A Simple and Efficient Method for Synthesis and Extraction of Ethyl Ferulate from γ-Oryzanol

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Abstract: Ethyl ferulate (EF) is a ferulic acid (FA) derivative with high commercial value. It is not found naturally and is mostly synthesized from FA via esterification with ethanol. The present work aimed to synthesize the EF from γ-oryzanol, a natural antioxidant from rice bran oil via acid-catalyzed transesterification at refluxing temperature of ethanol. The reaction was optimized by central composite design (CCD) under response surface methodology. Based on the CCD, the optimum condition for the synthesis of EF from 0.50 g of γ-oryzanol was as follows: γ-oryzanol to ethanol ratio of 0.50:2 (g/mL), 12.30% (v/v) H₂SO₄, and a reaction time of 9.37 h; these conditions correspond to a maximum EF yield of 87.11%. Moreover, the optimized transesterification condition was further validated using 12.50 g of γ-oryzanol. At the end of the reaction time, distilled water was added as antisolvent to selectively crystallize the co-products, phytosterol and unreacted γ-oryzanol, by adjusting the ethanol concentration to 49.95% (v/v). The recovery yield of 83.60% with a purity of 98% of EF was achieved. In addition, the DPPH and ABTS assays showed similar antioxidant activities between the prepared and commercial EF.

Key words: antisolvent crystallization, central composite design, ethyl ferulate, γ-oryzanol, transesterification

1 Introduction

Ethyl ferulate (ethyl-4-hydroxy-3-methoxy cinnamate; EF) is a derivative of ferulic acid (4-hydroxy-3-methoxy cinnamic acid; FA), which has many beneficial health properties such as antioxidation¹⁻¹², anti-inflammation¹³⁻¹⁵, inhibiting neurodegenerative diseases¹⁶⁻¹⁸, inhibiting obesity and vascular disease¹⁹⁻²⁰, UV-protection¹¹,¹² and anti-microbial functions¹³. The exploration of EF has been reported in several patents and publications relating to its utilization for the synthesis of other value-added and bio-active compounds, such as FA phosphoglyceride, glyceride FA, ethyl palmitoyl ferulate, and feruloyl oleins²¹⁻²⁴. The hydrophobicity of EF is higher than FA, which makes it superior to FA in many beneficial properties such as higher antioxidant activity¹,¹² and greater permeability in the cell membrane and blood brain barrier¹,⁶,²⁰. Therefore, EF has a great potential to be used as a functional ingredient for the development of pharmaceutical, cosmetic, and food products²⁵,²⁶. However, the availability of natural EF is limited; commercial EF is mostly derived from the esterification of FA with ethanol using an enzyme²¹⁻²⁴ or acid²⁵,²⁶ as a catalyst, and the variation EF yield was observed. The EF yield varied approximately 20-87% when using commercial esterase (Novozym 435) and celite-immobilized lipase (Steapsin) as a catalyst under different reaction temperatures and time²¹,²²,²⁴. Enzymatic catalysis with moderate temperature is considered a suitable method for the synthesis of EF because it can minimize the oxidation of FA. However, that method has EF yield variations, complicated operational units, and is time consuming. A high yield of EF (80%) was also obtained in the acid-catalyzed esterification of FA using conventional heating at refluxing temperature²⁵. In addition,
the reaction time was reduced to 5 min and a 95% EF yield was obtained by applying microwave irradiation instead of conventional heating[26]. However, acid-catalyzed esterification at high temperatures may adversely lead to the oxidation of FA.

γ-Oryzanol is a group of various phytosterols and triterpene alcohols esterified to ferulic acid. It has a melting point of 137.5–138°C[27]. γ-Oryzanol is insoluble in water while slightly soluble in non-polar solvent such as hexane, heptane, and diethyl ether. It is practically soluble in more polar solvent such as isopropanol and ethyl acetate due to the presence of a hydroxyl group in the ferulate portion of its molecule[28]. The most accessible natural source of γ-oryzanol is rice bran (RB), rice bran oil (RBO) and its refining byproducts such as soapstock and rice bran acid oil (RBAO). The content of γ-oryzanol in RBAO is approximately 3.3-7.4%[29]. A simple and rapid method to recover and purify γ-oryzanol from RBAO using acid-base extraction followed by washing with Na2CO3 solution has been reported previously. The 69.9% recovery yield and 89.9% purity of γ-oryzanol were obtained[30]. The relevant property of γ-oryzanol for EF synthesis is its high stability under high temperature conditions[27]. γ-Oryzanol possess antioxidant activities due to its FA moiety that acts as radical scavenger[1][30]. However, Kikuzaki et al.[1] pointed out that antioxidant activity of compounds was dependent on the radical scavenging capacity of the antioxidant and the affinity between the antioxidant and the lipid substrate. These factors determine the selection of the phenolic antioxidant for its application in the lipid systems. The sterol moiety of γ-oryzanol also showed antioxidative effects on in vitro studies of NIH 3T3 fibroblast cells[30]; it also has a capacity for lowering blood cholesterol and decreasing the cholesterol absorption[31].

The use of γ-oryzanol for preparation of FA has been reported[32][30]. As previously mentioned, the esterification of FA is a common reaction to produce EF. However, to the best of the authors’ knowledge, a direct method for the preparation of EF from γ-oryzanol has never been reported. The aforementioned EF preparation from γ-oryzanol is practical for commercial production, especially if the γ-oryzanol can be recovered from the low-cost RBAO[26]. Thus, this study aimed to directly synthesize the EF from γ-oryzanol by acid-catalyzed transesterification. The operating condition was optimized using response surface methodology (RSM). The extraction of EF from the reaction mixture was conducted using a simple antisolvent crystallization process. In addition, antioxidant activities of the EF obtained from the present study were investigated and compared to commercial EF.

