Enrichment of Palmitoleic Acid by a Combination of Two-step Solvent Crystallization and Molecular Distillation

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Abstract: Palmitoleic acid shows a variety of beneficial properties to human health. In this study, enrichment of palmitoleic acid from sea buckthorn pulp oil by two-step solvent crystallization and molecular distillation was investigated. Sea buckthorn pulp oil was first converted to its corresponding mixed fatty acids (SPOMFs) containing 27.17% palmitoleic acid. Subsequently, the effects of various factors on crystallization (i.e., crystallization temperature, type of solvent, ratio of SPOMFs to solvent (w/v), crystallization time) and molecular distillation (distillation temperature) were assessed on a 5-g scale. It was found that optimal primary crystallization conditions were a 1:15 ratio of SPOMFs to methanol (w/v), −20°C and 12 h. Secondary crystallization conditions were set to a 1:4 ratio of methanol to palmitoleic acid product obtained from the first step crystallization to methanol (w/v), −40°C and 6 h. For further purification of palmitoleic acid by molecular distillation, the optimal distillation temperature was determined to be 100°C. After purification by crystallization and molecular distillation under the optimal conditions, the final product consisted of 54.18% palmitoleic acid with an overall yield of 56.31%. This method has great potential for adoption by the food and medical industries for the preparation of palmitoleic acid concentrate for nutritional studies.

Key words: palmitoleic acid, sea buckthorn pulp oil, solvent crystallization, molecular distillation

1 Introduction

Palmitoleic acid, or (9Z)-hexadec-9-enoic acid, is a 16-carbon omega-7 monounsaturated fatty acid¹. Previous study has demonstrated that the fatty acid has a wide range of applications in nutrition, medicine and chemical industries². For example, in animal models, adipose tissue has been shown to release palmitoleic acid, which suppresses hepatic steatosis and improves insulin sensitivity³. Consequently, palmitoleic acid has been in the spotlight as a promising anti-inflammatory lipid that may help ameliorate metabolic disorders⁴. In addition, palmitoleic acid is used in cosmetics to improve water retention and elasticity of the skin, delay the aging of skin, hair and nails, and improve eye health⁵. At present many biopharmaceutical and nutrition companies are vigorously developing palmitoleic acid enrichment.

Palmitoleic acid can be found in almost any oils of animal or plant origin, but usually in a very low concentration⁶. At present, wild plants are the main sources of palmitoleic acid. The seed oil of cat’s claw (Doxantha unguis-cati L.), a woody vine native to the Amazon rainforest, South America and Central America, comprises 64% palmitoleic acid in the oil⁷. Macadamia nut oil contains 24%-36% palmitoleic acid⁸, and the pulp oil from sea buckthorn contains up to 30% palmitoleic acid⁹. Of these three plants, only sea buckthorn is widely distributed and has good cultivation development potential in China. From other wild plants that contain high proportions of palmitoleic acid, only low extraction yields of palmitoleic acid are obtained, and the geographical distribution of these plants is narrow, making them less suitable for commercial cultivation compared to sea buckthorn. Therefore, sea buckthorn pulp oil (SPO) is considered to be the best raw material for palmitoleic acid enrichment.
Fatty acids can be separated by crystallization, urea complexation, supercritical fluid extraction, molecular distillation, enzymatic transesterification and preparative liquid chromatography\textsuperscript{[8--10]}. To date, however, very little attention has been paid to the preparation of palmoleic acid concentrate from natural sources. Chemical synthesis is commonly used to obtain highly pure palmoleic acid, but this creates partial trans-palm oleic acid\textsuperscript{[22]}. Klaas and Meurer\textsuperscript{[10]} reported the enrichment of palmoleic acid from natural sources, in which the palmoleic acid concentration increased by approximately 50\% after transesterification, distillation and urea crystallization. Although this led to a product rich in the ester of palmoleic acid (81.9\%), the overall yield of this method was very low (\textasciitilde 4\%), and carcinogenic ethyl or methyl carbamate may be formed during urea inclusion\textsuperscript{[44]}, limiting the application of the extract in the food and pharmaceutical industries. In another study, Gutiérrez and Belkacemi\textsuperscript{[23]} crystallized SPO containing 41.4\% palmoleic acid at 15°C in acetone for about 24 h, the crystallization resulted in the increase of palmitoleic acid content from 41.4\% to 53\% in the final product with a 20\% yield.

