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Optimization of high ultrasound-assisted extraction (INEFU) of active components from natural materials by response HPLC-PDA analysis

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High ultrasonic-assisted extraction technology (INEFU) was employed to extract the active components from natural materials (green coffee beans, *Citrus madurensis, Centella asiatica, Laminaria Japonica*). The extraction conditions were optimized by a response surface method and Box-Behnken design. The active component yields were obtained under the optimum parameters: ultrasound power (1800 watts), ultrasonication time, and particle size. After INEFU of natural materials (green coffee beans, *Citrus madurensis, Centella asiatica* and *Laminaria Japonica*) the products were analyzed with high performance liquid chromatography (HPLC). HPLC analysis showed that the four natural materials were composed of different combinations of vitamin C, polyphenols, chlorogenic acid, caffein, caffeic acid, asiaticoside and alginic acid. In addition, the INEFU results showed that natural materials can yield more active components during a simulated extraction process.

Keywords: INEFU, Natural materials, Green coffee beans, *Citrus madurensis, Centella asiatica, Laminaria Japonica,* HPL

INTRODUCTION

Currently, ultrasound-assisted extraction has been applied to effectively extract natural components from different kinds of materials [1,2]. Compared with the conventional hot water extraction method, ultrasound-assisted extraction can enhance the extraction yield, save operation time and streamline the operation process [3]. Response surface method is a practical statistical method used for optimizing processing parameters [4]. The major advantage of Response surface method is that it can reduce the number of experimental groups and investigate the relation between the response and variables [5]. Therefore, Response surface method has been widely applied to accelerate and optimize the operation process for saving time, energy, and raw materials [6]. Green coffee beans have been commonly used in functional foods or medicines, which was ascribed to the valuable functional components [7]. Green coffee beans are rich in caffeins, polyphenols, and trace elements [8]. Phenols are a class of plant compounds with the potential to eliminate free radicals because of their stable structure after free-radical capture as hydroxycinnamic acid, with chlorogenic acid and caffeic acid being the most abundant in coffee [9,10].

Moreover, these compounds, found mainly in green coffee, are important and biologically active dietary polyphenols. [11]. Calamondin orange (*Citrus madurensis, Citrofortunella microcarpa)*, which belongs to *Citrus Metacitrus Pseudofortunella,* ranges from tropical to subtropical areas, including China, Philippines, Central America, Japan, and Hawaii [12]. Calamondin orange contains vitamin C, including tangeretin, and sinensetin, in large quantities in the peel part of this citrus orange [13]. Pure juice extracted from calamondin orange resembles the juice from shiikuwasha in color and flavor [14,15]. Moreover, the adulteration of fruit juices has been a serious economic problem. This problem has been detrimental to consumers and the food industry for many years [16]. *Centella asiatica* has active compounds such as pentacyclic triterpenes and has been used in treating small wounds, burns, psoriasis, and scleroderma [17]. *Centella asiatica* is also called guta kola. The mechanism of guta kola action involves increasing fibroblast production, synthesis of collagen and fibronectin content [18]. Brinkhaus *et al.* reported that application of *Centella asiatica* showed a beneficial effect on reducing the progression of cellulite, with significant improvement in 85% of the participants with no adverse reactions [19]. Moreover, researchers reported that it can improve the tensile strength of newly formed skin and reduces the inflammatory phase of hypertrophic scars and keloids. *Laminaria Japonica*, also called kelp in Chinese medicines, is a kind of large marine plant [20]. *L. japonica*, a brown alga, is very popular in East Asia due to its unique flavor, taste and biological activities, in which polysaccharides are the major active components, e.g. alginates,

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fucoidan, and laminarian [21]. By comparing 4 types of edible seaweeds, it is found that *L. japonica* has potential anti-diabetic and anti-inflammatory effects in diet-induced obesity [22,23]. Some recent studies showed that *L. japonica* has many beneficial effects to the human health, such as antiviral, anticoagulant, anticancer, hypolipidemic, anti-inflammatory and immunomodulatory activities [24].

