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PRODUCTION AND APPLICATION OF HERBAL EXTRACTS CONTAINING ROSMARINIC ACID FOR DIETARY SUPPLEMENT AND FUNCTIONAL FOOD

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1 Introduction and aims

Nowadays, interest in herbal extracts with high rosmarinic acid content has considerably increased due to their favorable pharmacological and biological properties. Rosmarinic acid is a secondary metabolite belonging to phenolic acids, and it is often used as a marker compound in the Nepetoideae subfamily of the Lamiaceae family. From a chemical point of view, rosmarinic acid is a phenylpropanoid as the caffeic acid ester of 3,4-dihydroxyphenillactic acid. Primarily, the compound has an antioxidant effect. The results of recent research have shown that rosmarinic acid has a preventive or therapeutic effect on neurodegenerative disorders, diabetes mellitus, or cancer. Based on this approach, the goal of this study was to improve the extraction efficiency of rosmarinic acid from six Lamiaceae herbs (*Melissa officinalis* L., *Mentha x piperita*, *Thymus vulgaris* L., *Origanum vulgare* L., *Rosmarinus officinalis* L., *Salvia officinalis* L.) to develop dietary supplements and functional food. To attainment the objectives, the following tasks were fulfilled:

- Four independent variables were examined using the one-factor-at-a-time approach (OFAT) to extract rosmarinic acid from six Lamiaceae plants with three extraction techniques.
- The long-term stability of hydroethanolic extracts (tinctures) was studied at ambient temperature simulating standard “home” storage conditions.
- Lyophilized lemon balm extract rich in rosmarinic acid was used as a functional ingredient in chocolate.

2 Experimental

2.1 Materials

The leaves of lemon balm (*M. officinalis*), peppermint (*M x piperita*), rosemary (*R. officinalis*), sage (*S. officinalis*), thyme (*T. vulgaris*), and oregano (*O. vulgare*) were harvested from Hungary (Kisalföldi Mezőgazdasági Zrt., Nagyszentjános) in the summer of 2017. The Belgian dark chocolate drops (54.5%) were purchased in a Hungarian candy store (Édeni Édességek Boltja, Szentendre).

2.2 Extraction method development

Extraction yields for rosmarinic acid from different extracts of Lamiaceae plants were investigated in the present study through maceration with shaking (MAC_S), heat reflux extraction (HRE), and microwave-assisted extraction (MAE) techniques. The following independent variables were used in this study: solvent acidity (0, 0.1, and 1 v/v% HCl), type of solvent (H_2O, MeOH, EtOH), extraction time, and extraction temperature.

MAC_S was carried out in a closed Erlenmeyer flask at room temperature at five different extraction times (30, 60, 120, 240, and 1440 min) with a laboratory shaker (120 rpm). HRE was performed at the boiling point for 15, 30, and 60 min. After the extraction time, the samples were allowed to stand for 10 min.

The MAE procedure was carried out in a closed Teflon extraction vessel. The samples were heated to the desired temperature for 5 min, and four different extractions were carried out at 5, 10, 15, and 30 min at 50°C and 80°C with a maximum power of 400 W. After that, the

mixture in the vessel was cooled down for 15 min and then the vessels were put into a cold water bath for 5 min.

2.2.1 HPLC-DAD analysis

The mobile phase consisted of methanol (A), 0.25 v/v% aqueous trifluoroacetic acid (B), and water (C). Separation and quantification was carried out using a Purospher Star C18 (250 mm x 4.6 mm, 5 μm) analytical column at 35°C with the following gradient mode: 0-25 min: 15% A, 25-30 min: 15-75% A, 30-35 min: 75-15% A, 35-47 min: 15% A. The content of eluent B was kept constant at 20 v/v% during the chromatographic analysis. Chromatograms were evaluated at the absorption maximum (330 nm) of rosmarinic acid. The flow rate was 1 mL/min, and the injection volume was 2 μL. Before the determination by HPLC, each sample was centrifuged (2500 × g, 10°C, 10 min), and then the supernatant was filtered through a 0.22 μm PVDF membrane before being directly injected into the HPLC column.