## 2 Materials and Methods

### 2.1 Materials

A food-grade γ-oryzanol (98.1%) was obtained from Oryza Fat & Oil Chemical (Ichinomiya, Japan). EF, β-sitosterol, and FA were used as standards and purchased from Sigma-Aldrich (St. Louis, MO, USA). Potassium persulfate (K2S2O8) and DPPH (1,1-diphenyl-2-picyrylhydrazyl) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Absolute ethanol (99.9%) and acetic acid were of analytical grade, and hexane and ethyl acetate were of HPLC grade (RCI Lab Scan Co. Ltd., Bangkok, Thailand). All other chemicals used were of analytical grade and were used as received.

### 2.2 HPLC analysis

The transesterification reaction was monitored by normal phase HPLC. The HPLC system consisted of a pump model 515 (Waters Associates, Milford, MA, USA.), a Rheodyne 7125 six-port valve injector, a 10 µL loop, and a photodiode array detector (PDA; Shimadzu, Kyoto, Japan). Samples were prepared in the mobile phase and analyzed on a Mightysil Si60 column (250 × 4.6 mm ID., 5 µm) protected with a Mightysil Si60 guard column (10 × 4.6 mm ID., 5 µm) (Kanto Chemical Co. Inc., Tokyo, Japan). The mobile phase was hexane/ethyl acetate/acetic (50:50:0.15, v/v/v) with a flow rate of 1.0 mL/min. The UV absorbance was monitored at 319 nm. The HPLC control and data collection were performed by LC Solution Software (version 1.24, Shimadzu, Kyoto, Japan).

The purity of EF was analyzed under the same condition as described above but with a 20 µL loop and a Sedex 80 evaporative light scattering detector (ELSD; Sedere, Alfortville, France). The ELSD drift tube and Na2 gas pressure were maintained at 30°C and two bars, respectively. Chromatograms were analyzed with Clarity Data Apex version 4.0.3.876 software. The calibration curves of γ-oryzanol, FA, and EF showed good linearity, and their coefficient of determination (R²) was greater than 0.99.

### 2.3 Synthesis of ethyl ferulate by transesterification

The transesterification of γ-oryzanol was carried out in a three-necked 50-mL flat-bottomed flask equipped with a magnetic stirrer and a condenser. The reaction was operated under temperature control in a thermostatic oil bath. The 0.5 g of γ-oryzanol and a half-volume of the desired amount of absolute ethanol were stirred at refluxing temperature in the reaction flask until the γ-oryzanol was completely dissolved. The reaction time was started when the other half volume of ethanol containing a catalyst (H2SO4 or NaOH) was added. The reaction was performed at refluxing temperature and a stirring speed of 375 rpm. An aliquot (1 mL) of the reaction mixture was taken at a certain time and the reaction was stopped by adding Na2CO3 solution or HCl until the pH was neutral (pH 7.0). The sample was extract-
ed with 2 mL ethyl acetate, dried over anhydrous Na₂SO₄, and was diluted in HPLC mobile phase before analysis by HPLC-PDA. Identification and quantification of the chromatographic peaks were performed by comparing with the reference standards and using the external standard method, respectively. Percentage of γ-oryzanol conversion and the yield of EF and FA were calculated according to Eq. (1–3) as follows.

\[
\text{Conversion of Oryzanol (\%) } = \frac{[\text{Oryzanol}]_{f} - [\text{Oryzanol}]_{i}}{[\text{Oryzanol}]_{i}} \times 100
\]

\[
\text{Yield of EF (\%) } = 100 \times \frac{\text{Mole of EF}}{\text{Mole of initial Oryzanol}}
\]

\[
\text{Yield of FA (\%) } = 100 \times \frac{\text{Mole of FA}}{\text{Mole of initial Oryzanol}}
\]

where \([\text{Oryzanol}]_{i}\) and \([\text{Oryzanol}]_{f}\) represent the initial and final concentration of γ-oryzanol in the reaction mixture. The molecular weights of EF, FA, and γ-oryzanol were 222.24 g/mol, 194 g/mol, and 602.89 g/mol, respectively.

2.4 Experimental design

A central composite design (CCD) was used to investigate the effect of H₂SO₄ concentration (A) and reaction time (B) on the conversion of γ-oryzanol to EF. The ratio between γ-oryzanol and ethanol was 1:40 (w/v) according to a preliminary experiment. The CCD consisted of a full 2⁴ factorial design (4 runs), a replicated central point (3 runs), and axial points at a distance ± α from the central point (4 runs). The results of the experiment were conducted through a 2⁴ order equation. The importance of each coefficient was decided by the Student’s t-test and p-values. The variability in the dependent variable was demonstrated by R² and adjusted R².

The optimized condition was validated for the maximum percentage of EF yield based on the predicted values obtained using RSM. The validity of the model was examined by comparing the experimental and the predicted values as measured by the coefficient of variation (CV). The experimental design and analysis were performed using the Minitab⁶ statistical software version 18 (Minitab Inc., State College, PA, USA).