In this paper, high ultrasonic-assisted extraction technology (INEFU) of the natural active components of four natural materials was investigated. The effects of four variables (extraction time, extraction temperature, ultrasonic power, and liquid-to-solid ratio) on the yields of four natural materials were optimized by Response surface method. After HPLC-PDA analysis of green coffee beans, *Citrus madurensis*, *Centella asiatica*, and *Laminaria Japonica*, INEFU was applied to accelerate and optimize the operation process for saving time, energy, and raw materials

MATERIALS AND METHODS

Materials

Green coffee beans, *Citrus madurensis*, *Centella asiatica* and *Laminaria Japonica* were purchased from G-market (Gmarket, South Korea). Before extraction, the green coffee beans, *Citrus madurensis*, *Centella asiatica* and *Laminaria Japonica* were dried in a forced convection oven dry box (Jeio Co., Ltd., South Korea) at 60 °C, ground into powder in a universal high-speed smashing machine (Jeio Ltd., South Korea), and screened through a 80 mesh sieve. Dry powder of Green coffee bean, *Citrus madurensis*, *Centella asiatica* and *Laminaria Japonica* was stored for further natural active components extraction. Analytical standards of different compounds (vitamin C, polyphenol mixer, chlorogenic acid, caffein, caffeic acid, asiaticoside, and alginic acid) were purchased from Sigma Co. (USA). HPLC solvents and chemicals were purchased from Daejungchem Ltd. (Shiheung, South Korea). All other reagents were of analytical grade.

High ultrasonic and heat-assisted extraction of the active components

Active component extraction with high ultrasonic assisted extraction was performed in a high ultrasonic cartriage (HUC, Classys Co., Ltd., Seoul, South Korea). The dandelion powder (5.0 g) was placed into a beaker, soaked with deionized water at a ratio of 1:20. The pH value was adjusted to 5 (± 0.05). Then the mixture was placed in a high ultrasonic cartridge (INEFU: high ultrasonic cartridge power ranging from 1800 to 2000 watts) for a certain time (extraction time 42 min) at 35 °C, and heat-assisted extraction (HAE) for a certain time (extraction time 42 min) at 60 °C. The extract was concentrated and then precipitated with 80% (*v*/*v*) ethanol. The precipitate was collected by centrifugation (3000 rpm for 5 min) and washed by anhydrous ethanol and acetone. Then the crude active components were prepared after freeze drying process. The extraction yield of crude active components *Y* (%) was calculated according to the following equation:

$$Y(\%)=((c \times V)/m) \times 100$$

where *c* (mg/mL) represents the concentration of the active components solution, *V* (mL) is the volume of solution, and *m* (mg) is the mass of powder.

HPLC

HPLC instrument such as ultraviolet-PDA detector used a Shimadzu LC-6AD pump with DGU-20A5 degasser, communication module-20A, and PDA detector SPD-M20A with FRC-10A fraction collector (Kyoto, Japan).

Vitamin C: The HPLC was set at isocratic method at 254 nm with a reverse-phase ODS C18 column (LiChrospher® 100 RP-18; diameter, 5 μm) under a 20°C-controlled column chamber. Rheodyne® sample loops of 50 μL capacity were used. The mobile phase was 0.05M KH_2PO_4/ ACN (60:40) at pH 6.8. The flow rate of injection into the system of HPLC was 1 mL/min.

Polyphenol: The HPLC was set at 280 nm with a reverse-phase ODS C18 column (LiChrospher® 100 RP-18; diameter, 5 μm) under a 30°C-controlled column chamber. Rheodyne® sample loops of 20 μL capacity were used. The mobile phase consisted of 0.1% acetic acid (A) and 0.1% acetic acid in acetonitrile (B) filtered through a membrane filter (0.2 μm) prior to use. A gradient program starting at A:B (89:5, v/v) and ending at A:B (65:35, v/v) was applied over 60 min at a flow rate of injection into the system of HPLC of 1 mL/min.

