: diethyl ether 1:9; acetic acid: chloroform 1:9; acetic acid: ethylacetate 1:9; acetic acid: butanol 1:9). Dried plates were sprayed by reagent (Tris/acetate buffer pH 6.0 with $FeSO_4$) used for photometric analysis of rosmarinic acid. Positive reaction of rosmarinic acid with reagent can be detected as dark blue spot on yellow background.

2.5 Solid phase extraction

For solid phase extraction, silica gel was used as stationary phase for sample (lemon balm crude extracts) adsorption. The ratio between stationary phase and crude extract was 1:1, 1:2, 1:4 (w/v). Samples were dried in the oven 25 min at 105 °C and cooled in the desiccators. Silica gel with adsorbed extracts were put into the extraction column and washed by eluent (acetic acid: diethyl ether 1:9; v/v). The first addition of eluent was 500 μL and other four additions were 250 μL of eluent. Every fraction were collected into the test tubes and evaporated by rotary evaporator. Obtained fractions were concentrated by 2 mL methanol. In all samples was determined concentration of rosmarinic acid by photometric method and HPLC analysis.

3. Results and discussion

3.1 Detection of rosmarinic acid by photometric method

Rosmarinic acid can be determinated by two photometric methods. Both are based upon the complexation of rosmarinic acid with Fe^{2+} and Zr^{4+} ions which caused increasing of sample absorbance at 572 and 362 nm respectively. By comparison of these methods for determination of rosmarinic acid comparable results were obtained (ÖZTÜRK *et al*., 2010). However, determination of rosmarinic acid with Fe^{2+} is cheaper and environmental acceptable as using of Zr^{4+} ions. Moreover, interferences caused by absorbance of non-complexated compounds in tested extracts are higher at 362 nm (Zr^{4+}) than 572 nm (Fe^{2+}). Therefore, the application of photometric method of rosmarinic acid determination by Fe^{2+} complexation for control of rosmarinic acid extraction process in preparative scale was studied.

Studying of extraction process starts by selection of extraction solvent. For rosmarinic acid extraction, spectrum of organic aliphatic alcohols (such as methanol, ethanol, propan-2-ol) and their water solutions were used. Rosmarinic acid content was determined by photometric and HPLC analysis. The results (Fig. 1) indicated disproportion between concentration of rosmarinic acid determined by photometric and HPLC methods. All concentration of rosmarinic acid determined by photometric analysis is higher as concentration of rosmarinic acid determined by HPLC. This finding refers to presence of other compounds which interference in photometric analysis. The result differences between both analytical methods are higher in samples prepared with water solution of used organic aliphatic alcohols. Therefore interference compounds may be belonging to polar organic compounds.

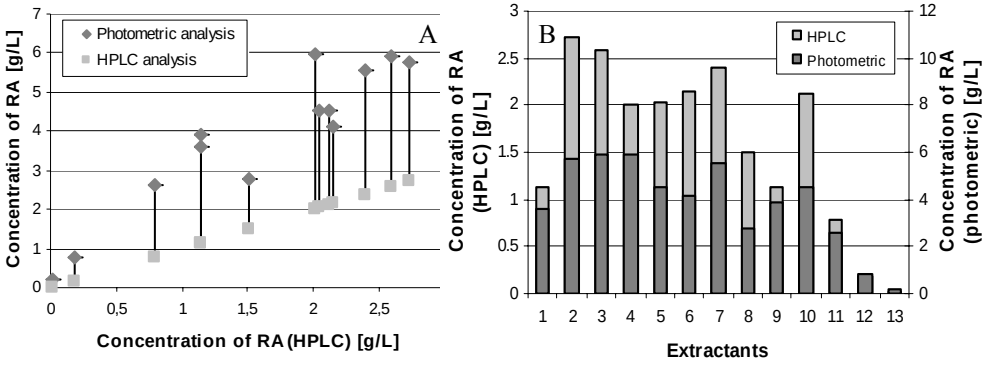

Fig. 1. Comparison of two methods of rosmarinic acid determination (RA); A - Dependence of RA concentration determined by photometric method and HPLC; B – Concentrations of RA determined by HPLC and photometric method in extracts from lemon balm prepared by extraction to various extraction solvents:1 – distilled water; 2 – 25 % methanol; 3 – 25 % ethanol; 4 – 25 % propan-2-ol; 5 - 50 % methanol; 6 - 50 % ethanol; 7 – 50 % propan-2-ol; 8 – 75 % methanol; 9 – 75 % ethanol; 10 – 75 % propan-2-ol; 11 – methanol; 12 – ethanol; 13 – propan-2-ol.

