Comparison of Extraction Methods and Extraction Optimization in Aqueous Two-Phase System for Ethyl Carbamate Analysis in Model Systems for Maesil Wine

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ABSTRACT: Ethyl carbamate (EC) is a known carcinogen, and therefore its intake is regulated internationally. The objectives of this study were to compare the EC recovery yields under different liquid-liquid extraction (LLE) conditions and to investigate the optimum conditions of the aqueous two-phase system (ATPS) for EC extraction. Our results showed that for the LLE method, addition of 15% NaCl improved the EC yield by 15%, and dichloromethane as the extraction solvent showed a slightly higher yield (about 5%) than chloroform. However, there was little difference in the yield when mixing was performed using an ultrasonic bath compared to a vortex mixer. Using response surface methodology with central composite design to analyze the ATPS results, optimal extraction was found to occur at 21.5°C for 2.8 h in the sample containing 70% alcohol and 15% phosphate, showing a recovery yield of 75.64%. This information can be applied to alcoholic beverages and other fermented food products to analyze EC with better extraction methods, depending on the types of food.

Keywords: aqueous two-phase system, central composite design, ethyl carbamate, liquid-liquid extraction, response surface methodology

INTRODUCTION

Ethyl carbamate (EC), commonly called urethane, is an ethyl ester of carbamic acid produced during fermentation processes. Today, EC is considered a carcinogenic substance that has the potential to harm the human body (Lachenmeier et al., 2006; Weber and Sharypov, 2009). This is due to the mechanism by which EC is metabolized to vinyl carbamate in the human body. Vinyl carbamate then undergoes epoxidation to form mutagenic DNA adducts (Ough, 1976). According to the International Agency of Research on Cancer, this carcinogenic mechanism affects the human body; therefore, EC was raised from “Group 2B”, which indicates a possible carcinogen, to “Group 2A”, which indicates a suspected human carcinogen (Hong et al., 2007). EC is mainly found in fermented foods, such as cheese, bread, and yogurt, as well as in alcoholic beverages, including wine and whiskey (Weber and Sharypov, 2009; Liao et al., 2013). EC is produced by the reaction of ethanol with urea, which is produced by enzymes present in food as well as from citrulline and carbamoyl phosphate in the urea cycle. Furthermore, cyanogenic glycosides contained in fruit seeds are decomposed into cyanide by enzymatic reactions and then oxidized to cyanate to react with ethanol, thereby generating EC (Hong et al., 2007). As a result of a survey of alcoholic beverages distributed by the Canadian government in 1985, it became the first international issue when it was announced that high EC was detected in alcoholic beverages made from fruit with seeds (Park et al., 2008). Since then, the United States, France, Germany, and the Czech Republic have set EC maximum residual standards for various alcoholic beverages and have promoted EC reduction. The United States Food and Drug Administration applies it as a voluntary standard, but for imported products, limits of 15 parts per billion (ppb) and 60 ppb or less are applied for table wines and fortified wines (Ryu et al., 2015). In Canada and the Czech Republic,
standards of 300 ppb and 400 ppb are applied for wines and fruit wines, respectively. In France, spirits are regulated to less than 150 ppb and fruit brandy to 1,000 ppb, whereas in Germany, only fruit brandy is regulated to less than 800 ppb (Jiao et al., 2014; Qin et al., 2021). In Korea, the maximum allowable EC value of wine (products with an alcohol content of less than 15% made from grapes) is 30 ppb or less (Ryu and Koh, 2015). Previously, EC was detected in 29 out of 218 cases of fermented foods (i.e., Korean soy sauce, kimchi, and salted fish); however, the levels were safe, with an average of 2 ppb, which was lower than that of foreign countries (Hong et al., 2007). However, very high levels of EC were detected in different alcoholic beverages, especially in distilled wines made from stone fruits (apricot, plum, cherry, etc.). Analysis of 33 types of plum wines (domestic and commercially available in Korea) detected 11~379 ppb of EC (Choi et al., 2017).