Chlorogenic acid, caffein, caffeic acid: The HPLC was set at isocratic method at 280 nm with a reverse-phase ODS C18 column (LiChrospher® 100 RP-18; diameter, 5 μm) under a 20°C-controlled column chamber. Rheodyne® sample loops of 20 μL capacity were used. The mobile phase was MeOH/ 5 mM KH_2PO_4 (pH 2.5, H_3PO_4) (30:70). The flow rate of injection into the system of HPLC was 0.4 mL/min.

Asiaticoside: The HPLC was set at Photo diode array detector/UV detector with a reverse-phase ODS C18 column (LiChrospher® 100 RP-18; diameter, 5 μm) under a 30°C-controlled column chamber. Rheodyne® sample loops of 20 μL capacity were used. The mobile phase consisted of 0.3% orthophosphoric acid (A)/ acetonitrile (B) filtered through a membrane filter (0.2 μm) prior to use. A gradient program starting at A:B (95:5, v/v) and ending at A:B (5:5, v/v) was applied over 40 min at a flow rate of injection of 1.8 mL/min into the system of HPLC.

Alginic acid: The HPLC was set at Photo diode array detector/UV detector with a reverse-phase ODS C18 column (LiChrospher® 100 RP-18; diameter, 5 μm) under a 30°C-controlled column chamber. Rheodyne® sample loops of 20 μL capacity were used. The mobile phase, consisting of 0.5 mL phosphoric acid in 1-L distilled water adjusted to pH 7.00 with sodium hydroxide, was applied over 30 min at a flow rate of 0.7 mL/min into the system of HPLC.

Data analysis

The ratio of the peak area of the standard (vitamin C, polyphenol mixer, chlorogenic acid, caffein, caffeic acid, asiaticoside, and alginic acid) was used as the assay parameter. Peak area ratios were plotted against analyte concentrations, and standard calibration curves were obtained from least-squares linear regression analysis of the data. The linearity of the method was confirmed *via* evaluation of the calibration y-intercept and correlation coefficients.

Statistical analysis

All data were expressed as mean ± standard deviation (SD) of three separate experiments. Design-Expert software (8.6 Statease Inc., Minneapolis, USA) was used for the experimental design and statistical analysis. Statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

Verification of the INEFU and HAE models

The suitability of the model equation was examined under the modified conditions: high ultrasound power(INEFU) of 1800 W, extraction time of 42 min, extraction temperature of 35°C, and particle size of 80 mesh, and the final mean values obtained from the experiments were 18.11 ± 0.42%, 19.51 ± 0.31%, 20.19 ± 0.12%, 31.23 ± 1.83%, (for green coffee beans, *Citrus madurensis*, *Centella asiatica* and *Laminaria Japonica*, respectively), which demonstrated that the model was suitable and precise for the actual extraction process. As shown in Table 1, different extraction methods including heat-assisted extraction (HAE) were also evaluated for comparison. Under the same extraction time, the active components of INEFU and HAE were 18.11 ± 0.42%, 6.34 ± 0.12% (n=3, green coffee beans), 19.51 ± 0.31%, 8.34 ± 0.14%, (n=3, *Citrus madurensis*), 20.19 ± 0.12%, 9.21 ± 0.91% (n=3, *Centella asiatica*), 31.23 ± 1.83%, 19.24 ± 1.09% (n=3, *Laminaria Japonica*), respectively. Apparently, INEFU was more efficient in comparison with HAE.

Identification and quantification of vitamin C in Citrus madurensis (Citrofortunella microcarpa)

Analytes were tentatively identified in *Citrus madurensis* samples by our combining the information obtained with PDA detectors and by comparison with literature data. When possible, the identification of compounds was confirmed by comparison with standards commercially available. The UV–vis chromatograms of the INEFU and HAE extracts at a wavelength of 254 nm are shown in Fig. 1.

Table 1. Comparison of the active component yields by different extraction methods.

