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Extraction and Purification of Kiwifruit Seed Oil Using Three-Phase Partitioning: An Efficient and Value-Adding Method for Agro-Industrial Residue Utilization

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Abstract: Kiwifruit seeds are usually discarded as waste in the kiwifruit processing industry. However, kiwifruit seeds are rich in oil, which implies their value as a potential resource. Therefore, three-phase partitioning (TPP) using ammonium sulfate and n-butanol has been developed to extract kiwifruit seed oil (KSO) from discarded kiwifruit seeds. The factors influencing the extraction process have been investigated. The highest extraction yield of 36.06% for KSO was obtained under the following conditions: an extraction time of 18 h, a liquid-solid ratio of 4:1, an amount of ammonium sulfate of 28.5 wt%, and a phase ratio (top phase/bottom phase) of 1/1. The composition of obtained KSO was analyzed by gas chromatography-mass spectrometry (GC-MS), and the results indicated the high content of α-linolenic acid. This simple and low-cost method can be used as an efficient approach for utilizing the value of kiwifruit seeds.

Keywords: kiwifruit seed oil; extraction and separation; three-phase partitioning; waste utilization

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1. Introduction

Kiwifruit (*Actinidia chinensis* Planch.) is a valuable agricultural produce that grows in the central and southern regions of China [1]. Kiwifruit contains a variety of essential nutrients, such as amino acids, proteins, polysaccharides, vitamin C, and minerals [2,3]. Kiwifruits are abundant in vitamin C, which can strengthen the immune system and promote wound healing and absorption of iron. The content of vitamin C in fresh kiwifruit is 3–14 times that of citrus, and 30–140 times that of pears. Kiwifruits are also abundant in inositol and amino acids, which can inhibit depression and supplement the nutrients consumed by mental work. The perfect ratio of low sodium and high potassium in kiwifruits can supplement the physical energy lost by staying up late and working overtime, and is beneficial for maintaining cardiovascular health [4]. In recent decades, there has been rapid progress in the industrial processing of kiwifruit. Kiwifruit has been processed to obtain various food products, including juice, jam, jelly, syrup, and candy [5–7]. Kiwifruit seeds, as a by-product of kiwifruit processing, are usually discarded as agro-industrial residue [8]. Kiwifruit seeds are abundant in a variety of unsaturated fat acids, lipids, flavonoids, phenols, vitamins, trace element selenium, and other bioactive substances. Linoleic acid, linolenic acid, and other main unsaturated fat acids account for more than 75% of the total quantity of unsaturated fat acids, especially linolenic acid content of 64.1% of the total quantity of unsaturated fat acids, which is currently the highest in natural vegetable oils except for perilla oil, and is also the premium resource for high-quality natural polyenoic acids. Modern research and clinical experiments have demonstrated that kiwifruit seed oil (KSO) possesses the ability to regulate blood lipids, soften blood vessels, improve obesity, and

delay aging [1,9]. Berry et al. conducted intervention tests on 399 male healthy subjects, and found that systolic pressure, diastolic pressure, and mean arterial pressure decreased by 5 mmHg [10], accompanied by α-linolenic acid increase of 1% in human adipose tissue. Ghafoorunissa et al. fed newly lactating rats with saturated fat acid, monounsaturated fatty acid, and polyunsaturated fatty acid, respectively, for 3 months, and induced insulin resistance with sucrose instead of starch [11]. The results showed that using α-linolenic acid instead of linoleic acid in food could improve insulin sensitivity of sucrose-induced rats. For these reasons, KSO has broad applications in medicine, health food, and beauty products [12–14]. Therefore, it is worthy to recover the KSO from discarded kiwifruit seeds.

In previous studies, organic solvent extraction, supercritical extraction, and subcritical extraction were commonly used to extract other oil and KSO [15–17]. Renata et al. used compressed propane as part of a liquid–liquid extraction system for separation of kiwi fruit seed oil and compared the total extraction rate with the Soxhlet extraction using n-hexane [2]. Most extraction conditions using compressed propane have high extraction rates and a total extraction rate of 31.7 ± 1.4%. Nedasadat et al. used supercritical extraction and mathematical modeling for Elaeagnus angustifolia oil [11]. The experimental design and optimization were completed through Box Behnken design (BBD) to analyze the impact of main operating parameters, including pressure, temperature, and particle size. The highest extraction rate (84.9% w/w) was achieved under the optimal conditions. However, these methods have some drawbacks. For example, the organic solvent extraction method is simple; however, additional purification steps are required to eliminate impurities such as pigments and proteins. Supercritical extraction and subcritical extraction are efficient, but require expensive equipment, which can be a significant cost factor. Therefore, it is necessary to find a simple, efficient, and low-cost method for the extraction and purification of KSO.