Currently, the most widely used method for analyzing EC is gas chromatography (GC)/mass spectrometry (MS) after isolating EC by liquid-liquid extraction (LLE) or solid phase extraction (SPE). Recently, some methods with improved accuracy and precision have been used by employing the latest analysis equipment, including high-performance liquid chromatography (HPLC) and GC-tandem mass spectrometry (MS/MS). Separation by LLE and direct injection into GC-MS/MS (Kim et al., 2013a), HPLC with fluorescence detection analysis based on derivation through 9-xanthydrol (Madrera and Valles, 2009), and GC-MS/MS using solid phase micro-extraction (SPME) (Lachenmeier et al., 2006) are representative examples of such methods. HPLC is mainly used for analysis of non-volatile substances, and since the mobile phase is liquid, it is an analytical method applicable to hydrophilic samples. HPLC has the advantages of no restrictions on the volatility or thermal stability of the sample and easy automation; however, it is time-consuming to determine the optimal state compared to GC because it has a great number of conditions to be considered, such as the mixing properties and viscosity of the mobile phase solvent. Conversely, GC has the advantages of a shorter analysis time than HPLC, high sensitivity, and low cost, thus GC is still widely used for analyzing volatile compounds. To analyze EC by GC/MS, a pretreatment to extract EC from the sample is required.

Some of the most well-known extraction methods are SPE using a Chem-Elut column, LLE, SPME, and aqueous two-phase system (ATPS). The method using a Chem-Elut column has high reproducibility but it has disadvantages, such as long extraction time and high cost. LLE has a short extraction time and high reproducibility, but the consumption of organic solvent is very high. SPME has disadvantages of low reproducibility along with low durability and the high cost of the fiber used. Although the extraction time for the ATPS method is lengthy, it is simple to operate and does not use an organic solvent; therefore, it has the advantages of being more cost-effective and environmentally friendly. In addition, if the ethanol and salt concentrations are properly adjusted, the extraction efficiency can be improved up to 90%. In the LLE method, if sodium chloride (NaCl) is added and chloroform is used as the organic solvent, the recovery yield can be increased to some extent, but the maximum value is about 62%. Consequently, the ATPS method is believed to be the most efficient in terms of yield.

Response surface methodology (RSM) is a statistical analysis method that can efficiently determine the optimal conditions in multivariate systems and determine the influence of independent variables on dependent variables (Fan et al., 2008). This method has been successfully applied to find the optimal conditions for extracting specific substances in various food analyses. For example, optimization of supercritical carbon dioxide extraction of carotene contained in carrots (Vega et al., 1996), optimization of anthocyanin extraction conditions in purple sweet potatoes (Fan et al., 2008), and optimization to improve the phenolic content for the antioxidant capacity in fruits (Belwal et al., 2016) were studied using RSM. Many studies have used various extraction methods to isolate EC, but there is no data comparing the EC yields using different LLE experimental methods or ATPS for Maesil wine, a fruit wine made by fermenting Prunus mume with alcohol and sugar. The objectives of this study are to compare the yields of different extraction methods and optimize the ATPS method to determine the optimal EC extraction conditions in model systems for Maesil wine using RSM.

MATERIALS AND METHODS

Materials
EC, butyl carbamate (BC), and dichloromethane were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Chloroform and NaCl were purchased from Samchun Chemical (Seoul, Korea). Dipotassium hydrogen phosphate (K2HPO4) and sodium sulfate (Na2SO4) were purchased from Junsei Chemical (Tokyo, Japan).