2.3 Analysis of tinctures

2.3.1 Tincture preparation

The hydroethanolic extracts (tinctures) were prepared from 3 g of plants and 60 mL 50:50 v/v EtOH-H_2O solution. The extraction was performed by MAC_S in a closed Erlenmeyer flask at room temperature for 2 hours. The extracts were filtered through Whatman No 1 filter paper (Sigma-Aldrich), and the filtrates were used as tinctures.

2.3.2 Stability testing

Each tincture was measured (4 mL) into six amber glass bottles and stored in a closed box at ambient temperature for six months

simulating standard “home” storage conditions. The tinctures were analyzed at the initial time and then monthly, which corresponds to the 27th, 55th, 86th, 113th, 141th, and 168th days. The limit of acceptance of rosmarinic acid as a marker compound of Lamiaceae plants was considered 90% from the initial assay value of the tinctures. Standard rosmarinic acid as control was dissolved in 50:50 v/v EtOH-H_2O similar to tinctures, to obtain a concentration of 500 µg/mL.

2.3.3 HPLC-DAD analysis (See Section 2.2.1)

2.3.4 Total phenolics assay

A 5-fold diluted tincture (50 µL) was mixed with 1.5 mL of water, 2.5 mL of Folin-Ciocalteu reagent (10 v/v%), and 2 mL of sodium carbonate solution (7.5 g/100mL) in a test tube. The samples were allowed to stand in the dark at room temperature for 90 min. The absorbances were measured at 725 nm versus the blank. Gallic acid was used as a standard (100-1000 µg/mL) for the calibration curve, and the results were expressed as mg of gallic acid equivalents per gram of dry plant material (mg GAE/g).

2.3.5 2,2-diphenyl-1-picrylhydrazyl scavenging assay

A solution of the radical is prepared by dissolving 3.9 mg of DPPH in 100 mL of methanol. An aliquot of each diluted extract (0.05 mL) was added to a DPPH solution (4.950 mL) and the reaction mixture was kept in the dark for 1 hour at room temperature and the absorbance was read at 517 nm. The half-inhibitory concentration (IC_{50}) was obtained through the interpolation of linear regression analysis. The $1/IC_{50}$ coefficient was also calculated to determine the

correlation between antioxidant activity and the rosmarinic acid and total phenolic content of the tested plant extracts. L-ascorbic acid was used as a standard (40-800 μg/mL) for the calibration curve. Rosmarinic acid and caffeic acid were used as the positive control.

2.4 Analysis of chocolate

2.4.1 Chocolate preparation

Chocolates containing rosmarinic acid (100 mg/25 g) were prepared using commercially available dark chocolate drops (54.5%) according to the following procedure. 100.34 g chocolate drops were slowly melted in a double boiler, taking care that the temperature of the melted chocolate did not get above 45°C. After that, 2/3 of the melted chocolate was poured onto a granite slab and cooled down to 27-28°C while 4.4936 g of freeze-dried lemon balm extract was added to it. The thickened chocolate was returned to the residue of melted chocolate and mixed until the temperature reached 31-32°C. Finally, the tempered chocolate was poured into a polycarbonate mold and stored in a refrigerator for 1 hour. This procedure was repeated with 0.4004 g of standard rosmarinic acid. The control chocolate contained neither dry plant extract nor standard rosmarinic acid.

Sample preparation consisted of two distinct steps. First, the cooled (+5°C) chocolates were grated and then pulverized and homogenized in a mortar and pestle. The second step was defatting. To remove the lipid phase, 5 mL of n-hexane was added into a Falcon tube containing 500 mg ground chocolates. Samples were shaken for 1 min using a vortex mixer, followed by sonication for 5 min in an ultrasonic bath at room temperature. Afterwards, the resulting mixtures were

centrifuged for 5 min at 2500 g, and the supernatants containing fat were removed. The defatted chocolates were oven-dried (35°C) for 1 hour.