Natural materials	Extraction methods	Extraction conditions			The yield of components (%)
		A	B	C	
Green coffee beans	INEFU	1800	42	35	18.11 ± 0.42%
	HAE	−	42	60	6.34 ± 0.12%
Citrus madurensis	INEFU	1800	42	35	19.51 ± 0.31%
	HAE	−	42	60	8.34 ± 0.14%
Centella asiatica	INEFU	1800	42	35	20.19 ± 0.12%
	HAE	−	42	60	9.21 ± 0.91%
Laminaria Japonica	INEFU	1800	42	35	31.23 ± 1.83%
	HAE	−	42	60	19.24 ± 1.09%

A: Ultrasound power (watt); B: Extraction time (min); C: Extraction temperature (°C).

The INEFU and HAE extracts of *Citrus madurensis* yielded similar qualitative HPLC profiles for each kind of sample. Vitamin C concentration is expressed as number of times for INEFU and HAE extract, and INEFU extracted 27 times more vitamin C than HAE for *Citrus adurensis* samples. *Citrus madurensis* juice usually contains vitamin C, between 3% (for INEFU extracts in *Citrus madurensis*) and 0.2% (for HAE extracts in *Citrus madurensis*) of the total concentration of detected analyte [25]. In addition, HAE extracts contained vitamin C at a very lower percentage than in INEFU extracts (range 0.12~0.23%).

Identification and quantification of polyphenols in green coffee beans

Analytes were tentatively identified in green coffee beans samples by our combining the information obtained with PDA detectors and by comparison with literature data. When possible, the identification of compounds was confirmed by comparison with standards commercially available. The UV–vis chromatograms of the INEFU and HAE extract at a wavelength of 280 nm are shown in Fig. 2. Six phenolic compounds belonging to different classes were found, and six of them were tentatively identified. Among them, the following compounds were identified: sinapic aldehyde, P-coumaric acids, rutin, feruric acid, epicatechin gallate and naringin. The INEFU and HAE extracts of the green coffee beans yielded similar qualitative HPLC profiles for each kind of sample. Polyphenols concentration is expressed as number of times for INEFU and HAE extract, and INEFU extracted 10.7 times (based on the feruric acid) more than HAE for green coffee beans samples. INEFU samples were quantitatively the richest ones in the phenolic compounds studied (medium total concentration of 121.1 mg/g), whereas HAE samples were the poorest ones in the molecules of interest (medium total concentration of 0.5 mg/mL) even though they had a large number of detected compounds.

Identification and quantification of chlorogenic acid, caffein and caffeic acid in green coffee beans

Analytes were tentatively identified in green coffee beans samples by our combining the information obtained with PDA detectors and by comparison with literature data. When possible, the identification of compounds was confirmed by comparison with standards commercially available. The UV–vis chromatograms of the INEFU and HAE extracts at a wavelength of 280 nm are shown in Fig. 3. The INEFU and HAE extracts of the green coffee beans yielded similar qualitative HPLC profiles for each kind of sample. Green coffee beans concentration is expressed as number of times for INEFU and HAE extract, and INEFU extracted 2.2 times (based on the caffein), 2.1 times (based on the chlorogenic acid) more than HAE for green coffee beans. Also, caffeic acid was only detected for INEFU. Green coffee beans usually contain caffeine between 0.7 and 1%, So, 80% of caffeine amounts from the green coffee beans can be extracted with INEFU methods. In addition, HAE extract contained caffein and chlorogenic acid at a very lower percentage than the INEFU extract.

Identification and quantification of asiaticoside and madecassoside in Centella asiatica

Analytes were tentatively identified in *Centella asiatica* samples by our combining the information obtained with PDA detectors and by comparison with literature data. When possible, the identification of compounds was confirmed by comparison with standards commercially available. The UV–vis chromatograms of the INEFU and HAE extracts at wavelengths of 254, 280 nm are shown in Fig. 4. The INEFU and HAE extracts of *Centella asiatica* yielded similar qualitative HPLC profiles for each kind of sample. Asiaticoside and madecassoside concentrations are expressed as μg/ml for INEFU and HAE extract, and INEFU extracted 30 μg/ml (madecassoside) 43 μg/ml (asiaticoside) for *Centella asiatica* samples. Asiaticoside and madecassoside can be extracted at 38~50 μg/ml and 28~39 μg/ml when methanol is used as a solvent, when water solvent is hardly extracting. In addition, HAE extract contained asiaticoside and madecassoside at a very lower percentage than INEFU extract.