Preparation of EC and BC solutions
A stock solution was prepared by dissolving 1,000 parts per million (ppm) EC in an organic solvent (dichloromethane or chloroform). For the internal standard, 1,000 ppm of BC was dissolved in the same organic solvent and diluted to 200 ppm. The stock solution was serially diluted to prepare EC standards with 8 different concentrations (500, 200, 100, 50, 10, 5, 2.5, and 1 ppm) for the standard curve. The samples were then injected into the
GC (6000 series, YoungIn Chromass, Anyang, Korea) with 200 ppm BC.

**Extraction of EC**

**LLE:** After adding 5 mL of BC as an internal standard into 5 mL of the sample containing 20% alcohol, 5 mL of organic solvent (dichloromethane or chloroform) was added. NaCl was then added to a sample for comparison with the sample without added NaCl. Then the sample was shaken vigorously for 10 s using a vortex mixer (GW92VM, Go Won Scientific Technology Co., Seoul, Korea) or 5 min using an ultrasonic bath (PowerSonic 400, Hwashin Technology, Yangsan, Korea). After centrifugation at 10,000 rpm and 4°C for 10 min, 1 mL of the organic layer was extracted using a syringe (J-S-5, Jung Rim Medical Industrial Co., Ltd., Jincheon, Korea). The extract was dried by adding a small amount of anhydrous sodium sulfate and filtering with a 0.45-mL syringe filter (5190-5263, Agilent Technologies Inc., Santa Clara, CA, USA). Finally, a 2-μL sample was removed using a syringe (549597, Hamilton Company, Reno, NV, USA) and injected into the GC-FID, flame ionization detection (6000 series, YoungIn Chromass).

**ATPS:** A 5-μL internal standard of 100 ppm BC was added to 4 mL of the sample, which was adjusted to a concentration of 50% alcohol. Then, 15% K2HPO4 was added to the sample, which was shaken for 10 s using a vortex mixer (GW92VM, Go Won Scientific Technology Co.). The solution was centrifuged at 3,600 rpm for 5 min and then placed in a 25°C water bath for 3.5 h. When the volume of the upper phase was equal to the volume of the lower phase, the phases were considered to be in equilibrium. A 1.5-mL sample of the upper phase was extracted using a syringe (J-S-5, Jung Rim Medical Industrial Co., Ltd.) and mixed with 150 mg of Na2SO4. This was centrifuged at 4,000 rpm for 1 min and then 1 mL of the upper layer was isolated for GC-FID injection.

**Analysis conditions**

The gas chromatograph was an ACME 6000 series (YoungIn Chromass), the column was a fused silica capillary column (SP-2560, 24056, Supelco Inc., Bellefonte, PA, USA), the carrier gas was nitrogen (N2) with a flow rate of 1.5 L/min, and the injection mode was split. The oven temperature was maintained at 150°C for 1 min, then heated at a rate of 15°C/min and maintained at 180°C for 2 min, and finally heated at a rate of 10°C/min and maintained at 250°C for 4 min. The temperatures of the injection and detection ports were set to 210°C and 260°C, respectively.

**Optimization experiments**

The factors affecting the extraction recovery rate of EC were evaluated through the Plackett-Burman design as the first statistical analysis. The Plackett-Burman design was established with 7 factors: alcohol concentration, phosphate concentration, centrifugation speed, centrifugation time, extraction temperature, extraction time, and extraction time after extraction. As the second statistical analysis, RSM was used to investigate the optimum conditions for EC extraction in ATPS. The RSM was designed with 4 factors: alcohol concentration, phosphate concentration, extraction temperature, extraction temperature, and extraction time. With these 4 factors for 5 levels, the central composite design method was established as 27 experimental runs by 16 factorial points, 8 axis points ($\alpha$=2), and 3 central points Table 1. Recovery yield (Y, %) was set as the dependent variable. It was measured twice and the average value was used for analysis. The Plackett-Burman and RSM results were obtained using Minitab ver. 17 (Minitab Inc., State College, PA, USA) and Design Expert (ver. 13, Stat-Ease, Inc., Minneapolis, MN, USA), respectively.