Various methods are available in the literature for the concentration or separation of unsaturated fatty acids, but only a few are feasible for scalable preparation\textsuperscript{[45]}. In this study, crystallization and molecular distillation were used to enrich palmoleic acid from sea buckthorn pulp oil mixed fatty acids (SPOMFs). The operating conditions, namely the crystallization temperature, type of solvent, ratio of SPOMFs to solvent (w/v), crystallization time, and the distillation temperature, were optimized to achieve an acceptable concentration and yield of palmoleic acid. Importantly, these methods are suitable for the scalable production of palmoleic acid from an inexpensive and accessible natural source.

2 Experimental
2.1 Materials
SPO was purchased from Qinghai Kangpu Co., Ltd. (Xining, Qinghai). Standards of 37 fatty acid methyl esters (FAMEs) were purchased from Sigma-Aldrich Chemical Co., Ltd. (Shanghai, China). Hexane, methanol, ethanol, acetone and isopropanol were provided by Sinopharm Chemical Regent (Shanghai, China). All of the other reagents were analytically pure and were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2 Free fatty acid preparation by saponification of SPO
SPO (20 g) was mixed with 2.2 g KOH and 40 mL 95\% ethanol, and the mixture was stirred at 75°C in a water bath for 2 h. After the completion of the reaction, distilled water (100 mL) and hexane (200 mL) were added, and the mixture was transferred to a separating funnel to stand for separation. The unsaponifiable matters in the hexane phase were removed, and the collected lower layer containing soaps was acidified with 3 M hydrochloric acid to pH 2 to release free fatty acids. Subsequently, the solution was extracted three times with total 300 mL hexane. The upper layer containing free fatty acids was collected, and trace water was removed using anhydrous sodium sulfate. Then, the extract was concentrated at 50°C on a rotary evaporator to obtain SPOMFs. The final product was stored in a sealed aluminum pot at \textasciitilde 20°C before use.

2.3 Two-step crystallization of SPOMFs
The crystallization of SPOMFs was performed at a controlled temperature in a 100 mL batch reactor. In general, SPOMFs of 5 g was mixed with a solvent at a certain ratio, and then the mixture was allowed to crystallize at a selected temperature. At the end of the crystallization, the crystals were removed by low-temperature filtration to obtain the liquid fraction 1 that was rich in palmoleic acid. Samples were obtained at regular time intervals, and the solvent in the sample was evaporated under reduced pressure at 50°C. The fatty acid composition was analyzed by GC after the samples were diluted to 2 mg SPOMF/mL.

Several parameters were optimized, which were crystallization temperature, type of solvent, substrate ratio of SPOMFs to solvent (w/v) and crystallization time. Five solvents were used to enrich palmoleic acid, and they were hexane, methanol, ethanol, acetone and isopropanol. Crystallization temperature was in the range of 0°C to \textasciitilde 40°C. Six different SPOMFs-to-solvent ratios in the range of 1:3 to 1:18 (w/v) were used, and it was found that the best range for the investigation was 1:9 to 1:18. The crystallization time ranged from 4 h to 16 h. While one of these parameters was being optimized, the others were held at a fixed level. After one parameter optimization was completed, the optimal value of this parameter was used for the optimization of other parameters. The design for these optimization experiments is outlined in Table 1. The yield and content of palmoleic acid were used as the main response variables for the selection of optimum conditions.

As a small amount of saturated fatty acids still remained in the liquid fraction 1, a secondary crystallization was conducted for further concentration of palmoleic acid. First, the solvent in the liquid fraction 1 was evaporated at 50°C to obtain palmoleic acid product. Subsequently, secondary crystallization was conducted at \textasciitilde 40°C by mixing 5 g palmoleic acid product obtained from the primary crystallization with 20 mL methanol for 6 h. The resulting crystals were separated from the liquid by filtration, giving liquid fraction 2. The fatty acid composition was analyzed by GC and the yield of palmoleic acid was based on the following equation by the solvent was removed:

\[
\text{Yield of palmoleic acid (\%)} = \frac{\text{mass of palmoleic acid}}{\text{mass of crude oil}} \times 100\%
\]
Table 1  Experimental design for optimization of palmitoleic acid purification by a combination of solvent crystallization and molecular distillation.