2.4.2 Extraction method development

Dried and defatted chocolates were used for MAC_S and MAE with the following sample weight: solvent ratio (1:20 g/mL). In each case, an OFAT methodology was used to define the best extraction condition considering two factors (independent variables): extraction time and solvent type. For both extraction methods, ethanol and methanol were used as solvents with the following compose: 100 and 50 v/v%. Treatment times of 30, 60, 120, 240, and 1440 minutes were used for the conventional method and 5, 10, 15, and 30 minutes for the MAE. MAC_S (120 rpm) was carried out in a closed Erlenmeyer flask at room temperature. The MAE procedure was performed in a closed Teflon extraction vessel as follows. The chocolate samples were heated to the desired temperature (60 °C) for 5 min and extracted with a maximum power of 400 W.

2.4.3 HPLC-DAD analysis

The mobile phase consisted of acetonitrile (A), 0.5 v/v% aqueous phosphoric acid (B), and water (C). Separation and quantification was carried out using an Ascentis Express C18 (150 mm x 3 mm, 5 μm) analytical column at 35°C with the following gradient mode: 0-15 min: 8% A, 15-22.8 min: 8-25% A, 22.8-24 min: 25-70% A, 24-35 min: 70-8% A. The content of eluent B was kept constant at 20 v/v% during the chromatographic analysis. The

quantification was done at a wavelength of 330 nm. The flow rate was 0.5 mL/min, and the injection volume was 2 μL.

2.4.4 Stability testing

For the stability study, a part of the fortified chocolate (40 g) was packed in aluminum foil and stored in a refrigerator at 5°C. Stored chocolates were evaluated for rosmarinic acid content for 29 days intervals for 6 months. The chocolate samples were extracted by MAC_S.

3 Results

3.1 Extraction method development

3.1.1 Effect of acidification on extraction

The addition of HCl contributes to an improvement of the extraction yield for all types of solvent studied, and the best results were obtained for a solvent of type EtOH-H_2O (70:30 v/v) with a content of 1 v/v% HCl. For lemon balm, a 129% (from 12.5 to 28.6 mg/g) increase in the rosmarinic acid yield was observed when the acidification was increased from 0 to 1% in 70:30 v/v EtOH-H_2O. A similar tendency was indicated for peppermint (116%, from 7.5 mg/g to 16.2 mg/g) and sage (85%, from 10.6 mg/g to 19.6 mg/g), but the acidification increased the efficiency significantly for oregano (64%, from 23.6 mg/g to 38.8 mg/g), thyme (57%, from 12.5 mg/g to 19.6 mg/g), and rosemary (37%, from 7.1 mg/g to 9.7 mg/g).

3.1.2 Effect of solvent type on extraction

The rosmarinic acid contents have shown that ethanol (7.9 ± 0.5 mg/g – 38.8 ± 0.5 mg/g) was significantly more effective than the water (2.5 ± 0.2 mg/g – 14.2 ± 1.7 mg/g) or methanol solvent

system (<LOQ – 37.1 ± 1.7 mg/g) used to extract rosmarinic acid from plants. The highest level of rosmarinic acid was measured when using 70:30:1 v/v/v EtOH-H_2O-HCl for lemon balm (28.6 ± 1.2 mg/g), peppermint (16.2 ± 1.5 mg/g), oregano (38.8 ± 0.5 mg/g), rosemary (9.7 ± 0.7 mg/g), sage (19.6 ± 1.2 mg/g), and thyme (14.6 ± 0.2 mg/g).