Three-phase partitioning (TPP) is a cheap and efficient separation technology first developed in 1972 [18]. Usually, TPP was performed by adding ammonium sulfate and t-butanol to the crude extract [19,20]. TPP has been widely used for the extraction and purification of various bioactive components, including polysaccharides, proteins, enzymes, oils, and other substances [21–27]. TPP had been previously applied in the separation and purification of proteins and enzymes by the formation of a solid middle phase to effectively preserve their biological activity. Subsequently, it was found that TPP also exhibited excellent performance in the extraction and purification of plant oils. For example, TPP based on t-butanol and ammonium sulfate was used to extract oil from *Crotalaria juncea* seeds to obtain a yield of 37%, which was much higher than that (13%) obtained by Soxhlet extraction [28]. In our previous study, enzyme-assisted TPP based on t-butanol and ammonium sulfate was also developed for the extraction of flaxseed oil [29], and 71.68% extraction yield was obtained under the optimal conditions. Through TPP was used for oil extraction, a preliminary purification can be achieved. In this process, impurities such as proteins or pigments are either dissolved in the salt phase or deposited in the intermediate phase, leaving the oil enriched and separated in the top phase.

In this study, TPP was initially performed using n-butanol and ammonium sulfate. This system was then employed for the extraction, separation, and purification of oil from discarded kiwifruit seeds. Single-factor experiments were conducted to investigate the influencing factors involved in the preparation of crude extract. These factors include the type of solvent, the extraction time, and the solid-liquid ratio. Then TPP was developed for the separation and purification of KSO by adding salts to the crude extract. The types of salts added to the system, as well as the effects of salt concentration and phase ratio (top n-butanol phase/bottom salt phase), were investigated. With these single factor experiments, the optimal conditions for separating kiwifruit seed oil through TPP were determined, and the final extraction yield of oil was 79.23% under the optimal conditions of the total oil content. Finally, the main components of KSO extracted by TPP were analyzed by GC-MS. The highest content of oil obtained is α-linolenic acid (51.26%), followed by Squalene

(15.07%), and then Linoleic acid (14.49%), which are more than 10% of all other components. This TPP method can provide some guidance for kiwifruit processing industry.

2. Materials and Methods

2.1. Materials and Reagents

The kiwifruit seeds were provided by Xiangxi Laodie Biotechnology Co., Ltd. (Jishou City, China). Kiwifruit seeds were crushed and passed through a 40-mesh sieve to obtain fine and uniform particles. Intermittent operation was employed during the crushing process to prevent excessive heat buildup. Kiwifruit seeds were dried to constant weight at 60 °C in an electric thermostatic drying oven. Ethanol (>98%), acetone (>98%), n-propanol (>98%), isopropanol (>98%), t-butanol(>98%), n-butanol (>98%), ammonium sulfate ($(NH_4)_2SO_4$) (>98%), sodium chloride (NaCl) (>98%), anhydrous sodium carbonate (Na_2CO_3) (>98%), anhydrous sodium sulfate (Na_2SO_4) (>98%), dipotassium hydrogen phosphate trihydrate ($K_2HPO_4 \cdot 3H_2O$) (>98%), and ammonium citrate ($C_6H_5O_7(NH_4)_3$) (>98%) were purchased from Shanghai Titan Technol. Co., Ltd. (Shanghai, China). All the reagents are of analytical reagent grade and used without further treatment.

2.2. Preparation of the Crude Extract

Kwifruit seeds of 2.0 g and an organic solvent (ethanol, acetone, n-propanol, isopropanol, t-butanol, or n-butanol) of 4.0 mL were added to a tube. The amount of kiwifruit seeds added was accurately weighed. For the extraction of KSO, various intensification methods were employed, including ultrasonic treatment, microwave irradiation, high-temperature conditions, or stirring. These techniques were used to enhance the extraction process and improve the yield of kiwifruit seed oil. After extraction, the supernatant was separated by centrifugation at 4000 r/min for 5 min. After determining the optimal conditions for the extraction stage, the crude extract of KSO was obtained under the optimal conditions, then stored in a refrigerator for further use.