2.5 Scale-up production and extraction of ethyl ferulate

The optimum condition that provided the maximum yield was selected for the scaled-up production of EF. The transethylation procedure was the same as described in Section 2.3. The reaction set-up consisted of a three-necked 1-L flat-bottomed flask equipped with a thermostatic oil bath, magnetic stirrer, and condenser; the γ-oryzanol to ethanol ratio was 12.500 (g/mL), H₂SO₄ was 12.3% (61.5 mL in 500 mL ethanol), the stirring speed was 750 rpm, and the reaction time was 11 h at a refluxing temperature. The reaction mixture was stopped by adding 500 mL of 14% Na₂CO₃ (w/v) solution and was left to stand overnight at room temperature. The final concentration of ethanol in the reaction mixture was assumed to be 49.95% (v/v). The water acted as an antisolvent to reduce the solubility of phytosterol and untransesterified γ-oryzanol. The crystallized compounds were separated by filtering through Whatman No. 1 filter paper. The EF which remained in the ethanol solution was recovered by rotary evaporation at 60°C under reduced pressure. Recovery of EF was determined from the content of EF obtained by HPLC-ELSD according to Eq. (4).

\[
\text{Recovery of EF (\%) } = \frac{\text{EF content dissolved in ethanol solution (g)}}{\text{Total EF content in the reaction mixture (g)}} \times 100
\]

2.6 Antioxidant activity of ethyl ferulate

2.6.1 DPPH radical scavenging assay

The antioxidant capacity of the EF was determined using DPPH radical scavenging assay described by Berber et al.⁴ with slight modifications. Briefly, the sample dissolved in ethanol was mixed with 0.1 mM ethanolic DPPH solution. The mixture was stirred and incubated in the dark at room temperature for 30 min. The absorbance of the sample \((A_s)\), blank \((A_b)\), and control \((A_c)\) were then measured at 517 nm (UV-1601, Shimadzu, Kyoto, Japan). The experiment was performed in triplicate and the antioxidant capacity was expressed as percent inhibition of DPPH radical relative to the control as calculated in Eq. (5).

\[
\text{DPPH radical scavenging activity (\%) } = \left( \frac{A_s - (A_s + A_b)}{A_b} \right) \times 100
\]

where \(A_s\) was the absorbance at 517 nm of 400 µL ethanol + 2 mL of 0.1 mM ethanolic DPPH solution, \(A_s\) was the absorbance at 517 nm of 400 µL of 100 µg/mL ethanolic sample + 2 mL of 0.1 mM ethanolic DPPH solution, and \(A_b\) was the absorbance at 517 nm of 400 µL of 100 µg/mL ethanolic sample + 2 mL of ethanol

2.6.2 ABTS⁺ radical scavenging assay

The methodology described by Turkylmaz et al.⁵ was used for the determination of antioxidant capacity against ABTS with some modifications. Briefly, the ABTS⁺ radical was generated by mixing 7 mM ABTS solution and 2.45 mM potassium persulfate solution (2:1, v/v); it was then kept in the dark for 16 h at room temperature. Before analysis, the ABTS⁺ radical solution was diluted with absolute ethanol to obtain an absorbance of 0.700 ± 0.02 at 734 nm. To determine antioxidant activity, 20 µL of EF (1000 µg/mL) was mixed with 3980 µL of ABTS⁺ solution. The mixture was kept in the dark for 6 min, and the absorbance was measured at 734 nm. The control solution of 99.80% ethanol

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was used as blank. The ABTS\textsuperscript{+} scavenging effect was calculated according to the Eq. (6):

\[
\text{ABTS\textsuperscript{+} radical scavenging activity}(\%) = \left(\frac{A_{\text{so}} - A_s}{A_{\text{so}}}\right) \times 100
\]

where \(A_s\) was the absorbance at 734 nm of diluted ABTS\textsuperscript{+} solution at the beginning of the analysis and \(A_s\) was the absorbance at 734 nm of the mixture after 6 min.

3 Results and Discussion

3.1 Single factor experiment

3.1.1 Effect of catalyst types on the transesterification of \(\gamma\)-oryzanol

Figure 1 shows the effect of catalyst types on the conversion of \(\gamma\)-oryzanol to EF via transesterification using NaOH and H\textsubscript{2}SO\textsubscript{4} as catalysts. The reaction was conducted at refluxing temperature using \(\gamma\)-oryzanol to ethanol ratio of 1:40 (w/v) at 0.02 mol of catalyst. The conversion of \(\gamma\)-oryzanol and EF yield were rapidly increased to 19.40\% ± 0.11 and 16.28\% ± 0.05, respectively and only 3.10\% ± 0.01 of FA was observed within the first 3 min of the reaction when NaOH was used as catalyst. The \(\gamma\)-oryzanol conversion was gradually increased along with FA yield and reached to 84.56 ± 0.03\% and 84.56 ± 0.03\%, respectively at 60 min. Whereas, EF was gradually decreased after the 3 min of reaction and only 1.86\% ± 0.01 was remained at 60 min (Fig. 1A).

On the other hand, conversion of \(\gamma\)-oryzanol slightly increased during the time course of the reaction when H\textsubscript{2}SO\textsubscript{4} was used as catalyst, although the maximum conversion was only 44.44 ± 0.49\% at 360 min of reaction time. However, under acidic catalysis, the yield of EF increased along with the conversion of \(\gamma\)-oryzanol and only 1.13 ± 0.04\% of FA occurred (Fig. 1B). The low hydrolysis reaction rate of acid catalysis was probably caused by the protonation of the carbonyl group of \(\gamma\)-oryzanol that was preferentially transesterified. However, ethanol is a weak nucleophile leading to slow attack on the molecules of protonated \(\gamma\)-oryzanol to produce EF\textsuperscript{36}. Accordingly, H\textsubscript{2}SO\textsubscript{4} was selected to be used as a catalyst in the transesterification of \(\gamma\)-oryzanol.

