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Analytical Methods

Response surface optimised extraction and chromatographic purification of rosmarinic acid from *Melissa officinalis* leaves

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ABSTRACT

The extraction of lemon balm (*Melissa officinalis*) leaves with aqueous methanol was optimised using response surface methodology. Fifteen runs were conducted following a Box-Behnken design (BBD) followed by ridge analysis using the concentration of methanol, the extraction temperature and time as the independent variables and taking the extraction yield of RA from lemon balm as the response variable. The optimal extraction conditions were a methanol concentration of 59.0% (v/v), a temperature of 54.8 °C and a time of 64.8 min, which gave a maximal RA yield of 46.1 mg RA/g dry materials. The RA extract was loaded onto a column packed with Sephadex LH-20 and then was eluted with 100% methanol, which resulted in RA with a purity of 38.8% and a yield of 43.8%. The purity of RA increased by 3.1-fold when compared to its initial purity in the extract obtained from extraction.

1. Introduction

Melissa officinalis (lemon balm) is a perennial herb that belong to the family *Lamiaceae* and is found throughout East Asia. *M. officinalis* has long been known as an effective medicine for the treatment of headache, rheumatism, hypersensitivities, and digestion disorder, as well as for its sedative properties (Reiter & Brandt, 1985; Tagashira & Ohtake, 1998). Furthermore, *M. officinalis* is used to treat Graves', Alzheimer's and thyroid diseases (Auf'mkolk, Ingbar, Kubota, Amir, & Ingbar, 1985; Auf'mkolk, Köhrle, Gumbinger, Winterhoff, & Hesch, 1984; Perry, Pickering, Wang, Houghton, & Perry, 1998). Lemon balm contains various potentially active compounds including phenolic acids (Caniova & Brandsteterova, 2001), which are widely known antioxidant. Phenolic acids are known to possess many physiological activities, including antibacterial, antiviral, and anti-fungal effects, as well as to stimulate the immune and blood circulatory systems (Dimitrova et al., 1993; Grange & Davey, 1990; Nikitina, Kuz'mina, Melent'ev, & Shendel', 2007; Potenza et al., 2007; Viollon & Chaumont, 1994).

In lemon balm, protocatechuic acid, caffeic acid and rosmarinic acid are representative phenolic acids. Protocatechuic acid has anti-inflammatory (Liu, Wang, Chu, Cheng, & Tseng, 2002) and anti-tumour (Tseng et al., 1998) activities. Caffeic acid has anti-tumour (Chlabicz, Paszkiewicz-Gadek, Grochowska, & Galasinksi, 1991) and anti-fungal activities (Ravn, Andary, Kovacs, & Molgaard, 1989). Rosmarinic acid (RA) has antioxidant (Erkan, Ayranci, & Ayranci, 2008; Lopez-Arnaldos, Zapata, Calderon, & Barcelo, 1997; Tepe, 2008), anti-allergic and immunosuppressive effects (Tanaka, Kojima, Suzui, & Mori, 1993; Yun et al., 2003). Therefore, the antioxidative activity of polyphenolic acids from lemon balm has recently been the subject of many studies (Capecka, Mareczek, & Leja, 2005; Triantaphyllou, Blekas, & Boskou, 2001).

To utilise lemon balm as a natural functional food material, effective methods of extracting and purifying phenolic acids are required. Aqueous organic solvents are predominantly used in extraction of not only RA but also other polyphenolic acids from plant materials (Wang, Provan, & Helliwell, 2004; Yilmaz & Toledo, 2004) since other alternative methods such as steam distillation and supercritical carbon dioxide (SC-CO_2) have drawbacks. Steam distillation can cause oxidation of polyphenolic acids due to the high operating temperature (Ammann, Hinz, Addleman, Wai, & Wenclawiak, 1998; Seidel, 2006), and SC-CO_2 can hardly extract polyphenolic acids since SC-CO_2 is strongly hydrophobic (Kim, Kim, Kim, Oh, & Lee, 2008; Park et al., 2007). Liquid membrane was used to selectively concentrate RA from aqueous extract of lemon balm (Boyadzhiev & Dimitrova, 2006). In another study, lemon balm was extracted with water and the aqueous extract

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was further processed through many steps including precipitation using hydrochloric acid and extraction using diisopropylether and drying for crystallization (Christ & Kesselring, 1981).