3.1.3 Effect of extraction time and temperature on extraction

My results revealed that the MAC_S method did not have a significant effect on the amount of rosmarinic acid after 30 min of extraction time. When comparing the best extraction time (120 min) for MAC_S that gave the best rosmarinic acid recovery with other techniques, it can be seen that, except for oregano, MAC_S or MAE give the best rosmarinic acid yield. Moreover, these rosmarinic acid recoveries are statistically ($p \leq 0.05$) the same. For instance, the MAC_S extraction for 60 and 120 min showed a similar rosmarinic acid yield compared to MAE at 80°C for 5 min for lemon balm, thyme, and sage. A similar trend was seen for peppermint extraction. Again, MAC_S exhibited the same efficiency (16.1 ± 1.0 mg/g) compared to MAE at 50°C for 10 min (16.1 ± 0.5 mg/g) and 15 min (16.0 ± 0.7 mg/g).

I found that the rosmarinic acid yield was, in many cases, lower at high temperatures with HRE and MAE, especially when the extraction time was longer. The decomposition effect was most noticeable for the MAE (80°C) method for oregano. In this case, rosmarinic acid decreased by 12.5 mg/g (34%). A comparison of HRE extraction between 15 and 60 min shows that the amount of rosmarinic acid decreased by 2.5 mg/g (>10%) for sage and thyme.

3.2 Analysis of tinctures

3.2.1 Stability of rosmarinic acid

The rosmarinic acid content in all tinctures started to decrease after 27-day storage, while the caffeic acid content increased. Basically, between the first and the last day of storage, the decreases in rosmarinic acid were 14.1%, 15.8%, 16.5%, 27.6%, and 41.4% for rosemary, peppermint, oregano, lemon balm, thyme, and sage, respectively. The highest change in caffeic acid concentration was observed for lemon balm (-54.8%), followed by oregano (-50.5%), peppermint (-37.9), sage (-27.0%), rosemary (-21.6%), and thyme (-19.7%). The degradation tendency of standard rosmarinic acid was also monitored during the 168-day of storage. In this case, I observed much lower decomposition values (9.6%). During the six-month storage period, the degradation of the rosmarinic acid did not exceed the limit of acceptance (10%).

3.2.2 Total phenolic and antioxidant characteristics

The initial total phenolic contents of the various Lamiaceae tinctures as determined using the Folin-Ciocalteu colorimetric method were in the range of 34.1 ± 2.7 mg GAE/g – 93.4 ± 5.5 mg GAE/g. The six tinctures showed significant activity ($IC_{50} \geq 19.2$ μg/mL) when compared with the values obtained for L-ascorbic acid (IC_{50}= 3.97 μg/mL), rosmarinic acid (IC_{50}= 4.02 μg/mL), and caffeic acid (IC_{50}= 4.24 μg/mL) standards. Among tinctures, oregano exhibited the strongest activity with an IC_{50} value of 19.2 μg/mL followed by lemon balm and peppermint at IC_{50} values of 30.2 μg/mL and 31.0 μg/mL, respectively. A weak activity was observed with the thyme (IC_{50}= 96.0 μg/mL) and rosemary (IC_{50}= 96.2 μg/mL). Similarly, a reduced

antioxidant effect was noticed in sage (IC_{50}= 77.2 μg/mL). The results of the study indicated a linear relationship between DPPH values and total phenolic (R^2=0.92) or rosmarinic acid (R^2=0.85) contents.

3.3 Analysis of chocolate

3.3.1 Effect of solvent type on extraction

Among the tested solvents, pure methanol was significantly ($p \leq 0.05$) the most efficient for extracting rosmarinic acid from chocolate by MAC_S (3.98 ± 0.07 mg/g), approximately more than five times the one of pure ethanol (0.72 ± 0.05 mg/g) which represent the lowest value. A similar tendency was observed with MAE. However, in this case, the highest extracted amount of rosmarinic acid was recorded as 50:50 v/v MeOH-H_2O (3.95 ± 0.25 mg/g), more than six times the one of pure ethanol (0.64 ± 0.02 mg/g).