3.1.2 Effect of H\textsubscript{2}SO\textsubscript{4} concentration on the transesterification of \(\gamma\)-oryzanol

The effect of H\textsubscript{2}SO\textsubscript{4} concentration on the \(\gamma\)-oryzanol conversion and EF yield is shown in Fig. 2. The acid-catalyzed protonation of the carbonyl group of \(\gamma\)-oryzanol and the transesterification reaction occurred by the attacking of ethanol\textsuperscript{36}. Therefore, the conversion of \(\gamma\)-oryzanol and the yield of EF increased as the concentration of H\textsubscript{2}SO\textsubscript{4} increased. In addition, this study operated at a low temperature (refluxing temperature of ethanol), the kinetic energy of the \(\gamma\)-oryzanol molecule was reduced, thus a greater number of catalyst molecules were needed to increase the reaction\textsuperscript{37}. However, a dark brown color was observed when greater than 12\% H\textsubscript{2}SO\textsubscript{4} was used. It has been reported that the formation of brownish color and degradation of bioactive compounds occurred under acid-catalyzed methanolysis of rice brain oil\textsuperscript{38}. Therefore, we limited the maximum concentration of H\textsubscript{2}SO\textsubscript{4} to 12\% to minimize undesired color formation and side reactions. The maximum EF yield at the maximum catalyst concentration was 58.52 ± 0.49\%. The results agree with a previous study, which reported that increasing catalyst loading led to an increase in reaction rate in the synthesis of feruloyl glycerols from methanolysis of rice brain oil\textsuperscript{14,15}.

3.1.3 Effect of \(\gamma\)-oryzanol to ethanol ratio

The effect of \(\gamma\)-oryzanol to ethanol ratio on the percentage of EF yield was studied by varying the ratio of \(\gamma\)-oryzanol to ethanol between 1:20 to 1:100 (w/v) using 12\% H\textsubscript{2}SO\textsubscript{4} (v/v) as a catalyst. The \(\gamma\)-oryzanol conversion
and EF yield were not significantly different when the γ-oryzanol to ethanol ratio was higher than 1:20 (Fig. 3). In this study, the ethanol acted as both a reactant and a reaction medium to dissolve the γ-oryzanol. The theoretical molar ratio of γ-oryzanol to ethanol is 1:1. In this study, we used 1:20 (w/v), or about 1:260 (mole/mole) of γ-oryzanol to ethanol, which far exceeded the theoretical value. Thus, a ratio higher than 1:20 may not enhance the rate of transesterification. The lower percentages of γ-oryzanol conversion and EF yield at a γ-oryzanol to ethanol ratio of 1:20 (w/v) than at other ratios may be due to the solubility limit of γ-oryzanol in the low amount of ethanol. Results show that the optimal ratio for γ-oryzanol to ethanol was 1:40 (w/v) or higher.

### 3.1.4 Effect of reaction time

The effect of reaction time between 0-8 h on the percentage of γ-oryzanol conversion and EF yield was observed. The H$_2$SO$_4$ and ratio of γ-oryzanol to ethanol were fixed at 12% (v/v) and 1:40, respectively. The percentage of γ-oryzanol conversion and EF yield increased along with the increase of reaction time (Fig. 4). However, it is interesting to note that the difference between the γ-oryzanol conversion and EF yield also increased as the reaction time increased implying that a competitive hydrolysis reaction
occurred. The maximum $\gamma$-oryzanol conversion and the yield of EF at 8 h were 80.78 ± 0.56% and 78.03 ± 0.24%, respectively. As mentioned above, since ethanol is a weak nucleophile, its reaction with $\gamma$-oryzanol is slow and has not yet reached equilibrium within 8 h. Results from the single factor experiment show that the $\text{H}_2\text{SO}_4$ concentration and reaction time significantly influenced the $\gamma$-oryzanol conversion and EF yield, whereas $\gamma$-oryzanol to ethanol ratio was not important. Therefore, the optimization of the method using response surface methodology (RSM) was further investigated, where the temperature and the $\gamma$-oryzanol to ethanol ratio were fixed at refluxing temperature and 1:40 (w/v), respectively.

3.2 Central composite design (CCD): model fitting

The coded and actual values of independent variables are presented in Table 1 with the experimental and model responses of the percentage of EF yield. The coded surface models of the EF yield percentage generated by the Minitab Version 17 software is shown in Eq. (7):

$$y = 89.28 - 2.507A + 4.143B - 3.529A^2 - 3.061B^2 + 1.305AB$$  (7)

where $y$ is the percentage of EF yield (% mol), A is the $\text{H}_2\text{SO}_4$ concentration (% vol), B is the reaction time (h), AB is the interaction of the parameters A and B, and $A^2$ and $B^2$ are the quadratic parameters. The calculated response values based on Eq. (7) agreed well with the experimental values (Table 1).