**Statistical analysis**

For data analysis of EC extraction, the Plackett-Burman and RSM results were obtained using Minitab ver. 17 (Minitab Inc., State College, PA, USA) and Design Expert (ver. 13, Stat-Ease, Inc., Minneapolis, MN, USA), respectively.

| Run no. | $X_1$ | $X_2$ | $X_3$ | $X_4$ | Response |
|---------|-------|-------|-------|-------|----------|
| 1       | 70 (+1) | 15 (+1) | 15 (+1) | 5 (+1) | 80.71    |
| 2       | 55 (0)  | 10 (0)  | 22.5 (0) | 3.5 (0) | 74.09    |
| 3       | 55 (0)  | 10 (0)  | 37.5 (+2) | 3.5 (0) | 54.28    |
| 4       | 40 (+1) | 15 (+1) | 15 (+1) | 2 (—1) | 60.36    |
| 5       | 55 (0)  | 10 (0)  | 22.5 (0) | 3.5 (0) | 60.00    |
| 6       | 40 (+1) | 5 (—1)  | 15 (+1) | 5 (+1) | 61.06    |
| 7       | 40 (+1) | 5 (—1)  | 30 (+1) | 2 (—1) | 50.94    |
| 8       | 55 (0)  | 0 (—2)  | 22.5 (0) | 3.5 (0) | 57.93    |
| 9       | 70 (+1) | 5 (—1)  | 30 (+1) | 5 (+1) | 61.57    |
| 10      | 70 (+1) | 15 (+1) | 30 (+1) | 2 (—1) | 62.56    |
| 11      | 70 (+1) | 5 (—1)  | 15 (+1) | 2 (—1) | 61.13    |
| 12      | 85 (+2) | 10 (0)  | 22.5 (0) | 3.5 (0) | 66.94    |
| 13      | 70 (+1) | 15 (+1) | 15 (+1) | 2 (—1) | 67.82    |
| 14      | 40 (+1) | 15 (+1) | 30 (+1) | 5 (+1) | 54.66    |
| 15      | 55 (0)  | 10 (0)  | 22.5 (0) | 0.5 (—2) | 62.63    |
| 16      | 70 (+1) | 15 (+1) | 30 (+1) | 5 (+1) | 68.16    |
| 17      | 55 (0)  | 20 (+2) | 22.5 (0) | 3.5 (0) | 73.74    |
| 18      | 55 (0)  | 10 (0)  | 7.5 (—2) | 3.5 (0) | 59.28    |
| 19      | 70 (+1) | 5 (—1)  | 15 (+1) | 5 (+1) | 81.93    |
| 20      | 70 (+1) | 5 (—1)  | 30 (+1) | 2 (—1) | 67.93    |
| 21      | 55 (0)  | 10 (0)  | 22.5 (0) | 6.5 (—2) | 49.09    |
| 22      | 40 (+1) | 5 (—1)  | 30 (+1) | 5 (+1) | 59.75    |
| 23      | 55 (0)  | 10 (0)  | 22.5 (0) | 3.5 (0) | 75.90    |
| 24      | 40 (+1) | 15 (+1) | 30 (+1) | 2 (—1) | 66.74    |
| 25      | 25 (—2) | 10 (0)  | 22.5 (0) | 3.5 (0) | 54.67    |
| 26      | 40 (+1) | 5 (—1)  | 15 (+1) | 2 (—1) | 46.07    |
| 27      | 40 (+1) | 15 (+1) | 15 (+1) | 5 (+1) | 46.39    |

$X_i$, alcohol concentration (%); $X_2$, K2HPO4 concentration (%); $X_3$, extraction temperature (°C); $X_4$, extraction time (h); response, recovery yield (%).
and RSM data were analyzed using Minitab version 17 (Minitab Inc., State College, PA, USA) and Design Expert (version 13, Stat-Ease, Inc., Minneapolis, MN, USA), respectively. P<0.05 was considered as a significant value.