RA, which is the predominant phenolic acid found in lemon balm, was extracted from *Coleus aromaticus* and purified using a Sephadex LH-20 with water as the eluant and then concentrated in methanol and evaluated for its antioxidant activity (Kumaran & Karunakaran, 2007). In addition, RA extracted from *Agastache rugosa* was purified by Sephadex LH-20 and its inhibitory effects against human immunodeficiency virus were evaluated (Kim, Lee, Shin, & Huh, 1999). However, these studies were intended to identify the active component or to investigate its biological function; therefore, process optimisation and quantitative chromatographic behaviour studies were not conducted. Accordingly, this study was conducted to optimise the extraction process of RA using response surface methodology (RSM) by evaluating the effects of concentration of methanol, extraction time and extraction temperature as independent variables. Following the extraction process, the chromatographic purification of RA in the extract was also studied under a variety of conditions.

2. Materials and methods

2.1. Materials

Lemon balm leaves were harvested from Korea University Arboretum (Seoul, Korea). The leaves were washed with water, after which they were dried in a vacuum-drying oven at 50 °C for 3–4 weeks. The dried lemon balm leaves with a final moisture content of 4.34% (w/w) were then ground to be able to pass through a sieve with mesh size of 250 μm using a cutting mill (IKA, Staufen, Germany), after which they were stored at −70 °C until use. All chemicals used in this study, including rosmarinic acid (purity > 97.0%), were obtained from Sigma (St. Louis, MO, USA), except for the methanol used as the extraction solvent, which was obtained from TEDIA (HPLC grade, Fairfield, USA).

2.2. Extraction of lemon balm

Because methanol is known to be an effective solvent for extracting polyphenolic acids from plants (Amakura, Okada, Tsuji, & Tonogai, 2000; Brolis et al., 1998; Gerothanassis et al., 1998; Zgorka & Kawka, 2000), lemon balm was extracted with methanol in this study. Briefly, 1 g of ground sample was placed in an Erlenmeyer flask, after which 20 ml of aqueous methanol solution was added to yield a ratio of dry weight of plant materials (g) to volume of solvent (ml) at 1:20. The extraction was then conducted by shaking the mixture in a water bath at 100 rpm for varying extraction times and temperatures. The extraction of RA from the lemon balm was then optimised by taking the concentration of the methanol, the extraction temperature and the extraction time as the variables for RSM as described later. After extraction, the extract was filtered with filter paper (110 mm, No. 2, Whatman, Brentford, UK) and then centrifuged for 10 min at 13,000 rpm using a microcentrifuge (Hanil, Seoul, Korea). The centrifugate was then filtered through a 0.45-μm syringe filter (hydrophilic PTFE, Advantec, Dublin, CA, USA) and transferred into a vial for HPLC analysis. The entire extraction procedure was performed in triplicate for each extraction condition.

2.3. HPLC analysis of RA

An Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a Hypersil ODS column (5 μm, 4.6 × 100 mm, Thermo Electron, Bellefonte, PA, USA) was used to analyse the RA. Twenty microlitres of the extract were injected into the HPLC, which was operated with two mobile phases, A (100% (v/v) methanol) and B (0.5% (v/v) acetic acid in water) at 25 °C. The gradient for mixing the mobile phases A and B was programmed as follows: for 0–10 min, 5% of A and then 5–75% A for 10–40 min. The flow rate of the mixed mobile phase was 1.0 ml min^{-1}. The concentration of RA was monitored using a UV/Vis detector at 280 nm.

2.4. RSM for extraction of RA

Optimisation of the extraction of RA from lemon balm using an aqueous methanol solution was conducted using RSM. Briefly, 15 experimental runs were conducted with three independent variables and three levels were developed according to the Box-Behnken design (BBD) as shown in Tables 1 and 2. The independent variables were X_1, the concentration of methanol (%, v/v), X_2, the extraction temperature (°C), and X_3, the extraction time (min), while the response variable was the amount of RA extracted from the lemon balm.

Data were analysed using the response surface regression (RSREG) procedure of the Statistical Analysis System (SAS, Version 8.2, SAS Institute, Cary, NC, USA). The mathematical relationship between the three independent variables and the response surface can be represented by the following second-order polynominal equation (Eq. (1)). In this case, the results of BBD analysis revealed a saddle point in the response surface analysis; therefore, the ridge analysis of SAS RSREG was used to predict the ridge of the optimal response.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X^2 + \beta_{33} X_3^2, \quad (1)$$

where Y is the amount of RA, and X_1, X_2 and X_3 are the independent variables for the concentration of methanol, the extraction temperature and the extraction time, respectively. In Eq. (1), β_0 is the

Table 1
Factor levels and design matrix in the Box-Behnken central composite design model and the response values obtained from experimental runs.