From the analysis of variance (ANOVA) using the $F$-test and $p$-value ($p < 0.05$) as shown in Table 2, A, B, $A^2$, and $B^2$ were significant, and the model $p$-value was $< 0.05$, indicating that the model was statistically significant. In addition, the $p$-value for lack of fit ($p = 0.335$) was insignificant, confirming that the model was a good fit. The $R^2$ and adjusted $R^2$ of the model were 93.91 % and 87.82 %, respectively; this indicated that the data were well-fitted by the model (Eq. 7). The interaction of $\text{H}_2\text{SO}_4$ concentration and reaction time on the percentage of EF yield can be better understood from the surface and contour plots (Fig. 5). The maximum percentage of EF yield was achieved when the $\text{H}_2\text{SO}_4$ concentration and reaction time were 12.10–12.50 (v/v) and 9.10–10.10 h, respectively. Moreover, the ANOVA showed that the effect of reaction time was more pronounced on the EF yield percentage than $\text{H}_2\text{SO}_4$ concentration.

![Fig. 4](image_url) Effect of reaction time on $\gamma$-oryzanol conversion and ethyl ferulate (EF) yield. Reaction condition: 12% (v/v) of $\text{H}_2\text{SO}_4$ and 1:40 (w/v) of oryzanol to ethanol at refluxing temperature.

### Table 1 The central composite design with coded and real variables and their experimental and predicted response.

| Run | Coded Value | Real value | Response |
|-----|-------------|------------|----------|
|     | A | B | A | B | EF<sub>(exp)</sub> (%) | EF<sub>(cal)</sub> (%) |
| 1   | -1 | -1 | 12.00 | 8.00 | 82.08 | 82.36 |
| 2   | 1 | -1 | 12.80 | 8.00 | 76.14 | 74.74 |
| 3   | -1 | 1 | 12.00 | 10.00 | 85.83 | 88.04 |
| 4   | 1 | 1 | 12.89 | 10.00 | 85.12 | 85.63 |
| 5   | -1.4142 | 0 | 11.83 | 9.00 | 87.36 | 85.77 |
| 6   | 1.4142 | 0 | 12.97 | 9.00 | 90.78 | 90.02 |
| 7   | 0 | -1.4142 | 12.40 | 7.59 | 76.35 | 77.30 |
| 8   | 0 | 1.4142 | 12.40 | 10.41 | 90.78 | 89.28 |
| 9   | 0 | 0 | 12.40 | 9.00 | 87.58 | 89.28 |
| 10  | 0 | 0 | 12.40 | 9.00 | 90.41 | 89.28 |
| 11  | 0 | 0 | 12.40 | 9.00 | 89.86 | 89.28 |

A = $\text{H}_2\text{SO}_4$ (% vol.); B = Reaction time (h)
Table 2  ANOVA analysis of the percentage of ethyl ferulate (EF) in the central composite design.

| Source          | DF | Adj SS   | Adj MS  | F value | p-Value |
|-----------------|----|----------|---------|---------|---------|
| Model           | 5  | 290.036  | 58.007  | 15.41   | 0.005*  |
| Linear          | 2  | 187.579  | 93.790  | 24.92   | 0.003*  |
| A               | 1  | 50.286   | 50.286  | 13.36   | 0.015*  |
| B               | 1  | 137.294  | 137.294 | 36.48   | 0.002*  |
| Square          | 2  | 95.643   | 47.822  | 12.71   | 0.011*  |
| AA              | 1  | 70.347   | 70.347  | 18.69   | 0.008*  |
| BB              | 1  | 52.910   | 52.910  | 14.06   | 0.013*  |
| 2-Way Interaction| 1 | 6.813    | 6.813   | 1.81    | 0.236   |
| AB              | 1  | 6.813    | 6.813   | 1.81    | 0.236   |
| Error           | 5  | 18.816   | 3.763   |         |         |
| Lack-of-fit     | 3  | 14.336   | 4.779   | 2.13    | 0.335   |
| Pure Error      | 2  | 4.480    | 2.240   |         |         |
| Total           | 10 | 308.852  |         |         |         |

$R^2 = 93.91\%, \ R^2 (adj) = 87.82\%$

Fig. 5  Surface (A) and contour (B) plots of the percentage of ethyl ferulate (EF) yield according to reaction time (h) and H$_2$SO$_4$ concentration.
3.3 Optimization and validation of the model

By using the response optimizer in the Minitab program, the optimum condition for the suggested model was found to be 12.30 (v/v)% H₂SO₄ and 9.37 h reaction time for the maximum predicted EF yield of 90.88%. Three replicates of the transesterification using the optimum condition were carried out to verify the model. An experimental result of 87.11 ± 1.41% EF yield was obtained, which agreed well with the predicted value, indicating that the model was adequate for the transesterification of γ-oryzanol.

Although the synthesis of EF by transesterification has not been reported, esterification of FA to EF using free and immobilized enzymes have been studied²¹, ²³, ²⁴. However, the EF yields were quite low, approximately 20–68% of EF yields (by mole of FA) were obtained. On the other hand, more than 90% of EF yield was obtained when H₂SO₄ was used as a catalyst for the esterification of FA under microwave irradiation²⁶.