RESULTS AND DISCUSSION

Comparison of extraction methods for EC measurement in references

Currently, the most widely used methods for extracting EC from alcoholic beverages are SPE, LLE, SPME, and ATPS. Table 2 shows the differences between these four extraction methods. Among them, the method used by the Korean Ministry of Food and Drug Safety to extract EC contained in plum wine is SPE, in which the sample to be measured is passed through a Chem-Elut column and concentrated (Choi and Koh, 2015). This method has high reproducibility, but it is expensive and the analysis is time-consuming. With LLE, phase separation is applied between an organic solvent and the sample. The method has high reproducibility and short analysis time (15 ∼ 30 min), but a large amount of organic solvent is consumed (Kim et al., 2013b). With SPME, the target material is extracted using an SPME fiber. Although this method requires less time, the fiber is highly likely to be damaged due to its poor durability. Furthermore, this method is costly and shows low reproducibility (Ye et al., 2011). ATPS is a method of extracting EC by separating the sample into two liquid phases (water and alcohol) with an excess of phosphate salt, which is added into the sample to saturate it. This method is time-consuming, but it is more environmentally friendly and cost-effective because it is simple to operate and does not require an organic solvent. Furthermore, if the ethanol and salt concentrations are properly adjusted, a maximum extraction efficiency of about 90% or more can be achieved (Ma et al., 2019).

Experimental comparison of extraction methods for EC measurement

Among the various extraction methods, LLE has the shortest analysis time, but its efficiency is low and consumption of organic solvents is very high. Some factors, such as ultrasonic strength for mixing, NaCl concentration, and type of organic solvents, are expected to affect the extraction efficiency of LLE. Table 3 shows a comparison of the recovery yield results of ATPS (Exp. No. IX) and LLE using different conditions (Exp. No. I ∼ VIII).

According to Woo et al. (2001) and Liao et al. (2013), more EC migrates into the organic solvent when the aqueous layer is saturated with NaCl; therefore, we compared the EC recovery rates of LLE with and without NaCl saturation. The maximum yield achieved without the addition of NaCl (Exp. No. I ∼ IV) was 47±4% and the minimum yield was 42±3%. The maximum yield achieved using NaCl (Exp. No. V ∼ VIII) was 62±11%, and the minimum yield was 53±8%. The difference in yield was about 15%, confirming that the addition of NaCl improves the extraction efficiency of EC.

Dichloromethane is generally used as the extraction solvent for LLE (Leça et al., 2014); however, Liao et al.

Table 2. Comparison of extraction methods for analyzing ethyl carbamate in alcoholic beverage

| Variable                      | SPE          | LLE          | ATPS          | SPME          |
|-------------------------------|--------------|--------------|---------------|---------------|
| Sample/solvent quantity       | 10 g/80 mL   | 5 mL/5 mL    | 15 mL         | 20 μL/300 μL acetone, 60 μL TFA |
| Absorbent                     | Chem Elut    | −            | −             | Fiber         |
| Mixer                         | −            | Vortex mixer | Vortex mixer  | −             |
| Concentration method          | Water bath, rotary evaporator | −          | −             | −             |
| Centrifugation setting        | −            | 4°C, 10,000 rpm, 10 min | 3,600 rpm, 5 min | −             |
| Extraction time               | 33.3 min     | 15.6 min     | 57 min        | 18 min        |
| Reference                     | Choi and Koh (2015) | Kim et al. (2013b) | Ma et al. (2019) | Ye et al. (2011) |

SPE, solid phase extraction; LLE, liquid-liquid extraction; ATPS, aqueous two-phase system; SPME, solid phase micro-extraction; TFA, trifluoroacetic acid.