Independent variable	Level		
	Low	Middle	High
Methanol concentration (%, v/v)	40	60	80
Extraction temperature (°C)	25	40	55
Extraction time (min)	30	60	90

Table 2
Box-Behnken design for the three independent variables for the extraction of RA from lemon balm using aqueous methanol.

Run	Concentration of methanol (%, v/v)	Temperature (°C)	Time (min)
1	−1	0	−1
2	−1	−1	0
3	−1	+1	0
4	−1	0	+1
5	0	−1	−1
6	0	+1	−1
7	0	0	0
8	0	0	0
9	0	0	0
10	0	−1	+1
11	0	+1	+1
12	+1	0	−1
13	+1	−1	0
14	+1	+1	0
15	+1	0	+1

regression coefficient for the intercept, β_1, β_2, and β_3 are the linear coefficients, β_{12}, β_{13}, and β_{23} are the interactive coefficients and β_{11}, β_{22}, and β_{33} are the quadratic coefficients.

2.5. Elution profile of RA on Sephadex LH-20

To study the elution profile of RA in the chromatographic separation, three chromatographic columns (10 × 250 mm) were filled with 2.5 g of Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) each. Next, 10 ml of 2 mg ml^{-1} RA solution in 10% aqueous methanol (v/v) were loaded into each column at a constant flow rate of 0.2 ml min^{-1}. Elution was then conducted on each RA-loaded column loaded using various concentrations of aqueous methanol (100%, 80% and 60% (v/v)) with volumes in the range 40–50 ml. The concentration of RA in each fraction was determined at 280 nm using a UV/Vis spectrophotometer (Uvmini-1240, Shimadzu, Kyoto, Japan).

2.6. Purification of RA extract by Sephadex LH-20

The extract of RA, which was obtained at the optimised extraction conditions, proceeded to the following purification steps. The extract was then evaporated in a rotary vacuum evaporator (Eyela, Tokyo, Japan) at 30 °C for 5 h to concentrate the extract, after which the concentrated extract was lyophilised using a freeze dryer (Il Shin, Seoul, Korea). One gramme of freeze-dried sample dissolved in 10 ml of 10% (v/v) aqueous methanol solution and 1 ml of the solution was then loaded into a chromatographic column (10 × 250 mm) filled with 2.5 g of Sephadex LH-20 resin at a constant rate of 0.2 ml/min. The sample was then eluted using an aqueous methanol solution with concentrations of 100%, 80% and 60% (v/v). The RA concentration in each fraction was determined by UV/Vis spectrophotometry and HPLC, and the purity and yield were calculated after each step.

3. Results and discussion

3.1. Optimisation of the extraction of RA from lemon balm using RSM

The independent variables for the extraction of RA from lemon balm leaves, which were the concentration of methanol, extraction temperature and extraction time, were optimised using BBD of RSM. Following the experimental design by BBD, 15 runs of the extraction were conducted (Table 3). The extraction yields of RA varied significantly depending on the extraction conditions from 30.78 to 50.74 mg RA/g dry material. Analysis of the experimental data for the extraction yield of RA from lemon balm using SAS produced the following parameters for the mathematical model (Eq. (2)).

$$Y = 47.311 + 0.5736X_1 - 1.2884X_2 - 0.3227X_3 - 0.0047X_1^2 - 0.0009X_1X_2 + 0.0206X_2^2 - 0.0002X_1X_3 + 0.0026X_2X_3 + 0.0022X_3^2 \quad (2)$$

where Y is the yield of RA, and X_1, X_2, and X_3 are the methanol concentration, the extraction temperature, and the extraction time, respectively. The relationship between the extraction yield of RA and the solvent concentration, extraction temperature and time was expressed in the quadratic equation. Although the R^2 value was relatively high (0.8953), ridge analysis was conducted because the predicted stationary point was at the saddle point. In other words, the optimal points were beyond the experimental conditions shown in Table 2. The maximum yield of RA obtained by the ridge analysis was 46.1 mg RA/g dry materials, which was obtained using a methanol concentration of 59.0% (v/v), an extraction temperature of 54.8 °C and an extraction time 64.8 min.