| Level | Solvent crystallization | Secondary crystallization | Molecular distillation |
|-------|-------------------------|---------------------------|-----------------------|
|       | X₁ (°C) | X₂ | X₃ (w/v) | X₄ (h) | X₁ (°C) | X₁ (°C) |
| 1     | 0       | methanol | 1:9 | 4 | -40 | 95 |
| 2     | -20     | ethanol | 1:12 | 8 | 100 |
| 3     | -40     | acetone | 1:15 | 12 | 105 |
| 4     | Hexane  | 1:18 | 16 | 110 |
| 5     | isopropanol | | | |

X₁ = temperature, X₂ = solvent type, X₃ = ratio of SPOMFs to solvent, X₄ = time.

2.4 Molecular distillation of liquid fraction 2

The palmitoleic acid concentrate obtained from the two-step crystallization was further enriched using molecular distillation in a standard glass evaporator (KDL 1, UIC GmbH, Alzenau-Hoerstein, Germany). Molecular distillation was performed varying the evaporation temperature from 95°C to 110°C to obtain a liquid containing a high proportion of palmitoleic acid in the distillate. The other operation parameters were fixed [17], and were as follows: a rotation speed of 400 rpm, a feed temperature of 65°C, a feed rate of 1 mL/min, a vacuum level of 10⁻³ mbar and a condenser temperature of 55°C. Finally, the composition of fatty acid was analyzed by GC and the yield of palmitoleic acid was calculated after molecular distillation.

2.5 Fatty acid composition analysis

Esterification of SPOMFs (50 mg) was achieved using 25% boron trifluoride (BF₃) in methanol (2 mL) at 70°C for 3 min. The resulting FAMEs were extracted with hexane (2 mL). The upper layer was separated and dried over anhydrous sodium sulfate, centrifuged at 8000 rpm for 5 min, and then used for GC analysis.

The FAMEs were separated by GC (7820A, Agilent, USA) equipped with a flame-ionization detector (FID) using a DB-Fast FAME column (30 m × 0.25 mm × 0.25 μm). The injector and FID temperatures were 250°C. The initial column temperature was maintained at 80°C for 0.5 min and then increased to 165°C at a rate of 40°C/min, following which it was maintained at 165°C for 1 min. Finally, the temperature was increased to 230°C (at a rate of 4°C/min) and held at 230°C for 4 min. The running time of the entire program was 23.875 min. The FAMEs were identified and quantified by comparison of the retention times of the sample peaks with those of a mixture of the FAME standards. The content of a specific fatty acid was calculated based on its peak area relative to the total peak area of a particular sample.

2.6 Statistical analysis

All analyses were carried out in duplicates, and the results were expressed as means ± standard deviations (SD). Statistical analyses were performed using the SPSS 24.0 (SPSS, Chicago, IL, USA), and one-way analysis of variance (ANOVA, Tukey’s test) was performed to identify differences (p < 0.05, significant difference).

3 Results and Discussion

The goal of this study was to enrich palmitoleic acid. As shown in Table 2, palmitic acid is the most abundant saturated fatty acid in the SPOMFs, accounting for 33.59% of total fatty acids, followed by oleic acid (32.16%), and palmitoleic acid (27.17%).

First, temperature for low-temperature crystallization was evaluated, and then solvent type, substrate ratio of SPOMFs to solvent (w/v) and crystallization time during the first crystallization stage were optimized to increase palmitoleic acid content in the concentrates. Some residual saturated fatty acids still remained in the collected liquid fraction 1, thus a secondary crystallization was performed at -40°C to remove these, and this resulted in the formation of the liquid fraction 2. Then, the liquid fraction 2 was subject to molecular distillation to produce a final palmitoleic acid concentrate.