3.3.2 Effect of extraction time on extraction

Results showed that the amount of rosmarinic acid extracted by MAC_S increased when extraction time was increased from 30 min to 120 min. This duration allowed the extraction of 3.98 ± 0.07 mg/g of rosmarinic acid from fortified chocolate, which corresponded to an extraction efficiency of 99.5%. After 120 min, increasing the extraction time decreased the recovery. The amount of rosmarinic acid extracted by MAE did not show a significant difference ($p \leq 0.05$) with the increase of extraction time from 5 min to 30 min. Moreover, the MAE extraction for 10 min showed the same rosmarinic acid yield (3.98 ± 0.05 mg/g) when compared to MAC_S at room temperature for 120 min.

3.3.3 HPLC method optimization and validation

The mobile phase was optimized by examining the effect of pH, thus avoiding the ionization of rosmarinic acid during identification. For this reason, the pH was controlled by the addition of different concentrations of phosphoric (0.1 and 0.5 v/v%) and formic acid (0.1 v/v%). The best separation performance was observed when the phosphoric acid solution of 0.5 v/v%, i.e., 0.07 M concentration (pH=1.72) was used. In addition, the eluent flow rate was also investigated. The optimum value was determined to be 0.5 mL/min. The developed method was found to be precise as the RSD% values for repeatability of intra-day and inter-day precision studies were below 2.3%. A recovery study has been conducted to confirm the accuracy of the method developed. The recovery percentages were 95.4 ± 1.2 and 94.6 ± 0.9% for MAC_S and MAE, respectively.

3.3.4 Stability of rosmarinic acid in chocolate

Based on one measurement before storage, the initial rosmarinic acid value of fortified chocolate was 3.98 ± 0.07 mg/g. There was no significant effect ($p \leq 0.05$) of storage time on the rosmarinic acid content of chocolate. These results indicate that the chocolate sample constituted a feasible matrix for the addition of lemon balm extract as the bioactive compound (rosmarinic acid) of freeze-dried lemon balm extract was not affected.

4 New scientific results (thesis)

1. I have pointed out that the use of appropriate extraction parameters for conventional (maceration with stirring, heat-reflux extraction) and microwave-assisted extraction can increase the rosmarinic acid content of plant extracts, thereby improving their quality. I have demonstrated that the extraction efficiency is dependent on the solvent type, solvent acidity, and extraction temperature. I have proved that extraction with acidified aqueous ethanol (70:30:1 v/v/v, EtOH-H_2O-HCl) was the best choice for the recovery of rosmarinic acid.

2. My studies have shown that microwave-assisted extraction is only superior to conventional extraction methods (maceration with stirring (120 min), heat-reflux extraction (15 min)) with respect to extraction time (5 min).
3. I have proved that rosmarinic acid from plants is quite unstable at ambient temperature, therefore the component can convey the expected physiological effects in the form of a tincture only with a short quality preservation time (≤113 days). At the same time, the results of the study indicated a linear relationship between DPPH values and total phenolic (R^2=0.92) or rosmarinic acid (R^2=0.85) contents.
4. I have developed a solid-liquid extraction technique for rosmarinic acid extraction from dark chocolate fortified with freeze-dried lemon balm. I have demonstrated that the maceration with stirring (120 min, ambient temperature, 120 rpm) and microwave-assisted

extraction (5 min, 400 W, 60 °C) may successfully apply to rosmarinic acid extraction from lemon balm-fortified chocolate.

5. I have developed a reversed-phase high-performance liquid chromatography method (RP-HPLC-DAD) for the selective determination of rosmarinic acid in fortified chocolate. The validity of the method was demonstrated by evaluating the system suitability, linearity (5-800 μg/mL), precision (0,5-2,4 RSD%), accuracy (R%= ≥94.6), and the limit of quantification (LOQ= 0,0021 mg/mL).
6. I have confirmed by the developed RP-HPLC-DAD method that the functionality of dark chocolate may increase with freeze-dried lemon balm extract. I have pointed out that due to the excellent active ingredient distribution and stability (≥6 months), chocolate could play an ideal carrier matrix for the delivery of the bioactive plant ingredients to the human body in the future.