Fig. 1 shows the effects of the extraction temperature and time on the extraction yield of RA when the methanol concentration was fixed at 59.0% (v/v). At this fixed methanol concentration, the maximal RA yield, 46.1 mg RA/g dry materials, was obtained when a temperature of 54.8 °C and a time of 64.8 min were used. In Fig. 1, increasing the extraction temperature or time within the ranges tested in this study led to an increase in the yield of RA. Therefore, the higher temperature and longer extraction time were advantageous to the extraction of RA from lemon balm. The higher mass transfer rate and solubility and the increased extraction time could be the primary factors responsible for these changes.

In Fig. 2, when the extraction temperature was fixed at 54.8 °C, it was revealed that the maximum yield of RA of 46.1 mg RA/g dry materials was achieved when the extraction was conducted with 59.0% methanol for 64.8 min. At all concentrations of methanol, the extraction yield of RA increased as the extraction time in-

Table 3
Extraction yields of RA obtained when RA was extracted from lemon balm using aqueous methanol according to the Box-Behnken design.

Run	Concentration of methanol solution (%, v/v)	Temperature (°C)	Time (min)	Extraction yield (mg RA/g dry materials)
1	40	40	30	35.08 ± 0.27
2	40	25	60	30.78 ± 0.91
3	40	55	60	43.96 ± 1.98
4	40	40	90	36.47 ± 1.17
5	60	25	30	33.56 ± 0.41
6	60	55	30	46.15 ± 1.57
7	60	40	60	34.22 ± 0.47
8	60	40	60	34.19 ± 0.60
9	60	40	60	34.76 ± 0.06
10	60	25	90	33.48 ± 0.25
11	60	55	90	50.74 ± 6.32
12	80	40	30	32.76 ± 0.17
13	80	25	60	30.92 ± 0.09
14	80	55	60	42.98 ± 5.50
15	80	40	90	33.61 ± 0.35

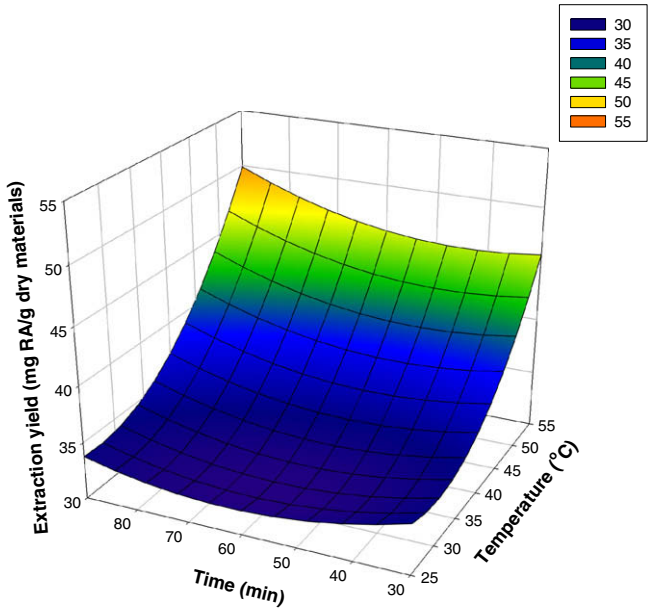

Fig. 1. Three-dimensional response surface plot for the effects of temperature and time on the yield of RA in mg/g dry weight of lemon balm at a fixed methanol concentration of 59.0% (v/v).