3.1 Effect of the crystallization temperature

First, the effect of crystallization temperature (0°C, -20°C, and -40°C) on palmitoleic acid content and yield was investigated. Crystallization conditions were selected to be 1:12 ratio of SPOMFs to solvent (w/v) and 12 h. When the free fatty acids are mixed with a selected solvent and then stored at a low temperature, saturated fatty acids, which have higher melting points and lower solubility in solvent, will undergo crystallization and unsaturated fatty acids will remain in the liquid fraction [18]. The content of palmitic acid in SPOMFs was 33.59%, and the total contents of saturated fatty acids were 35.48% (Table 2). Therefore, the main goal of the crystallization process was...
to remove saturated fatty acids. In this study, two solvents (methanol and acetone) were selected to examine the effect of crystallization temperature. As shown in Figs. 1a and 1b, when the crystallization was conducted at 0°C in methanol and acetone, the yield of palmitoleic acid was 93.21% and 94.88%, and the content of palmitoleic acid was 30.75% and 28.73%, respectively. Therefore, the increase of palmitoleic acid content in the liquid fraction 1 was limited at 0°C. When the crystallization was conducted at −20°C, the levels of palmitoleic acid using methanol and acetone as solvents increased to 38.55% and 33.82%, respectively, which were significantly higher than the initial content. In addition, the palmitoleic acid product obtained at −20°C had a significantly higher content compared to 0°C regardless of which solvent was used. However, when the crystallization temperature was further decreased to −40°C, the content of palmitoleic acid decreased slightly compared to that at −20°C. The results might be due to the crystallization of palmitoleic acid from the solution at a lower temperature, causing the removal of palmitoleic acid from the liquid fraction.

The yield of palmitoleic acid, as shown in Fig. 1b, was the lowest at −40°C compared to yields at other temperatures (−20 and 0°C). The yield of palmitoleic acid showed a significant reduction with decreased temperature because more fatty acid crystals formed at a very low temperature. The results are in agreement with those in a previous study. Taking both of palmitoleic acid content and yield into account, −20°C was determined as the optimum temperature, and this temperature was used in the following crystallization process.

3.2 Effect of various solvent types

In this study, hexane, methanol, ethanol, acetone and isopropanol were examined for the palmitoleic acid enrichment.

### Table 2  Fatty acid composition of SPOMFs, liquid fraction 1, liquid fraction 2 and the distillate.

| Fatty acid     | SPOMFs     | Liquid fraction 1 | Liquid fraction 2 | The distillate |
|----------------|------------|-------------------|-------------------|---------------|
| Myristic acid  | 0.37 ± 0.01<sup>a</sup> | 0.44 ± 0.02<sup>b</sup> | ND                | 0.74 ± 0.01<sup>c</sup> |
| Palmitic acid  | 33.59 ± 0.12<sup>a</sup> | 9.37 ± 0.06<sup>b</sup> | 2.13 ± 0.08<sup>c</sup> | 7.92 ± 0.22<sup>d</sup> |
| Palmitoleic acid| 27.17 ± 0.01<sup>a</sup> | 40.55 ± 0.16<sup>b</sup> | 45.22 ± 0.12<sup>c</sup> | 54.18 ± 0.27<sup>d</sup> |
| Stearic acid   | 1.52 ± 0.05<sup>b</sup> | 0.40 ± 0.11<sup>c</sup> | ND                | ND            |
| Oleic acid     | 32.16 ± 0.02<sup>d</sup> | 41.33 ± 0.48<sup>a</sup> | 45.06 ± 0.18<sup>b</sup> | 31.66 ± 0.38<sup>c</sup> |
| Linoleic acid  | 3.50 ± 0.05<sup>b</sup> | 5.02 ± 0.28<sup>c</sup> | 5.11 ± 0.13<sup>d</sup> | 3.76 ± 0.06<sup>a</sup> |
| Linolenic acid | 1.70 ± 0.05<sup>b</sup> | 2.34 ± 0.04<sup>c</sup> | 2.50 ± 0.11<sup>d</sup> | 1.76 ± 0.05<sup>a</sup> |
| SFA            | 35.48 ± 0.23<sup>b</sup> | 10.21 ± 0.03<sup>c</sup> | 2.13 ± 0.08<sup>d</sup> | 8.66 ± 0.23<sup>a</sup> |
| UFA            | 64.53 ± 0.04<sup>c</sup> | 89.24 ± 0.32<sup>a</sup> | 97.52 ± 0.11<sup>b</sup> | 91.36 ± 0.22<sup>d</sup> |

SFA, saturated fatty acids, SFA = myristic acid + palmitic acid + stearic acid; UFA, unsaturated fatty acids. UFA = palmitoleic acid + oleic acid + linoleic acid + linolenic acid; ND, not detected;

