OPTIMIZATION OF ROSMARINIC ACID EXTRACTION FROM LEMON BALM (*Melissa officinalis*)

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Abstract: The aim of this study was optimization of rosmarinic acid extraction from lemon balm (*Melissa officinalis*). The optimal conditions for the extraction of rosmarinic acid from lemon balm were determined using response surface methodology (RSM). A center composide design (CCD) was used to investigate the effects of three independent variables, namely solid-liquid radio, solvent composition (%) and extraction temperature (°C). Dependent variable was yield of rosmarinic acid. A second-order polynomial model was used for predicting the response. Optimized conditions for rosmarinic acid were: PEME 1:29 (w/v), temperature 66 °C a % propan-2-ol 34 %. The experimental values agreed with predicted within a 95 % confidence interval. Yield of rosmarinic acid extraction by these optimized conditions was achieved 72.6 mg of rosmarinic acid / g of dry extraction matter.

Keywords: rosmarinic acid, optimization, *Melissa officinalis*, extraction

1. Introduction

Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid (PEREIRA *et al*., 2005; PETERSEN, 1996). It has a number of interesting biological activities, e. g. antimicrobial (PAL BAIS *et al*., 2002), antiallergic (ITO *et al*., 1998; MATSUNO *et al*., 2002), antiviral and anti-inflammatory (GEORGIEV *et al*., 2006; HUANG *et al*., 2006; KAMATOU *et al*., 2005). Rosmarinic acid is commonly found in species of the *Boraginaceae* and *Lamiaceae*. The plants with rosmarinic acid content were used in traditional medicine. Today, plant extracts found some application in food and cosmetic industry.

The aim of the present study was to evaluate the effect of changing solvent polarity characterisation using dielectric constant, liquid to solid ratio, temperature and time of extraction on the rosmarinic acid yield in extracts.

2. Material and methods

2.1 Plant material

Adult leaves of *Melissa officinalis* were harvested with the twigs and dried at 40°C. Thereafter, the leaves were separated from the twigs and cut in small squares of 1 – 1.5 cm^2. This extraction material was stored in powder flask at laboratory temperature.

2.2 Extraction procedures

In all experiments, the extractions were done in experimental microtubes and stopped by a rapid decantation of extracts. The impact of solvent composition on the rosmarinic acid content from propanol, ethanol, methanol and their water solution (33 and 66 %; v/v) to water were explored using extraction by solid-liquid ratio 1:30 (w/v), at 40 °C during 1 hour. The influence of the solid-liquid ratios on the extraction was investigated, by considering four ratios (1:10, 1:20, 1:30, 1:40; w/v) with 33 % (v/v) water solution of propan-2-ol as extraction solvent. A kinetic study was performed to select reach an asymptotic region in the extraction. The axial (40 and 80 °C) and central (60 °C) temperatures were used in this kinetic study. The solid-liquid ratio was 1:100 and extraction solvent was again 33 % (v/v) water solution of propan-2-ol.

2.3 Experimental design for the response surface methodology (RSM)

The RSM used a three-factor and central composite design in three blocks consisting of 17 experimental runs with three replicated at the centre point. The design variables were the solid-liquid ratio (0.019 – 0.099 g/ml; X_1), the propan-2-ol proportion (propan-2-ol: water, 1 – 95 %, v/v; X_2) and the temperature (20 – 80 °C; X_3). The variables particle size (< 0.5 mm) and extraction time (1 hour) were kept at constant values.

Experimental data were fitted to the following second-order polynomial model (Eq. 1) and regression coefficients were obtained.

$$Y = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ii} X_i^2 + \sum_{\substack{i=1 \\ i<j}}^{k-1} \sum_{j=2}^{k} b_{ij} X_i X_j \qquad (1)$$

where X_1, X_2, ..., X_k are the independent variables affecting the response Y; b_0, b_i (i = 1, 2,.., k), b_{ii} (i = 1, 2, ..., k) and b_{ij} (i = 1, 2,...k − 1; j = 2, 3,..., k) are regression coefficients for intercept, linear, quadratic, and interaction terms, respectively; k is the number of variables (SILVA *et al.*, 2007).