2.3. Extraction of KSO Using TPP

TPP was performed by adding certain concentration of ammonium sulfate solution to the crude extract of KSO. This led to the formation of a three-phase system comprising a solvent top phase, a solid middle phase, and a salt bottom phase. The solvent top phase, which contained the desired kiwifruit seed oil, was collected from the TPP system. Subsequently, the solvent was removed using rotary evaporation and then dried in a vacuum oven until reaching a constant weight, resulting in the isolated oil as the final product. The oil weight was measured, and the extraction yield (Y, %) of KSO was calculated using Equation (1).

$$Y = \frac{Mass\ of\ obtained\ KSO}{Mass\ of\ Kiwifruit\ seeds} \times 100\% \quad (1)$$

2.4. Low Field Nuclear Magnetic Resonance Relaxation Measurements

KSO is measured using low field nuclear magnetic resonance instrument (MesoMR23-060H-I, Suzhou Niumag Analytical Instrument Co., Ltd., Suzhou, China) [30]. The analyzer operates at 32 °C and has a proton resonance of 20 MHz, with a sample tube diameter of 25 mm. The sequence measurement of spin lateral relaxation of the Carr Purcell Meiboom Gill (CPMG) sequence was used. This sequence has the following parameters: 90° pulse width (P1) = 5.52 μs,180 ° pulse width (P2) = 12.48 μs, waiting time (TW) = 2000 ms, number of scans (NS) = 32. The T2 distribution curve is fitted with multiple exponents using the Simultaneous Iterative Reconstruction Technique (SIRT) algorithm. The relaxation time, peak area, and ratio of peak area were recorded.

2.5. GC-MS Analysis of KSO

The components of KSO were analyzed by using a GC-MS (an Agilent 7890B GC coupled with a 5977A MS). For this purpose, the KSO was first esterified to fatty acid methyl ester according to previous reports [31]. The specific details are as follows:100 µL KSO were taken on centrifuge tube and 2 mL of n-hexane were added into the centrifuge tube, then 2 mL of 0.4 mol/L NaOH methanol solution were added to the centrifuge tube. The centrifuge tube was sealed and vibrated violently. Then it was placed at 70 °C water bath for heating and refluxing for 10 min. These solutions were then transferred to another graduated centrifuge tube, and saturated salt water was added to 10 mL, shaken and centrifuged. The top layer of solution was taken and subjected to 0.22 µm membrane filtration. The fatty acid methyl ester was analyzed by GC-MS. The detailed analysis conditions are listed in Table 1.

Table 1. The GC-MS conditions for the analysis of KSO.

GC Conditions		MS Conditions	
Chromatographic column	HP-5MS (30.0 m × 250 µm, 0.25 µm)	Tune	Autotune
Carrier gas	He	Acquisition mode	scan mode
Carrier gas flow rate	1.0 mL/min	Electron energy	70 eV
Oven program	Initial 50 °C (hold 5 min); 150 °C (hold 0 min) at 15 °C/min; 180 °C (hold 0 min) at 5 °C/min; 325 °C (hold 10 min) at 8 °C/min; max 350 °C (1 min equilibrium time)	Solvent delay	3.5 min
Inlet temperature	320 °C	MS temperature	230 °C (Source); 150 °C (Quad)
Transmission line temperature	320 °C	Scan range	20–600 amu
Injection mode	Split (2:1)		
Injection volume	0.2 µL		

2.6. Statistical Analysis

Determination was repeated three times in this study. The statistical analysis was done using the SPSS 26.1 software. The one-way analysis of variance (ANOVA) was used for statistical significance analysis. A "p-value" of less than 0.05 ($p < 0.05$) was regarded as statistical significance. Different letters in all the figures represent the statistically significant difference.