3.4 Scale-up production and extraction of ethyl ferulate

A scale-up transesterification using 12.50 g of γ-oryzanol was conducted to verify the feasibility of EF production under the determined optimum condition of 12.30 (v/v)% H₂SO₄ and 9.37 h reaction time. The EF yield from the scale-up process was 79.34 ± 0.26% which was 8.92% lower than that obtained from the lab-scale (87.11 ± 1.41%). Lower yield in the large-scale compared to the lab-scale production was probably due to a poor mixing of the reaction mixture and inhomogeneity of temperature in the scale-up process³⁹, ⁴⁰. Meullemiestre et al.⁴⁰ reported that large-scale extraction of phenolic compounds from Maritime pine sawdust yielded 32% lower than that of the optimized lab-scale extraction. The biodiesel yield from large-scale enzymatic synthesis was reported 10% lower than that of the yield from its optimized lab-scale³⁹. From the model fitting in Eq.(7) and ANOVA analysis in (Table 2), it is shown that reaction time was the most impact parameter on the EF yield. To improve the yield of EF close to the optimized lab-scale production (87.11 ± 1.41%), thus parameters under the optimum condition were maintained except for the reaction times between 9.37 h and 12 h were re-investigated. It was found that the maximum EF yield of 82.27 ± 0.07% was obtained at the reaction time of 11 h. According to the results, the model and factor affecting to the EF yield from RSM was reliable. In addition, the optimal condition obtained from the RSM could be used as a guidance for re-evaluating any factor that had a large impact on reaction in the large-scale production to achieve a desired yield.

The EF was extracted from the reaction mixture using a simple antisolvent crystallization process by adding water into the ethanol solution. The solubility of phytosterol and unreacted γ-oryzanol were reduced and became crystallized at about 49.95% ethanol. The EF solubilized in the ethanol solution was recovered and identified using HPSEC. The recovery and purity were 83.60 ± 0.13% and 98.00 ± 0.00%, respectively (Fig. 6). The results confirmed that the proposed method for synthesis and extraction of EF from γ-oryzanol can be used for large-scale production.

![Fig. 6](image)

**Fig. 6** HPLC chromatogram of γ-oryzanol, ethyl ferulate, β-sitosterol, and ferulic acid standards (A) and the ethyl ferulate (EF) synthesized by transesterification reaction with a simple antisolvent crystallization process (B).
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3.5 Antioxidant activity test

The antioxidant activity of EF with 98% purity obtained from the scale-up production by transesterification and antisolvent extraction was compared with those of the commercial EF (98% purity). The DPPH radical scavenging activities of the prepared and commercial EF were 51.85 ± 0.16% and 54.40 ± 0.32%, respectively. The radical inhibitions based on the ABTS assay were 76.35 ± 1.50% and 77.55 ± 0.24% for the prepared EF and commercial EF, respectively. Results show that antioxidant activities of the prepared EF were close to commercial EF. Their differences were 2.55% and 1.20% for DPPH and ABTS assays, respectively.

4 Conclusion

To our knowledge, this is the first time that EF has been prepared from γ-oryzanol via transesterification reaction using H2SO4 as a catalyst. The RSM was used to optimize the influence of the factors on the transesterification. At the optimum ratio of γ-oryzanol and ethanol of 1:40 w/v, H2SO4 concentration of 12.30% (v/v), and a reaction time of 9.37 h at refluxing temperature, an 87.11% EF yield was obtained. This process can be scaled up and operated at optimum conditions obtaining 82.27% EF yield. In addition, a high recovery yield of 83.60% with 98.00% purity of EF were obtained under a fast and effective method using water as an antisolvent to crystallize the co-product phytosterol from EF. The purity and antioxidant activity of the prepared EF was comparable to the commercial analytical grade EF.

Author Contributions

Piraporn Sombutsuwan analyzed data and wrote the original manuscript. Apiwat Jirattisakul performed research. Akkaradech Nakornsadet contributed analytic tools. Saengchai Akepratumchai advised in statistical data. Salisa Chumsantea performed visualized data. Siriluck Pujjanapornpun analyzed data. Supathra Lilitchan discussed and commented data. Kanit Krisangkura provided conceptualization. Kornkanok Aryusuk supervised the work and reviewed the manuscript.

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Conflict of Interest

All authors have no conflict of interest.