Table 3. Comparison of recovery yields in several extraction methods for isolating ethyl carbamate

| Treatment   | I   | II  | III | IV  | V   | VI  | VII | VIII | IX (ATPS) |
|-------------|-----|-----|-----|-----|-----|-----|-----|------|-----------|
| Solvent     | DCM | DCM | CF  | CF  | DCM | DCM | CF  | CF   | −         |
| Mixing      | Vortex | Ultrasonic | Vortex | Ultrasonic | Vortex | Ultrasonic | Vortex | −         |
| Yield       | 43±5 | 42±3 | 47±4 | 43±3 | 53±8 | 57±2 | 62±11 | 58±6 | 90±2      |

Values are presented as mean±standard deviation for triplicate.
Both concentrations of NaCl and K2HPO4 are 15%. A vortex is used for 10 s and ultrasonic is operated for 30 min in a bath. ATPS, aqueous two-phase system; EC, ethyl carbamate; BC, butyl carbamate; DCM, dichloromethane; CF, chloroform.
(2013) showed that chloroform results in a higher extraction yield. We compared the results of dichloromethane (Exp. No. I, II, V, and VI) and chloroform (Exp. No. III, IV, VII, and VIII). The maximum yield using dichloromethane as the solvent was 57±2% and the minimum yield was 42±3%. The maximum yield using chloroform was 62±11% and the minimum yield was 43±3%. The difference between the two methods was low (about 5%); therefore, the effect of chloroform in improving the yield is considered insignificant.

As for the mixing method, Liao et al. (2013) reported that ultrasonic waves produced in an ultrasonic bath induce emulsification; hence, less organic solvent is required compared to the conventional LLE method. Additionally, the sample is more homogeneous, making batch processing easier. In order to assess these effects, experiments using an ultrasonic bath (Exp. No. II, IV, VI, and VIII) were compared with those using a vortex mixer (Exp. No. I, III, V, and VII). The result of using an ultrasonic bath was a maximum yield of 58±6% and a minimum yield of 42±3%. The vortex mixer resulted in a maximum yield of 62±11% and a minimum yield of 43±5%. The two methods of mixing showed almost no difference in yield; therefore, the effect of ultrasonic mixing was considered insignificant.

Finally, EC was extracted using ATPS (Exp. No. IX). According to Ma et al. (2019), when the concentration of ethanol is about 55%, the extraction efficiency is highest (≥90%). In this experiment, after adjusting the ethanol concentration to 50%, the extraction efficiency of EC was about 90%. Among the different extraction methods, the EC extraction yield was found to be the highest using ATPS.

**Extraction optimization in ATPS**

The extraction conditions of ATPS were studied to maximize the extraction yield. The influences of alcohol and phosphate concentrations, centrifugation speed and time, extraction temperature and time, and centrifugation speed after extraction on the rate of EC recovery were measured through the Plackett-Burman design. The results are shown in Fig. 1. Three of the factors, the alcohol and phosphate concentrations and the extraction temperature, had a positive effect on the extraction yield of EC, while the extraction time showed the opposite trend. In addition, since the centrifugation speed and time showed a gentler slope than the other factors, their effect on the recovery rate was somewhat reduced.

The central composite design was established with alcohol and phosphate concentrations, extraction temperature, and time, which have a high influence on the yield. These four factors and their response values are shown in Table 1. The extraction yield ranged from 46.07% to 74.09%. Among the experimental values for the extraction yield, the lowest yield (46.07%) occurred when 5% phosphate was added to 40% alcohol and extracted for 2 h at 15°C. Conversely, the highest yield (74.09%) occurred when 10% phosphate was added to 55% alcohol and extracted for 3.5 h at 22.5°C. The quadratic equation for the relationship between the extraction yield (Y) and the four factors [alcohol concentration (X₁), phosphate concentration (X₂), extraction temperature (X₃), and time (X₄)] is as follows:

\[
Y = 71.81 + 4.19X₁ + 2.9X₂ - 0.6729X₃ + 0.2654X₄
- 0.4081X₁X₂ - 1.17X₁X₃ - 0.4394X₁X₄
- 0.3056X₂X₃ - 2.84X₂X₄ - 2.18X₃X₄
- 2.22X₁² - 1.55X₂² - 3.81X₃² - 2.71X₄²
\]