Values in the same row with different letters are significantly different at $p < 0.05$.
ment. These solvents are permitted at parts-per-million (ppm)/residual concentrations in pharmaceuticals according to the United States Pharmacopoeia (USP). Methanol is a Class 2 solvent associated with an allowable concentration limit of 3000 ppm\(^{21}\). Acetone, a polar solvent, is commonly selected as crystallization solvent for the separation of triglycerides and free fatty acids\(^{22}\). It is a permitted solvent in food industry. Hexane, a nonpolar solvent, is less toxic than methanol and widely used as the extraction solvent in oilseed crushing plants\(^{23}\). Isopropanol is a relatively cheap solvent and it can mix with water at any ratios. It has higher stronger solubility to lipophilic substances than ethanol, so it may be a suitable solvent for the purification of palmitoleic acid. In addition, these solvents were chosen based on their polarity. Hexane has the lowest polarity, followed by isopropanol, acetone, ethanol and methanol. Thus, both of polar and nonpolar solvents were considered in the enrichment of palmitoleic acid.

**Figure 2a** shows the effect of solvent type on the content and yield of palmitoleic acid. Initially, crystallizations were performed with 5 g SPOMFs and 60 mL solvent at \(-20^\circ C\) for 12 h to investigate the effect of solvent type on palmitoleic acid enrichment. When methanol was used as the crystallization solvent, the palmitoleic acid content in the concentrate was up to 38.55\%, which was significantly higher than those with other solvents. Therefore, methanol gave the highest content of palmitoleic acid in the liquid fraction 1, followed by hexane, acetone, isopropanol and ethanol. For acetone, isopropanol and ethanol, the increase of palmitoleic acid in the fraction was very limited and insignificant differences in palmitoleic acid level were found among these three solvents. The results obtained in a previous study is consistent with those of this experiment, suggesting that hexane was not the most suitable solvents for the concentration of free fatty acids by low-temperature crystallization\(^{22}\).

Subsequently, the effect of solvent type on palmitoleic acid yield was also investigated. The results are shown in **Fig. 2a**. The yield ranged from 88.47\% to 92.57\% when different solvents were used to enrich palmitoleic acid. However, there were no statistical differences observed in the palmitoleic acid yield among solvents due to a big deviation. Thus, taking both purity and yield into account, methanol was selected as an optimal solvent for further crystallization treatments. Under these optimized conditions, 38.55\% palmitoleic acid was obtained with an 88.47\% yield after crystallization.

### 3.3 Effect of the ratio of SPOMFs to solvent (w/v)

Subsequently, the effects of the ratio of SPOMFs to solvent (w/v) on purity and yield of palmitoleic acid were investigated, and the results are shown in **Fig. 2b**. When crystallization was carried out at \(-20^\circ C\), our preliminary study showed that SPOMFs: methanol ratios from 1:3 to 1:6

![Figure 2a](image-url)  
**Figure 2** Optimization of crystallization conditions at the first crystallization stage. Crystallization conditions  
(a): 5 g SPOMF, 60 mL solvent [SPOMF:solvent ratio 1:12 (w/v)], crystallization temperature of \(-20^\circ C\), crystallization time of 12 h;  
(b): 5 g SPOMF, SPOMF to methanol ratio of 1:9 to 1:18 (w/v), crystallization temperature of \(-20^\circ C\), crystallization time of 12 h;  
(c): 5 g SPOMF, 75 mL methanol [SPOMF to methanol ratio 1:15 (w/v)], crystallization temperature of \(-20^\circ C\), crystallization time of 4 h to 12 h.

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were inappropriate, because the solution was froze too quickly to allow monitoring. Therefore, the examined SPOMF to methanol ratios were set to be 1:9, 1:12, 1:15 and 1:18 (w/v), and the methanolic SPOMF solutions were stored at −20°C for 12 h.

At shown in Fig. 2b, when the ratio of SPOMFs to methanol (w/v) was changed from 1:9 to 1:15, the purity of the palmitoleic acid increased from 36.32% to 40.43%. This is because saturated fatty acid was effectively crystallized out from the solution and palmitoleic acid remained in the liquid fraction at the ratio range26. However, as the SPOMFs to methanol ratio was beyond 1:15, the proportion of palmitoleic acid in solution decreased, indicating increased solubilization of saturated fatty acids with increased solvent volume.