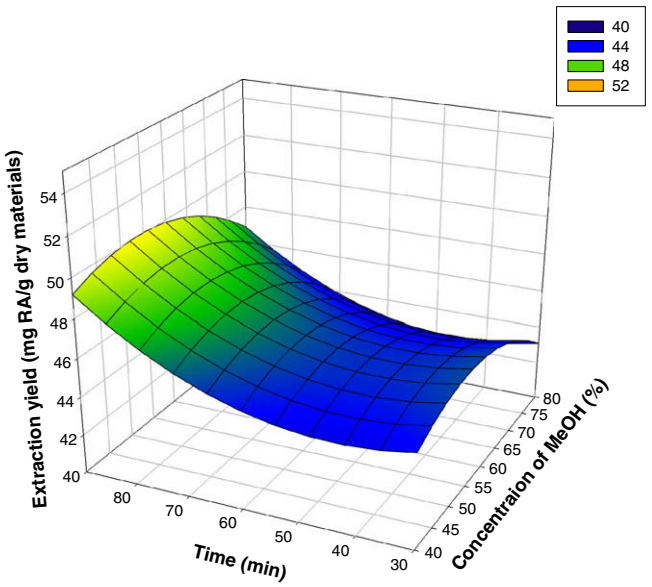

Fig. 2. Three-dimensional response surface plot for the effects of concentration of methanol and time on the yield of RA in mg/g dry weight of lemon balm at a fixed temperature of 54.8 °C.

creased. In addition, at all extraction times, the maximal yield of RA was attained when a methanol concentration of 55–65% was used, while concentrations of methanol greater than 65% caused a decrease in the extraction yield. These results imply that the polarity of the aqueous methanol solution needs be optimised to ensure effective extraction of RA.

As shown in Fig. 3, when a fixed extraction time of 64.8 min was used, the extraction yield of RA increased as the extraction temperature increased, regardless of the concentration of methanol. Although the maximal extraction yield of RA for each extraction time was obtained when the methanol concentration was 55–65%, at the concentration of methanol greater than 65%, the extraction yield of RA decreased. These findings confirmed that there is an optimal aqueous methanol solution for the effective extraction of RA, which is probably due to the polarity of the solvent. As shown in Fig. 3, the maximum extraction of RA (46.1 mg RA/g dry materials) was obtained when the methanol concentration was 59.0% and the temperature was 54.8 °C.

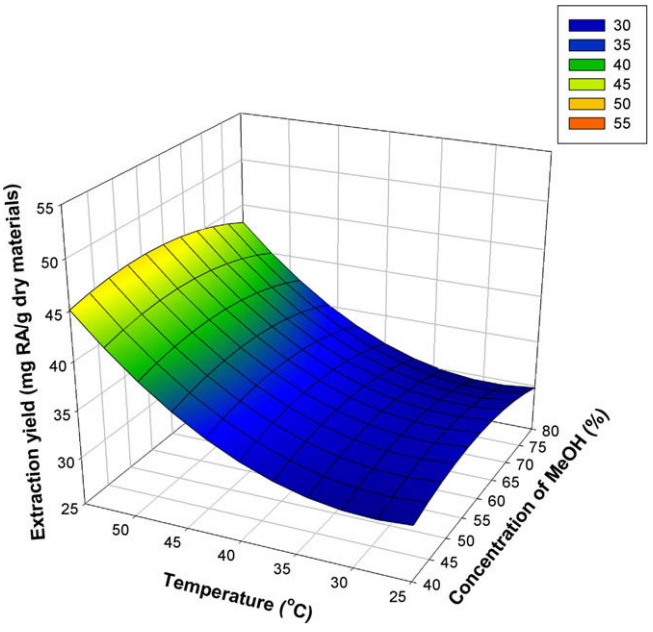

Fig. 3. Three-dimensional response surface plot for the effects of concentration of methanol and temperature on the yield of RA in mg/g dry weight of lemon balm at a fixed extraction time of 64.8 min.

3.2. Elution profile of RA on Sephadex LH-20

Sephadex LH-20 resin is widely used to purify phenolic acids (Nakahara et al., 1993; Rsch, Krumbein, Mgge, & Kroh, 2004; Singleton, Timberlake, & Lea, 1978). In this study, Sephadex LH-20 resin was used to purify RA extracted from lemon balm, and the adsorption and elution behaviours of RA were then evaluated. RA loaded onto the column packed with Sephadex LH-20 resin was eluted using concentrations of aqueous methanol of 60%, 80% and 100% (v/v). The elution profiles differed in response to differing concentrations of methanol (Fig. 4). Although initiation of the elution of RA occurred in the order of 60%, 80% and 100% methanol, finalisation of the elution was conducted in the order of 100%, 80% and 60% methanol. The shape of the RA peak became sharper as the concentration of the methanol solution increased. Therefore, the best resolution of the RA elution was obtained when 100% methanol was used. This may have occurred because the solubility of RA in the 100% methanol solution was greater than the solubility in the 60% and 80% methanol solutions. Georgiev, Kovacheva, Marcheva, and Ilieva (2006) also reported that 80% (v/v) ethanol was a better eluant when using XAD resins than 40% ethanol for obtaining a higher yield of RA from the extract of *Lavandula vera*. As shown in Table 4, the highest recovery yield, 85.5% (w/w), was obtained when 100% methanol was used as an eluant. Conversely, the widest RA peak and lowest recovery yield (74.0%) was obtained when 60% methanol was used. Based on these results, 100% methanol was chosen as the eluant for the elution of RA on Sephadex LH-20.