References

1) Kikuzaki, H.; Hisamoto, M.; Hirose, K.; Akiyama, K.; Taniguchi, H. Antioxidant Properties of Ferulic Acid and Its Related Compounds. J. Agric. Food Chem. 50, 2161-2168 (2002).
2) Cunha, F.V.M.; Gomes, B.D.S.; Neto, B.D.S.; Ferreira, A.R.; De Sousa, D.P.; Martins, M.D.C.D.C.; Oliveira, F.D.A. Ferulic acid ethyl ester diminished Complete Freund’s Adjuvant-induced incapacitation through antioxidant and anti-inflammatory activity. N-S Arch. Pharmacol. 389, 117-130 (2016).
3) Calabrese, V.; Calafato, S.; Puleo, E.; Cornelius, C.; Sapienza, M.; Morganti, P.; Mancuso, C. Redox regulation of cellular stress response by ferulic acid ethyl ester in human dermal fibroblasts: role of vitagenes. Clin. Dermatol. 26, 358-363 (2008).
4) Cunha, F.V.M.; Do Nascimento Caldas Trindade, G.; Da Silva Azevedo, P.S.; Côelho, A.G.; Braz, E.M.; De Sousa Neto, B.; De Rezende, D.C.; De Sousa, D.P.; De Assis Oliveira, F.; Nunes, L.C.C. Ethyl ferulate/β-cyclodextrin inclusion complex inhibits edema formation. Mater. Sci. Eng. C 115, 111057 (2020).
5) Nazare, A.C.; de Faria, C.M.; Chiari, B.G.; Petronio, M.S.; Regasini, L.O.; Silva, D.H.; Correa, M.A.; Isaac, V.L.; da Fonseca, L.M.; Ximenes, V.F. Ethyl ferulate, a component with anti-inflammatory properties for emulsion-based creams. Molecules 19, 8124-39 (2014).
6) Sultana, R. Ferulic acid ethyl ester as a potential therapy in neurodegenerative disorders. Biochim Biophys Acta Mol. Basis Dis. 1822, 748-752 (2012).
7) Sultana, R.; Ravagna, A.; Mohammad-Abdul, H.; Calabrese, V.; Butterfield, D.A. Ferulic acid ethyl ester protects neurons against amyloid β-peptide-(1-42)-induced oxidative stress and neurotoxicity: relationship to antioxidant activity. J. Neurochem. 92, 749-758 (2005).
8) Joshi, G.; Perluigi, M.; Sultana, R.; Agrippino, R.; Calabrese, V.; Butterfield, D.A. In vivo protection of synaptosomes by ferulic acid ethyl ester (FAEE) from oxidative stress mediated by 2,2′-azobis(2-amidino-propane)dihydrochloride (AAPH) or FeSO4/H2O2: Insight into mechanisms of neuroprotection and relevance to oxidative stress-related neurodegenerative disorders.
9) Cunha, F.V.M.; Coelho, A.G.; Azevedo, P.S.d.S.; da Silva, A.A.; Oliveira, P.d.A.; Nunes, L.C.C. Systematic review and technological prospection: ethyl ferulate, a phenylpropanoid with antioxidant and neuroprotective actions. *Expert Opin. Ther. Pat.* 29, 73-83 (2019).

10) Tsai, Y.C.; Lee, Y.M.; Hsu, C.H.; Leu, S.Y.; Chiang, H.Y.; Yen, M.H.; Cheng, P.Y. The effect of ferulic acid ethyl ester on leptin-induced proliferation and migration of aortic smooth muscle cells. *Exp. Mol. Med.* 47, e180 (2015).

11) Rodrigues, N.D.N.; Staniforth, M.; Young, J.D.; Peperstraete, Y.; Cole-Filipiak, N.C.; Gord, J.R.; Walsh, P.S.; Hewett, D.M.; Zwier, T.S.; Stavros, V.G. Towards elucidating the photochemistry of the sunscreen filter ethyl ferulate using time-resolved gas-phase spectroscopy. *Faraday Discuss.* 194, 709-729 (2016).

12) Horbury, M.D.; Baker, L.A.; Rodrigues, N.D.N.; Quan, W.-D.; Stavros, V.G. Photoisomerization of ethyl ferulate: A solution phase transient absorption study. *Chem. Phys. Lett.* 673, 62-67 (2017).

13) Shi, Y.G.; Bian, L.Q.; Zhu, Y.J.; Zhang, R.R.; Shao, S.Y.; Wu, Y.; Chen, Y.W.; Dang, Y.L.; Ding, Y.; Sun, H. Multifunctional alkyl ferulate esters as potential food additives: Antibacterial activity and mode of action against Listeria monocytogenes and its application on American sturgeon caviar preservation. *Food Control* 96, 390-402 (2019).

14) Sun, S.; Chen, X. Kinetics of enzymatic synthesis of monoferuloyl glycerol and diferuloyl glycerol by transesterification in [BMIM][PF6]. *Biochem. Eng. J.* 97, 25-31 (2015).

15) Sun, S.; Chen, X.; Bi, Y.; Chen, J.; Yang, G.; Liu, W. Functionalized Iodine-Liquid-Catalyzed 1-Feruloyl-α-glycerol Synthesis. *J. Am. Oil. Chem. Soc.* 91, 759-765 (2014).

16) Sun, S.; Shan, L.; Jin, Q.; Liu, Y.; Wang, X. Solvent-free synthesis of glycerol ferulate using a commercial microbially lipase. *Biotechnol. Lett.* 29, 945-949 (2007).

17) Laszlo, J.A.; Compton, D.L. Enzymatic glycerolysis and transesterification of vegetable oil for enhanced production of feruloylated glycerols. *J. Am. Oil. Chem. Soc.* 83, 765-770 (2006).

18) Xin, J.Y.; Chen, L.L.; Zhang, Y.X.; Zhang, S.; Xia, C.G. Lipase-catalyzed transesterification of ethyl ferulate with triolein in solvent-free medium. *Food Bioprod. Process.* 89, 457-462 (2011).

19) Chalas, J.; Claise, C.; Edeas, M.; Messaoudi, C.; Vergnes, L.; Abella, A.; Lindenbaum, A. Effect of ethyl esterification of phenolic acids on low-density lipoprotein oxidation. *Biomed. Pharmacother.* 55, 54-60 (2001).

20) Scapagnini, G.; Butterfield, D.A.; Colombrita, C.; Sultana, R.; Pascale, A.; Calabrese, V. Ethyl ferulate, a lipophilic polyphenol, induces HO-1 and protects rat neurons against oxidative stress. *Antioxid. Redox Sign.* 6, 811-8 (2004).

21) Compton, D.L.; Laszlo, J.A.; Berhow, M.A. Lipase-catalyzed synthesis of ferulate esters. *J. Am. Oil. Chem. Soc.* 77, 513-519 (2000).

22) Lee, G.S.; Widjaja, A.; Ju, Y.H. Enzymatic Synthesis of Cinnamic Acid Derivatives. *Biotechnol. Lett.* 28, 581-585 (2006).