The optimum conditions of rosmarinic acid extraction were looked by using Statgraphic, 5.1. The verification of the validity and adequacy of the predictive extraction model was carried out in these optimum conditions of solid-liquid ratio, solvent composition and extraction temperature. Three experimental replicates were performed at the optimized conditions and the experimental and predicted values were compared.

2.4 Determination of rosmarinic acid content

The rosmarinic acid content in prepared extracts was determined by HPLC analysis using Purospher Star RP18e, 250 x 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 %, with detection at 325 nm. Final

rosmarinic acid content was expressed as mg of rosmarinic acid per g of dry material (DM).

3. Results and discussion

3.1 Selection of optimized extraction parameters

Extraction efficiency of natural compounds is impacted by multiple parameters such as temperature, time and solvent polarity, among others, and their effects may be either independent or interactive. At the beginning of this study, the factors extraction solvent, solid-liquid ratio, temperature and time of extraction were investigated to determination the appropriate experimental ranges to be considered during the optimization process.

As stated in the literature, for preparation of crude extracts with rosmarinic acid content, authors used methanol, ethanol, acetone and their water solutions (AKOWUAH *et al.*, 2005; ALMELA *et al.*, 2006; AL *et al.*, 1994; ZELIĆ *et al.*, 2005). The best of rosmarinic acid yields was reached with 50 % (v/v) water solution of methanol or ethanol. Yield of extracted compounds is dependent on their solubility in solvent, which dependents on polarity of solvent. For the evaluation of solvent polarity the dielectric constant is used. Dependence of rosmarnic acid yields on dielectric constant (Fig. 1) was developed on the base evaluation extraction yields using methanol, ethanol, propan-2-ol and their water solutions (33 and 66 %; v/v) as extraction solvents. Dielectric constants were calculated according to Equation 2:

$$\varepsilon_{watersolution} = \varepsilon_{water} * v_{water} + \varepsilon_{alcohol} * v_{alcohol} , \qquad (2)$$

where ε is dielectric constant of extraction solvents and v is volume coefficient of resulting solution used as extraction solvent.
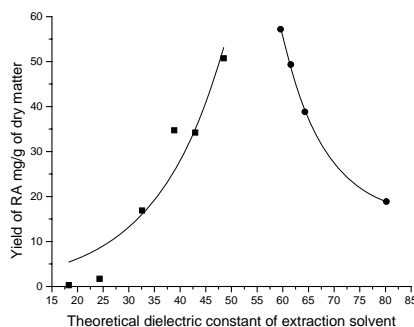

Fig. 1. Dependence of rosmarinic acid yield on theoretical dielectric constant of extraction solvent.

Results indicated that the biggest rosmarinic acid yields were obtained using solvent with dielectric constant in the range from 48 to 59, which represented 33 – 47 % water solution of propan-2-ol. Therefore, one of optimized parameter was concentration of propan-2-ol in water solution used as extraction solvent. Dependence

of isolated compound yields on theoretical dielectric constant of extraction solvent is well-know from literature. Similar course of this dependence was observed at various natural compounds such as antibiotics (BORA and DUTTA, 2000) or pigments from paprika (KISS *et al.*, 2000).

The impact of the solid-to-liquid ratio on the extraction of rosmarinic acid from *M. officinalis* was conducted with four ratios (1:10, 1:20, 1:30 and 1:40; w/v) during 1 hour at 80 °C with 33 % (v/v) water solution of propan-2-ol as extraction solvent. The results of the one-way analysis of variance showed that there was a significant difference among the ratios studied. There was no statistically significant difference between the ratios 1:30 and 1:40 (w/v). The solid-liquid ratio optimal for rosmarinic acid extraction is between the ratios 1:20 and 1:30 (w/v). This survey is consonant with literature (AKOWUAH *et al.*, 2005; GEORGIEV *et al.*, 2006; BASKAN *et al.*, 2007).
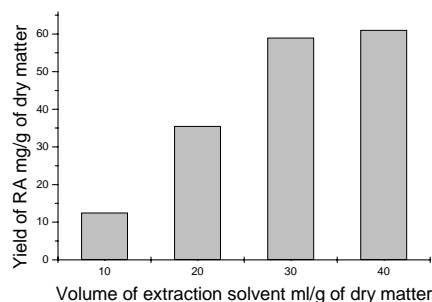