In terms of yield, the yield of palmitoleic acid increased with the increase of SPOMFs to methanol ratio from 1:9 to 1:15. Increasing SPOMFs to methanol ratio further from 1:15 to 1:18 led to the decrease of palmitoleic acid. However, no significant difference was found between SPOMFs to methanol ratios of 1:12 and 1:15. Considering both purity and yield of palmitoleic acid, an SPOMFs to methanol ratio of 1:15 (w/v) gave the best result. Hence, an SPOMF to methanol ratio of 1:15 (w/v) was used for further crystallization studies. Under these conditions, a solution with 40.43% palmitoleic acid was obtained with an 88.47% palmitoleic acid yield.

3.4 Effect of crystallization time

Time has an influence on the mass transfer between the solid and liquid phases in the crystallization process25. Thus, four crystallization times (4 h, 8 h, 12 h and 16 h) were investigated with the SPOMFs to methanol ratio of 1:15 at −20°C. It was observed that the change of crystallization time had a significant influence on the purity and yield of palmitoleic acid as shown in Fig. 2c. Overall, the purity of palmitoleic acid increased with time, whereas the yield decreased with time. This is because the target fatty acid (palmitoleic acid) was also crystallized with saturated fatty acids with time, resulting in a gradual decrease in the yield of palmitoleic acid25. When the crystallization time ranged from 4 to 16 h, the purity of palmitoleic acid ranged from 27.17% to 41.55%. There were only slight increases in palmitoleic acid purity from 12 h to 16 h, suggesting that crystallization was almost complete at 16 h. Longer time caused the crystallization of the palmitoleic acid, indicated a significantly lower palmitoleic acid yield. Thus, 12 h was selected as an optimal time.

3.5 Secondary crystallization of liquid fraction 1

After completing the optimization of crystallization conditions at the first stage, the fatty acid composition of the liquid fraction 1 was obtained under the optimal conditions. As shown in Table 2, the liquid fraction 1 contained low contents of saturated fatty acids, with palmitic acid being the most abundant, accounting for 9.37% of the total fatty acids. It is important that the fatty acids having ≤ 16 carbons are removed as much as possible from the liquid fraction because subsequent molecular distillation can only separate palmitoleic acid from the C18 fatty acids, but cannot separate palmitoleic acid from palmitic acid. However, it has been proven that multistage fractionation can further improve the palmitoleic acid purity20. Based on these considerations, secondary crystallization of the liquid fraction 1 was performed to remove palmitic acid.

Considering that methanol has a good effect on the crystallization of palmitic acid in the primary crystallization process, we chose to use methanol for secondary crystallization. The supersaturation of the substance in the solvent is closely related to the temperature. With the decrease of temperature, the solubility of fatty acids in solvent also decreased gradually. Therefore, we selected a lower temperature for palmitic acid to reach supersaturation and form crystals in methanol. In this way, a small amount of palmitic acid was further removed by secondary crystallization. A liquid fraction 2 was collected after re-crystallization at −40°C. As shown in Table 2, the purity of palmitoleic acid in the liquid fraction 2 by secondary crystallization was 45.22%, with a yield of 95.49%. The content of palmitic acid was significantly reduced, from 9.37% to 2.13% in this fraction. Therefore, re-crystallization led to a significant increase of palmitoleic acid content in the liquid fraction, from 40.55% to 45.22%. In addition, the oleic acid content in the liquid fraction 1 significantly differed from that in the liquid fraction 2, and no statistically significant differences were found in the proportions of linoleic acid and linolenic acid in solution after secondary crystallization. It should be noted that these contents are relative percentages. If one fatty acid is removed, others will tend to increase in relative percentage values.

Overall, secondary crystallization significantly decreased the proportion of saturated fatty acids in the liquid phase, and thus the unsaturated fatty acid contents in the liquid fraction 2 reached 97.52% after two-step crystallization after the solvent was removed. Finally, palmitoleic acid-enriched material was enriched further by molecular distillation.

