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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