Identification and quantification of alginic acid in Laminaria Japonica

Analytes were tentatively identified in *Laminaria Japonica* samples by our combining the information obtained with PDA detectors and by comparison with literature data. When possible, the identification of compounds was confirmed by comparison with standards commercially available. The UV–vis chromatograms of the INEFU and HAE extracts at a wavelength of 200 nm are shown in Fig. 5. The INEFU and HAE extracts of *Laminaria Japonica* yielded similar qualitative HPLC profiles for each kind of sample. Alginic acid concentration is expressed as number of times for INEFU and HAE extract, and INEFU extracted 1.72 times more than HAE for *Laminaria Japonica* samples. *Laminaria Japonica* juice usually mainly contains alginic acid.

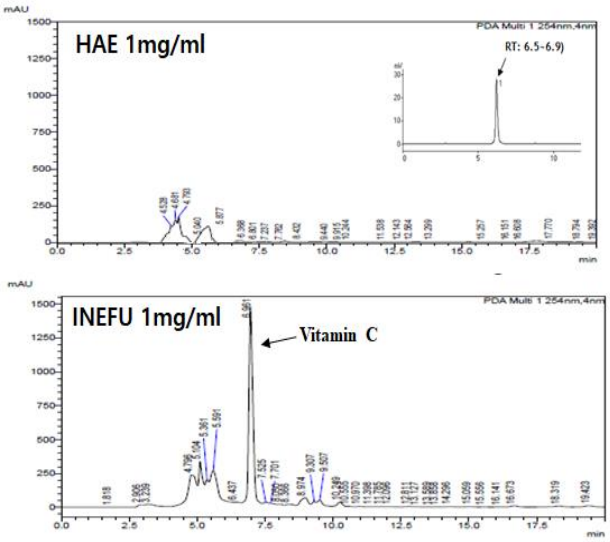

Fig. 1. High-performance liquid chromatography–photodiode array (HPLC–PDA) chromatogram of vitamin C in *Citrus madurensis* (*Citrofortunella microcarpa*).

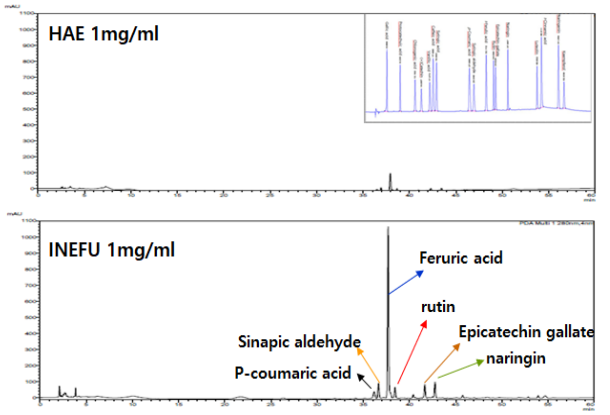

Fig. 2. High-performance liquid chromatography–photodiode array (HPLC–PDA) chromatogram of polyphenols in *Citrus madurensis* (*Citrofortunella microcarpa*).

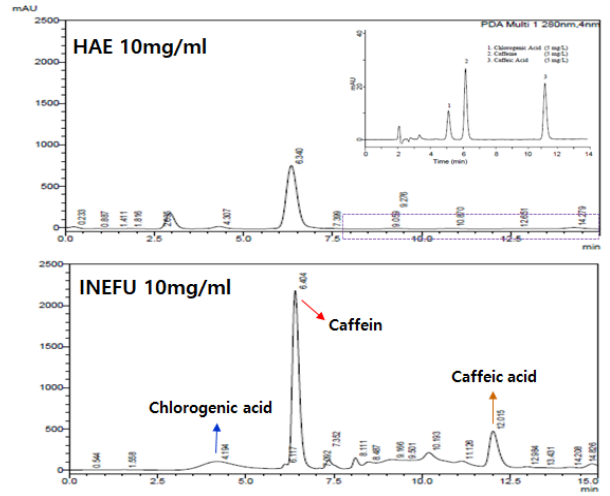

Fig. 3. High-performance liquid chromatography–photodiode array (HPLC–PDA) chromatogram of chlorogenic acid, caffein, caffeic acid in green coffee beans.