For determination of compounds interfered at photometric analysis of crude extracts from lemon balm, the TLC (thin-layer chromatography) analysis on the normal stationary phase by method of multiple elution was used. This method allows analyzing hydrophobic and hydrophilic compounds together. After visualization of separated compounds by spraying of reagent containing of Fe^{2+} ions on TLC layer was observed several blue spots (see R_f factors in Table 1). It is evident that prepared extracts contain more compounds with positive reaction with Fe^{2+} ions and higher differences between concentrations of rosmarinic acid determined by photometric and HPLC analysis correlate with number of positive spots determined by TLC analysis.

3.2 SPE purification of plant extracts

3.2.1 Choice of eluent

From the TLC analysis, it is possible to roughly estimate the polarity of ballasts interfered at determination of rosmarinic acid by photometric analysis. Because the

spot of rosmarinic acid was upper than another spots, the solid-phase extraction can be used for pretreatment of sample before the photometric determination of rosmarinic acid. In the literature, solid-phase extraction of rosmarinic acid was realized on the reverse stationary phase such as C18 (EXARCHOU et al., 2003; STEVENS et al., 2007), but also normal stationary phase is suitable for separation of rosmarinic acid from lemon balm extracts and it is cheaper than reverse stationary phase.

Table 1. Concentration of interfering compounds (ballasts) and retention factors of positive spots visualized on TLC plates after spraying with detection reagent (Fe^{2+}) in various extracts form lemon balm prepared by extraction with various extraction solvents.

Extraction solvents	**Concentration of ballasts [g/L]**	**Retention factors of positive spots**						
water	2.46	0	0.04	0.13	0.27	0.39	0.45	0.69
25% methanol	3.03	0	0.04	0.13	0.27	0.39	0.45	0.69
25% ethanol	3.34	0	0.04	0.13	0.27	0.39	0.45	0.69
25% propan-2-ol	3.94	0	0.04	0.13	0.27	0.39	0.45	0.69
50% methanol	2.47	0	0.04	0.13	0.27	0.39	0.45	0.69
50% ethanol	1.96	0	0.04	0.13	0.27	0.39	0.45	0.69
50% propan-2-ol	3.18	0	0.04	0.13	0.27	0.39	0.45	0.69
75% methanol	1.25	-	0.04	0.13	-	0.39	-	0.69
75% ethanol	2.75	-	0.04	0.13	0.27	0.39	0.45	0.69
75% propan-2-ol	2.40	-	0.04	0.13	0.27	0.39	0.45	0.69
methanol	1.83	-	0.04	0.13	-	0.39	-	0.69
ethanol	0.61	-	-	-	-	-	0.45	0.69
propan-2-ol	0.19	-	-	-	-	-	0.45	0.69

From the point of view of method application during technological production of rosmarinic acid, pretreatment of lemon balm extracts before photometric rosmarinic acid determination on normal stationary phase is more suitable.

In order to choose a suitable eluent for the elution of rosmarinic acid, crude extract from lemon balm was adsorbed on the stationary phase (silica gel) and organic solvent was evaporated. Thereafter the stationary phase with adsorbed crude extract without possible interference caused by extraction solvents was eluted by various organic solvents such as ethyl acetate, dichloromethane, chloroform, diethyl ether. The rosmarinic acid is found in the plant material as its salts (HÄUSLER et al., 1993). Since, extracts prepared by extraction solvent without acids contain rosmarinic acid salts. Therefore, all mobile phases contain also acetic acid in the ratio between organic solvent/acetic acid 9:1 (v/v). Prepared fractions were analysed by TLC after spraying by detection reagent with Fe^{2+} ions. Rosmarinic acid was found in all of selected eluents, but the spot of rosmarinic acid without all interference compounds was only in the diethyl ether fractions. Therefore, diethyl ether was selected as suitable component of mobile phase and it was used in another experiments.

3.2.2 Crude extract – stationary phase amount ratio

In order to determinate the suitable ratio between crude extract and amount of stationary phase, various crude extracts from lemon balm was adsorbed by evaporation of extraction solvent in the presence of silica gel with subsequent elution by the evaporated solvent (Fig. 2). The results indicated that the best ratio between crude extracts from lemon balm and amount of stationary phase was 1:1 (v/w). This ratio was used in next experiments.

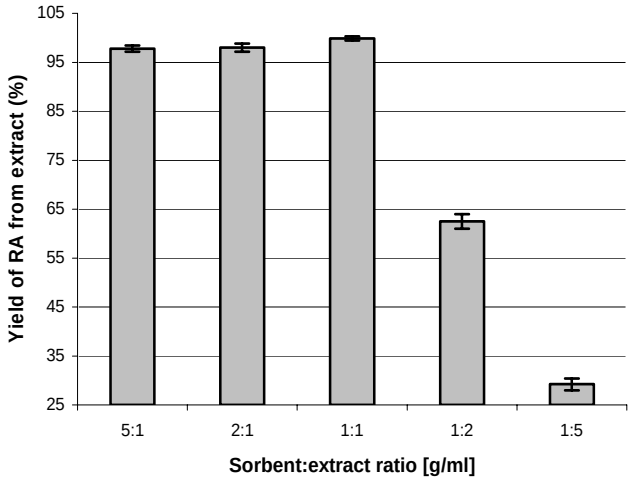

Fig. 2**.** Yield of rosmarinic acid eluted by mixture diethyl ether: acetic acid (9:1; v/v) from silica gel with adsorbed crude extract of lemon balm by various ratio between mass of silica gel and volume of extract.