5 List of publications

5.1 Publications directly related to the PhD thesis

5.1.1 Papers published in a foreign-language peer-reviewed journal

Sik B, Kapcsándi V, Székelyhidi R, Lakatos E, Ajtony Zs. (2019) Recent advances in the analysis of rosmarinic acid from herbs in the Lamiaceae family. *Nat. Prod. Comm.*, 14, 1-10. https://doi.org/10.1177/1934578X19864216

Impact factor: 0.468 (Q3) Citations: 10

Sik B, Lakatos E, Kapcsándi V, Ajtony Zs. (2020) Conventional and nonconventional extraction techniques for optimal extraction processes of rosmarinic acid from six Lamiaceae plants as determined by HPLC-DAD measurement. *J. Pharm. Biomed. Anal.*, 184, 113173. https://doi.org/10.1016/j.jpba.2020.113173

Impact factor: 3.209 (Q1) Citations: 14

Sik B, Lakatos E, Kapcsándi V, Székelyhidi R, Ajtony Zs. (2021) Exploring the rosmarinic acid profile of dark chocolate fortified with freeze-dried lemon balm extract using conventional and non-conventional extraction techniques. *LWT-Food. Sci. Technol.*, 147, 111520. https://doi.org/10.1016/j.lwt. 2021.111520.

Impact factor: 4.006 (D1) Citations: -

5.1.2 Paper published in a Hungarian-language conference proceedings

Sik B. (2020) Lamiaceae családba tartozó tradicionális gyógynövények fő bioaktív hatóanyagának kinyerésére szolgáló extrakciós eljárások kidolgozása. *Új Nemzeti Kiválósági Program 2019/2020 Konferencia Tanulmánykötet,* 181-188.

5.2 Publications not related to the dissertation

Kapcsándi V, Lakatos E, **Sik B**, Linka LÁ, Székelyhidi R. (2021) Characterization of fatty acid, antioxidant, and polyphenol content of grape seed oil from different *Vitis vinifera* L. varieties. OCL, 28:30. https://doi.org/10.1051/ocl/2021017.

Kapcsándi V, Barabás A, **Sik B**, Ajtony Zs. (2018) Különböző laktóz koncentrációk hatása *Kluyveromyces* élesztőtörzsek szaporodási

tulajdonságaira és etanoltermelési képességére. *Acta Agronomica Óváriensis*, 59, 27-43.

Sik B, Kapcsándi V, Lakatos E, Ajtony Zs. (2018) Flavonolignánok máriatövisből (*Silybum marianum* L. Gaertner) történő oldószeres kinyerésének optimalizálása. In: Szalka É. (szerk.) XXXVII. Óvári Tudományos Napok, 2018. november 9-10.: Fenntartható agrárium és környezet, az Óvári Akadémia 200 éve – múlt, jelen, jövő. SZE-MÉK, Mosonmagyaróvár, 81-90.

Sik B, Lakatos E, Kapcsándi V, Ajtony Zs. (2018) Gyógy-és fűszernövények illóolaj tartalmának vizsgálata vízgőz-desztillációval. In: Szalka É. (szerk.) XXXVII. Óvári Tudományos Napok, 2018. november 9-10.: Fenntartható agrárium és környezet, az Óvári Akadémia 200 éve – múlt, jelen, jövő. SZE-MÉK, Mosonmagyaróvár, 70-80.

Sik B, Kovács AJ, Kapcsándi V, Lakatos E. (2016) *Kluyveromyces* élesztőtörzsek alkoholtermelésének vizsgálata a laktóztartalom függvényében. In: Szalka É, Bali, Papp Á. (szerk.) XXXVI. Óvári Tudományos Nap: Hagyomány és innováció az agrár-és élelmiszergazdaságban I-II. SZE-MÉK, Mosonmagyaróvár, 143-152.