3.6 Effect of molecular distillation temperature on palmitoleic acid enrichment

Molecular distillation can be used for the separation of mixtures with different molecular weight and partial vapor pressure, and for the separation of homologs27,28,29. In general, molecular distillation is conducted under vacuum conditions, which decreases the evaporation temperature and the residence time27,29. Table 2 shows that the oleic acid in the liquid fraction 2 was 45.06% of the total fatty acids, which was approximately the same as the proportion of palmito-
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Table 3  Fatty acid composition of the distillate obtained by molecular distillation.

| Fatty acid     | 95°C     | 100°C    | 105°C    | 110°C    |
|----------------|----------|----------|----------|----------|
| Myristic acid  | 0.75 ± 0.01<sup>a</sup> | 0.74 ± 0.01<sup>a</sup> | 0.71 ± 0.05<sup>a</sup> | 0.67 ± 0.00<sup>a</sup> |
| Palmitic acid  | 8.09 ± 0.06<sup>b</sup> | 7.92 ± 0.22<sup>b</sup> | 7.66 ± 0.14<sup>b</sup> | 7.57 ± 0.01<sup>b</sup> |
| Palmitoleic acid | 58.4 ± 0.13<sup>b</sup> | 54.18 ± 0.27<sup>b</sup> | 53.56 ± 0.61<sup>b</sup> | 53.49 ± 0.51<sup>b</sup> |
| Stearic acid   | ND       | ND       | ND       | ND       |
| Oleic acid     | 28.47 ± 0.06<sup>b</sup> | 31.66 ± 0.38<sup>b</sup> | 32.44 ± 0.71<sup>b</sup> | 32.61 ± 0.47<sup>b</sup> |
| Linoleic acid  | 2.91 ± 0.03<sup>b</sup> | 3.76 ± 0.06<sup>b</sup> | 3.86 ± 0.08<sup>b</sup> | 3.87 ± 0.06<sup>b</sup> |
| Linolenic acid | 1.46 ± 0.07<sup>b</sup> | 1.76 ± 0.05<sup>b</sup> | 1.79 ± 0.00<sup>b</sup> | 1.8 ± 0.01<sup>b</sup> |
| Yield (%)      | 17.79 ± 1.56<sup>b</sup> | 63.64 ± 1.43<sup>b</sup> | 65.65 ± 1.74<sup>b</sup> | 64.14 ± 1.13<sup>b</sup> |

ND, not detected;
Values in the same row with different letters are significantly difference at <i>p</i> < 0.05.

Palmitoleic acid. Oleic acid is similar in structure to palmitoleic acid, with the former having two more carbons but with both containing one double bond. These two fatty acids are difficult to separate by crystallization. Thus, molecular distillation was applied to separate palmitoleic acid from oleic acid.

The chain length and the number of double bonds of a fatty acid affect its boiling point, and the distillation order of palmitoleic acid > oleic acid = linoleic acid<sup>27</sup>. Thus, oleic acid is expected to remain in the residue while palmitoleic acid would be collected in the distillate under a proper temperature. In a study of Solaesa <sup>14</sup>, saturated fatty acids such as myristic acid and palmitic acid were evaporated in the distillate at 125°C under a vacuum level of 10<sup>−3</sup> mbar. Thus, considering the fatty acid composition difference in the feedstock in this study, evaporation temperature was first set at 120°C to separate palmitoleic acid from other fatty acids. Almost all of the fatty acids were evaporated at such a temperature. All products went to the distillate, resulting in a poor enrichment of palmitoleic acid. At 95°C, palmitoleic acid was enriched into the distillate in a high concentration, but the yield of the distillate was only 17.79%.

Table 3 shows that as the temperature increased, a greater yield (about 65%) of palmitoleic acid was obtained. The purity of the distillate was ~54% in temperature range of 100°C to 110°C. There were no significant differences in palmitoleic acid content and yield among the selected temperatures. Accordingly, 100°C was considered to be the best temperature for molecular distillation. Thus, by combining the above steps of crystallization and distillation, a 54.18% content of palmitoleic acid was obtained, with an overall yield of 56.31%.

4 Conclusion
In this study, palmitoleic acid was enriched by combining crystallization and molecular distillation, leading to a high yield of a liquid containing a high proportion of palmitoleic acid. Methanol was found to be the optimum organic solvent to increase palmitoleic acid concentration in solution by crystallization. The optimal crystallization conditions were SPOMFs:methanol ratio of 1:15 (w/v), a crystallization temperature of ~ 20°C, and incubation time of 12 h. Then, molecular distillation performed at 100°C led to a distillate that contains 54.18% palmitoleic acid in a yield of 56.31%, which was twice as enriched as the raw material. This demonstration of an effective method for obtaining a concentrated palmitoleic acid fraction from a natural source provides basis for industrial scale-up process of this unique fatty acid for the food and pharmaceutical industries.

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Conflict of Interest
The authors declare that they have no conflict of interest.

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