3.3. Isolation and purification of RA from lemon balm

Plant extract obtained from lemon balm under the optimal extraction conditions determined by RSM was purified by column chromatography using Sephadex LH-20. As shown in Table 5, when the extract containing the RA was filtered, concentrated and freeze-dried to remove the methanol solvent before loading it onto the Sephadex LH-20 column, the purities of the RA extract were

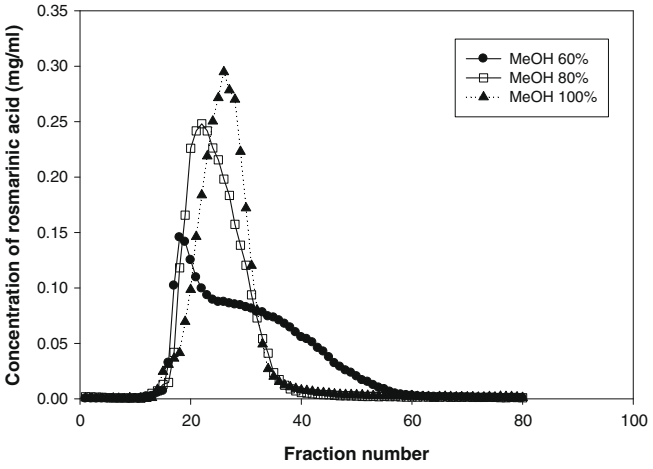

Fig. 4. Elution profile of RA (a total of 20 mg) loaded onto a Sephadex LH-20 resin column and then eluted with aqueous methanol solutions at different concentrations at a constant rate of 0.2 ml min^{-1}.

Table 4
Recovery yield of RA (total of 20 mg) loaded onto a Sephadex LH-20 resin column and then eluted with aqueous methanol solutions at different concentrations.

Solvent	Recovery yield of RA (%, w/w)
MeOH 60% (v/v)	74.0
MeOH 80% (v/v)	81.8
MeOH 100% (v/v)	85.5

Table 5
Purification of RA from lemon balm extract in Sephadex LH-20 resin using various solutions containing different concentrations of methanol as eluents.

	Purity (%)	Yield of RA (%)
Extraction and filtration	12.7	100.0
Evaporation	13.5	98.7
Freeze drying	11.0	80.3
Sephadex LH-20 eluted with 100% methanol	38.8	43.8
Sephadex LH-20 eluted with 80% methanol	27.8	52.3
Sephadex LH-20 eluted with 60% methanol	21.4	48.4

12.7%, 13.5% and 11.0% (w/w), respectively. The decrease in the purity of RA after the freeze-drying step was probably due to the loss of RA under the vacuum conditions in the freeze dryer. The freeze-dried extract was loaded onto a chromatographic column packed with Sephadex LH-20 to purify the RA and then eluted using 60%, 80% and 100% (v/v) aqueous methanol. The purity of RA in the extract increased as the concentration of methanol in the eluents increased, with the highest purity of RA, 38.8%, being obtained when 100% methanol was used. These results agree well with the elution profiles of RA in the Sephadex LH-20 column, in which the RA peak with the highest resolution was obtained when 100% methanol was used as the eluant. After purification with Sephadex LH-20, the purity increased 3.1-fold but the recovery yield decreased by 43.8%.

4. Conclusion

The extraction of lemon balm (*M. officinalis*) with aqueous methanol was optimised by RSM by using the concentration of methanol, the extraction temperature and the extraction time as independent variables. The optimal extraction conditions obtained by ridge analysis were a methanol concentration of 59.0% (v/v), an extraction temperature of 54.8 °C and an extraction time of 64.8 min, and the resulting maximal yield of RA was 46.1 mg RA/g dry materials. When the RA extract was purified using column chromatography and Sephadex LH-20 resin with 100% methanol as the eluant, the purity and recovery yield of RA were 38.8% and 43.8%, respectively.

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