3.2.3. Effect of volume of eluent

In order to investigate the eluent volume (diethyl ether: acetic acid; 9:1; v/v) for quantitative extraction of rosmarinic acid from stationary phase, rosmarinic acid in concentration varied from 0.03 to 3.0 mg/g of stationary phase was adsorbed on the silica gel by addition 0.25 ml of standard solution of rosmarinic acid to 0.25 g of silica gel. Used concentration range of rosmarinic acid can be expected in lemon balm crude extracts. Thereafter, rosmarinic acid from stationary phase by 1 ml of selected eluent was eluted. In first ten fractions, the rosmarinic acid was presented in detectable values and their elution yield was varied from 98 to 99 % of adsorbed rosmarinic acid. From results verified on lemon balm crude extract pretreatment, the lemon balm extracts for determination rosmarinic acid by photometric methods could be eluted by selected eluent from stationary phase minimally 40 times of volumes of adsorbed crude extract.

3.2.4. Effect of flow rate

The influence of flow rate was studied on the base of analysis of the retention and recovery of rosmarinic acid in the range of 0.5 – 5 mL/min. The retention of the

rosmarinic acid on the stationary phase was not considerably affected by the sample solution flow rate. The recovery of rosmarinic acid by the elution was dependent just on the volume of selected eluent. Therefore in another experiments the flow rate of 5 mL/min was used.

3.3 Validation of method

For validation of suggested method, concentration of rosmarinic acid in crude extract from lemon balm was determined by photometric analysis with SPE sample pretreatment and HPLC. In the Fig. 4, concentrations determined by photometric analysis and HPLC method are correlated and it is evident that differences between results achieved by both methods are minimal.

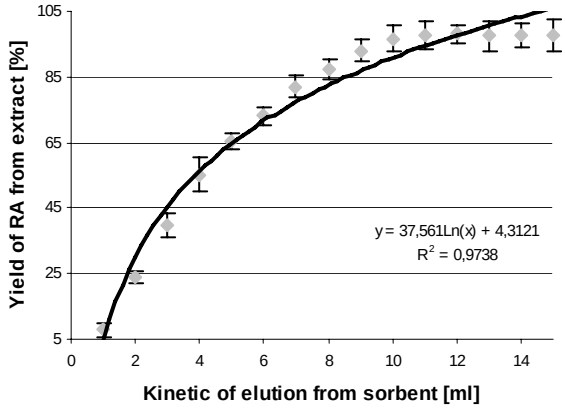

Fig. 3. Yield of rosmarinic acid obtained from silica gel with adsorbed crude extract of lemon balm.

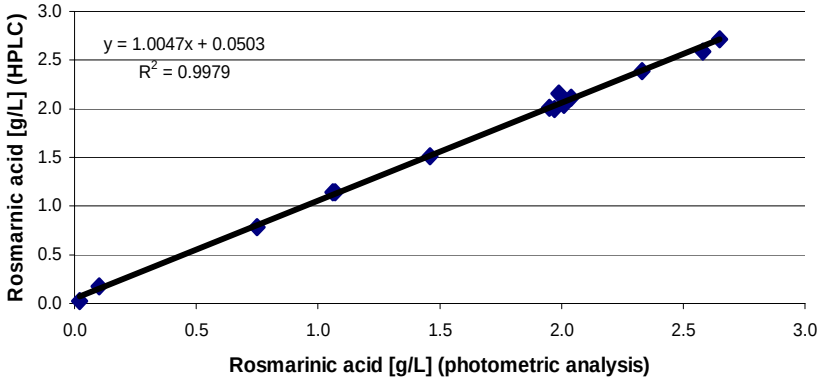

Fig. 4. Comparison of rosmarinic acid concentrations determined by photometric method with SPE sample pretreatment and HPLC in crude extracts form lemon balm prepared by various extraction solvents.

4. Conclusion

In this study, the solid-phase extraction was demonstrated to be very efficient for elimination of interfering compounds for precise photometric determination of rosmarinic acid from lemon balm extracts. The lemon balm extracts should be pretreated by adsorption on normal stationary phase (silica gel) in ratio sample volume to silica gel weight 1:1 (v/w), elution by mobile phase – diethyl ether: acetic acid (9:1; v/v) – volume - 40 times of initial crude extract volume – with flow rate 5 mL/min. After selection of SPE conditions, the method was validated by comparison to HPLC analysis. The results indicated that this method may be useable for determination of rosmarinic acid by photometric method based on the complexation of Fe^{2+} ions with rosmarinic acid.

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