Fig. 2. Effect of the solid-liquid ratio on the extraction of rosmarinic acid from *M. officinalis* leaves over 1 hour with a 33 % (v/v) water solution of propan-2-ol at 80 °C.
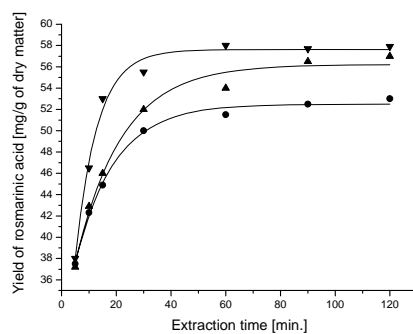

Fig. 3. Kinetics of rosmarinic acid extraction with a 33 % (v/v) water solution of propan-2-ol at various temperatures (● – 40 °C; ▲ – 60 °C; ▼– 80 °C) by solid-liquid ratio 1:100.

From kinetics of rosmarinic acid extraction represented in Fig 3, it is evident, that maximum yield of rosmarinic acid was reached after 60 min of extraction for the three temperatures studied. The best bits were achieved using the exponential model, for the three temperatures and the range of time used, as expressed by the coefficients of determination (R^2) (Tab. 1).

Table 1. Exponential models for the kinetic of rosmarinic acid extraction from *M. officinalis* leaves.

Temperature (°C)	$y = a + b*exp(-x/c)$			
	a	b	c	R^2
40	53.82 ± 0.37	-34.78 ± 3.23	16.76 ± 1.67	0.9752
60	57.34 ± 0.59	-25.75 ± 1.47	18.95 ± 2.46	0.9788
80	58.93 ± 0.54	-34.03 ± 1.06	9.22 ± 1.15	0.9556

3.2 Optimization of extraction by RSM

The rosmarinic acid extraction from *M. officinalis* was optimized through the RSM approach. A fixed extraction time (1 hour) and a fixed particle size (< 0.5 mm) were chosen. Results of rosmarinic acid yields for all runs are reported in Table 2.

Multiple linear regressions using the second-order polynomial model (Eq. 1) were performed in the results of Table 2. The response variability was explained by the model, the coefficient of multiple determination (R^2) was 0.9253.

3.2.1 Analyses of the regression coefficients and the response surface

The regression coefficients of the model for rosmarinic acid yield obtained by the multiple linear regression are reported in Table 3. Variables in their coded form (Table 2) permitted a direct interpretability of effects (linear, quadratic and interaction) of independent variables, and the surface plot (Fig. 4) facilitated the visualisation of the statistically significant factors (denoted by the superscript letters on the regression coefficients of Table 3) derived from the statistical analysis.

Table 2. Experimental results for the response variable (rosmarinic acid yield) and independent variables (X_1, X_2 and X_3) in original and coded form.

Standard order	Factor 1 (X_1)	Factor 2 (X_2)	Factor 3 (X_3)	Factor Y
	Solid-liquid ratio (g/ml DM)	Propan-2-ol (%)	Temperature (°C)	RA yield mg/g DM
1	0.035 (-1)	20 (-1)	32 (-1)	38.71
2	0.035 (-1)	20 (-1)	68 (1)	62.54
3	0.083 (1)	20 (-1)	32 (-1)	36.65
4	0.083 (1)	20 (-1)	68 (1)	33.52
5	0.035 (-1)	76 (1)	32 (-1)	21.04
6	0.035 (-1)	76 (1)	68 (1)	55.98
7	0.083 (1)	76 (1)	32 (-1)	17.88
8	0.083 (1)	76 (1)	68 (1)	30.59
9	0.059 (0)	48 (0)	20 (-1.68)	21.68
10	0.059 (0)	48 (0)	80 (1.68)	55.79
11	0.019 (-1.68)	48 (0)	50 (0)	62.62
12	0.099 (1.68)	48 (0)	50 (0)	17.37
13	0.059 (0)	1 (-1.68)	50 (0)	53.73
14	0.059 (0)	95 (1.68)	50 (0)	0.5
15	0.059 (0)	48 (0)	50 (0)	62.62
16	0.059 (0)	48 (0)	50 (0)	63.5
17	0.059 (0)	48 (0)	50 (0)	65.34

Table 3. Regression coefficients of second-order model for dependent variable – rosmarinic acid yield.