The P-values for the quadratic polynomial equation are shown in Table 4. R² (coefficient of determination), which represents the fit of the response model regression analysis, was 0.920, and the P-value of this quadratic equation was 0.005, which was significant. Through this, it was determined that the quadratic equation was suitable for predicting the response value. The yield tended to increase when the alcohol and phosphate concentrations were close to 70% and 15%, respectively, the temperature was about 21.5°C, and the time was 2.8 h. In particular, it was predicted that the alcohol and phosphate concentrations were the most influential factors in the response model. The three-dimensional response surface graph for the extraction yield is shown in Fig. 2. This graph showed a tendency to increase as the alcohol and
phosphate concentrations increased, and the optimum recovery yield (75.64%) occurred at 69% alcohol and 15% phosphate concentrations.

The optimal extraction conditions for each independent variable in ATPS are shown in Table 5. The actual extraction experiment was performed under the optimal extraction conditions. The difference between the derived observed value and the predicted value was expressed through an error through which the accuracy of the model was verified. The error was obtained by dividing the absolute value of the difference of the experimental value minus the predicted value with the predicted value. The error of the recovery yield of the experiment under the optimal conditions was as low as 3.4%, and it was determined that the reaction model was reasonable.

Using RSM, this study compared the yield of several extraction methods and optimized the ATPS approach to discover the optimal extraction conditions of EC in model systems for Maesil wine. The ATPS had high extraction efficiency than other extraction techniques. The GC seems to be a promising technique for EC analysis in alcoholic beverages. The intended study will help the society in analyzing EC with better extraction methods based on the types of food, as well as knowing the recommended procedures for lowering EC levels in alcoholic drinks and fermented foods before they enter the market.

Table 4. Results of statistical analysis a regression model of central composite design on extraction yield from the model system for Maesil wine

| Source   | Yield  |
|----------|--------|
| Linear   | 0.005* |
| $X_1$    | <0.001*|
| $X_2$    | <0.001*|
| $X_3$    | 0.193  |
| $X_4$    | 0.672  |
| Quadratic| 0.002* |
| $X_1X_2$ | 0.596  |
| $X_1X_3$ | 0.143  |
| $X_1X_4$ | 0.568  |
| $X_2X_3$ | 0.690  |
| $X_2X_4$ | 0.003* |
| $X_3X_4$ | 0.013* |
| $X_1^2$  | 0.005* |
| $X_2^2$  | 0.035* |
| $X_3^2$  | 0.001* |
| $X_4^2$  | 0.013* |

Residual
Lack of fit 0.577
Fitness of model ($R^2$) 0.920

Statistically significant at *P<0.05.

Fig. 2. Response surface plot showing the effects of alcohol concentration ($X_1$), $K_2HPO_4$ concentration ($X_2$), extraction temperature ($X_3$), and time ($X_4$) on extraction yield (Y).
Table 5. Optimum conditions and comparison of predicted and observed values for verification

| Response                           | Optimum extraction condition |
|------------------------------------|------------------------------|
|                                    | Alcohol concentration (%) | K2HPO4 concentration (%) | Extraction temperature (°C) | Extraction time (h) | Predicted | Observed | Error (%) |
| Recovery yield (%)                 | 69                          | 15                       | 21.5                        | 2.8                 | 75.6      | 78.2±0.2 | 3.4       |

Values are presented as number only or mean±standard deviation.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Data interpretation and writing: TMM. Statistical analysis: JYK. Data analysis and collection: HRK, SYP, SYK, YKK, and JHL. Concept and design: YSS and JYY. Responsibility: YBL. All authors have read and agreed to the published version of the manuscript.

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