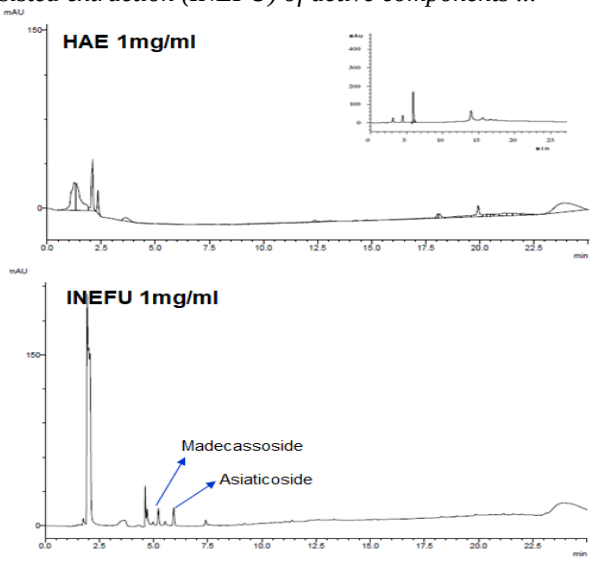

Fig. 4. High-performance liquid chromatography–photodiode array (HPLC–PDA) chromatogram of asiaticoside and madecassoside in *Centella asiatica*.

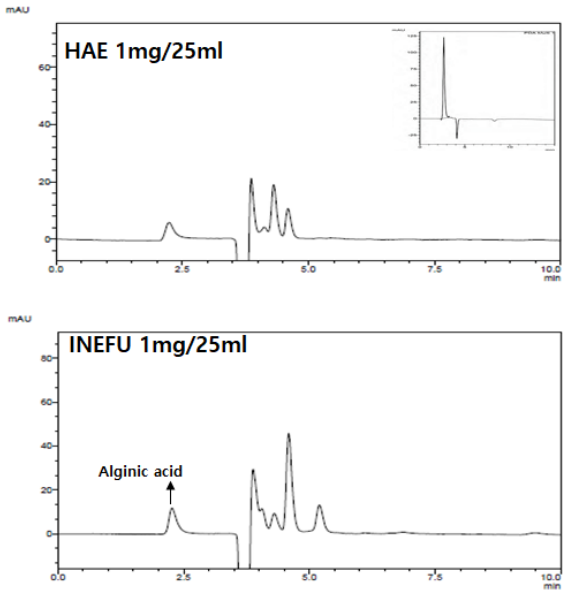

Fig. 5. High-performance liquid chromatography–photodiode array (HPLC–PDA) chromatogram of alginic acid in *Laminaria Japonica*.

CONCLUSION

A method for the analysis of natural materials (green coffee beans, *Citrus madurensis*, *Centella asiatica* and *Laminaria Japonica*) belonging to different classes was developed and validated. The analyses were performed by HPLC–PDA directly after INEFU and HAE extractions of the natural materials and after freeze drying for five important active index components. The method was fully validated and applied to the analysis of the active index components in samples from natural materials varieties and one well-known international cultivar, Wonderful. The method allowed qualitative and quantitative analysis of the principal active index component in the different

extraction methods (INEFU and HAE) of green coffee bean, *Citrus madurensis*, *Centella asiatica* and *Laminaria Japonica*. Differences in active index component profile and concentration can be evidenced, allowing green coffee beans, *Citrus madurensis*, *Centella asiatica* and *Laminaria Japonica* to be distinguished on the basis of the concentration of compounds from INEFU extraction methods.

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