	Model parameters	RA yield
Intercept		-89.391
Linear	Temperature	**3.52489**
	Solid-liquid ratio	**1837.34**
	Propan-2-ol	**0.78097**
Quadratic	Temperature x temperature	**-0.02493**
	Solid-liquid ratio x solid-liquid ratio	**-13245.3**
	Propan-2-ol x propan-2-ol	**-0.01559**
Interaction	Temperature x solid-liquid ratio	-14.2332
	Temperature x propan-2-ol	0.00668
	Solid-liquid ratio x propan-2-ol	0.47061

Regression coefficients with a statistically significant at $p < 0.05$ are printed in bold.

As indicated by the p value, regarding the propan-2-ol proportion (X_2), negative linear and quadratic effects were verified to be statically significant for rosmarinic acid yield. This result indicated that rosmarinic acid yield increases with decreasing of propan-2-ol proportion but quadratic effect of this variable parameter suggested forming of specific area whit maximal yield of rosmarinic acid. These results supported by results in first part of this study (an appropriate extraction solvent with dielectric constant between 48 and 55) and results of another study (DURLING *et al.*, 2007). Negative linear and quadratic effects of solid-liquid ratio (X_1) suggested that the increase of extraction solvent volume to extraction material mass improves the rosmarinic acid yield. Positive effect of temperature (X_3) was detected, which confirms that the increase in temperature improves the rosmarinic acid yield. Indeed, a higher temperature increases the solubility and diffusion coefficient of rosmarinic acid, allowing higher extraction rate.
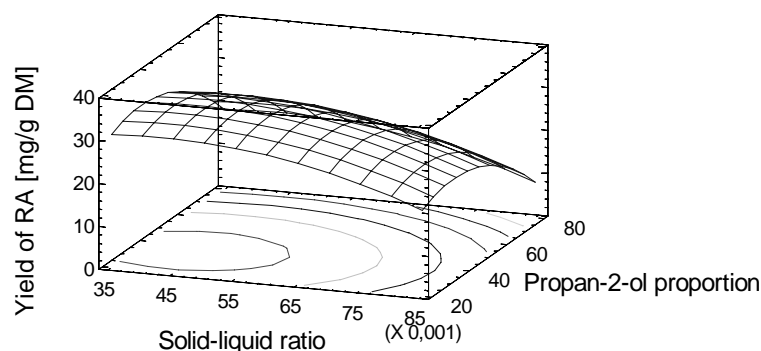

Fig. 4. Response surface plot for yield of rosmarinic acid, in function solid-to-liquid ratio and proportion of propan-2-ol in extraction solvent at 50 °C.

3.3 Determination and experimental validation of the optimal conditions

In order to verify the predictive capacity of the model, an optimum condition was determined using the simplex method and the maximum desirability for the rosmarinic

acid yield, and it was used for an extraction test (Table 4). The measured values lay within a 95 % mean confidence interval of the predicted value for rosmarinic acid yields. These results confirm the predictability of the model for the extraction of rosmarinic acid from *M. officinalis* leaves in the experimental condition used.

Table 4. Comparison of predicted and experimental values at optimal conditions of rosmarinic acid extraction.

Optimized conditions	
Propan-2-ol	39
Temperature [°C]	66
Solid-liquid ratio	0.035
Predicted value of rosmarinic acid yield [mg/g]	74.4 ± 2.59
Experimental value of rosmarinic acid yield [mg/g]	72.6 ± 3.12

4. Conclusions

The response surface methodology was successfully employed to optimize the rosmarinic acid extraction from *M. officinalis* leaves. The second-order polynomial model gave a satisfactory description of the experimental data. An optimized condition for maximum extraction of rosmarinic acid was determined. The optimal conditions were − propan-2-ol proportion 39 %, extraction temperature 66 °C and solid-liquid ratio 1:29 (w/v). This study can by useful for the development of industrial extraction processes, including further studies concerning the optimal number of sequential steps to enhance the efficiency of a large-scale extraction system.

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