23) Saun, N.K.; Narwal, S.K.; Dogra, P.; Chauhan, G.S.; Gupta, R. Comparative study of free and immobilized lipase from *Bacillus aerius* and its application in synthesis of ethyl ferulate. *J. Oleo Sci.* 63, 911-9 (2014).

24) Kumar, A.; Kanwar, S.S. Synthesis of ethyl ferulate in organic medium using celite-immobilized lipase. *Bioresour. Technol.* 102, 2162-2167 (2011).

25) Voisin-Chiret, A.S.; Bazin, M.A.; Lancelot, J.C.; Rault, S. Synthesis of new L-ascorbic ferulic acid hybrids. *Molecules* 12, 2533 (2007).

26) Li, N.G.; Shi, Z.H.; Tung, Y.P.; Li, B.Q.; Duan, J.A. Highly efficient esterification of ferulic acid under microwave irradiation. *Molecules* 14, 2118 (2009).

27) Nyström, L.; Achenius, T.; Lampi, A.M.; Moreau, R.A.; Piironen, V. A comparison of the antioxidant properties of steryl ferulates with tocopherol at high temperatures. *Food Chem.* 101, 947-954 (2007).

28) Juliano, C.; Cossu, M.; Alamanni, M.C.; Piu, L. Antioxidant activity of gamma-oryzanol: Mechanism of action and its effect on oxidative stability of pharmaceutical oils. *Int. J. Pharm.* 299, 146-154 (2005).

29) Islam, M.S.; Matsuki, N.; Nagasaka, R.; Ohara, K.; Takanatsu, H.; Ozaki, H.; Ushio, H.; Hori, M. Chapter 34 - γ-oryzanol from rice bran acid oil. *In: Rice bran antioxidants in health and wellness. in Health A2 - Watson, Ronald Ross. in Health A2 - Watson, Ronald Ross.* Academic Press, pp. 453-465 (2014).

30) Sombutsuwon, P.; Nakornsadet, A.; Aryusuk, K.; Akeprasumchai, S.; Jeyashoke, N.; Lilitchan, S.; Krisnangkura, K. Recovery of γ-oryzanol from rice bran acid oil by an acid-base extraction method with the assistance of response surface methodology. *J. Oleo Sci.* 67, 1405-1415 (2018).

31) Srikaeo, K. Chapter 35 - Organic rice bran oils in health A2 - Watson, Ronald Ross. in *Rice and Rice in Disease Prevention and Health* (Watson, R.R.; Preedy, V.R.; Zibadi, S. eds.). Academic Press, pp. 453-465 (2014).

32) Truong, H.; Do Van, M.; Duc Huynh, L.; Thi Nguyen, L.; Do Tuan, A.; Le Xuan Thanh, T.; Duong Phuoc, H.; Takenaka, N.; Imamura, K.; Maeda, Y. A method for ferulic acid production from rice bran oil soapstock using a homogenous system. *Appl. Sci.* 7, 796 (2017).

33) Truong, H.T.; Van Do, M.; Duc Huynh, L.; Thi Nguyen, L.; Tuan Do, A.; Thanh Xuan Le, T.; Phuoc Duong, H.;
Takenaka, N.; Inamura, K.; Maeda, Y. Ultrasound-assisted, base-catalyzed, homogeneous reaction for ferulic acid production from γ-orizanol. *J. Org. Chem.* **2018**, *3132747* (2018).

34) Bersuder, P.; Hole, M.; Smith, G. Antioxidants from a heated histidine-glucose model system. I: Investigation of the antioxidant role of histidine and isolation of antioxidants by high-performance liquid chromatography. *J. Am. Oil. Chem. Soc.* **75**, 181-187 (1998).

35) Türkyılmaz, M.; Tağ, Ş.; Dereli, U.; Özkan, M. Effects of various pressing programs and yields on the antioxidant activity, antimicrobial activity, phenolic content and colour of pomegranate juices. *Food Chem.* **138**, 1810-1818 (2013).

36) Reyes, L.; Nicolás-Vázquez, I.; Mora-Diez, N.; Alvarez-Idaboy, J.R. Acid-catalyzed nucleophilic additions to carbonyl groups: Is the accepted mechanism the rule or an exception? *J. Org. Chem.* **78**, 2327-2335 (2013).

37) Bhatti, H.N.; Hanif, M.A.; Faruq, U.; Sheikh, M.A. Acid and base catalyzed transesterification of animal fats of biodiesel. *Iran. J. Chem. Chem. Eng.* **27**, 41-48 (2008).

38) Ju, Y.H.; Zullaikah, S. Effect of acid-catalyzed methanolysis on the bioactive components of rice bran oil. *J. Taiwan Inst. Chem. Eng.* **44**, 924-928 (2013).

39) Lee, M.; Lee, D.; Cho, J.; Lee, J.; Kim, S.; Kim, S.W.; Park, C. Optimization of enzymatic biodiesel synthesis using RSM in high pressure carbon dioxide and its scale up. *Bioproc. Biosyst. Eng.* **36**, 775-780 (2013).

40) Meullemiestre, A.; Petitcolas, E.; Maache-Rezzoug, Z.; Chemat, F.; Rezzoug, S.A. Impact of ultrasound on solid-liquid extraction of phenolic compounds from maritime pine sawdust waste. Kinetics, optimization and large scale experiments. *Ultrason. Sonochem.* **28**, 230-239 (2016).

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