3. Results and Discussion

3.1. Factors Influencing the Preparation of Crude Extract

3.1.1. Screening of the Extractant

The choice of organic solvent plays a crucial role in the extraction of KSO using TPP. The type of solvent used can significantly influence the efficiency and effectiveness of the extraction process. Different solvents may exhibit varying abilities to dissolve and separate the components of kiwifruit seed oil, thereby affecting the overall extraction yield and purity. Therefore, six common solvents were chosen as the extractants with stirring assisted extraction. According to the data presented in Figure 1, it is evident that n-butanol used as the extractant resulted in the highest extraction yield of 19.6% for KSO, surpassing the commonly used t-butanol by a significant margin. This difference in extraction efficiency can be attributed to the non-polar nature of n-butanol, which appears to be better suited for extracting KSO. While t-butanol has demonstrated efficient performance in TPP, the similarity in structure and properties between n-butanol and t-butanol suggests that n-butanol is the more appropriate choice for subsequent studies. n-propanol and isopropanol have the lowest extraction efficiency for KSO. This is because the polarity of propanol series solvents is greater than that of butanol; their viscosity is higher than that of methanol and ethanol at the same time. After careful consideration of these factors, n-butanol was selected for use in the following experiments.

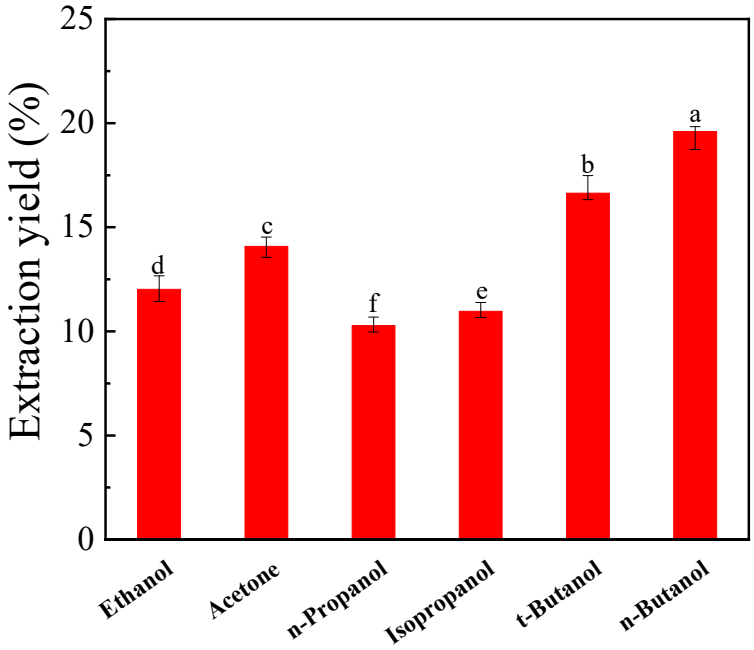

Figure 1. Extraction yields of KSO based on TPP using different organic solvents with stirring assisted extraction. The extraction conditions were as follows: 3:1 liquid–solid ratio, 6 h extraction time, 4/3 phase ratio (top/bottom phase), and 25 wt% ammonium sulfate. Different letters represent the statistically significant difference.

3.1.2. Effect of the Intensification Methods for Extraction

In this study, four intensification methods, namely, ultrasonic treatment, microwave irradiation, high-temperature, and stirring, were utilized to aid the extraction of KSO. The static extraction was used for comparison. The conditions for ultrasound-assisted extraction in this study were as follows: 200 W power ultrasound was applied for 10 min, followed by a resting period of 10 min. This process was repeated six times to complete the extraction procedure. Then, it was allowed to rest for 6 h. The conditions for microwave assisted extraction were as follows: 200 W power microwave was used for 5 min and then stood for 2 min; the procedure was repeated 6 times. The total duration of ultrasound assisted extraction was about 6 h. The high-temperature assisted extraction was performed by placing a tube in a water bath at 60 °C for 6 h using n-butanol as the extractant. The stirring assisted extraction was performed for 6 h at a stirring rate of 200 r/min using n-butanol as the extractant. The static extraction was performed for 6 h using n-butanol as the extractant. As shown in Figure 2, the stirring method has the highest extraction yield of 26.22%. This is because that stirring can make n-butanol efficiently contact with kiwifruit seeds, which is beneficial to the extraction. Therefore, the stirring assisted method was chosen for further experiments.

3.1.3. Effect of Extraction Time

The extraction time was a crucial factor in the solid-liquid extraction process. In this study, a range of 1 to 24 h was selected to investigate its impact on the efficiency and yield of the extraction of KSO from kiwifruit seeds. As shown in Figure 3, the extraction yield of KSO significantly increased in the interval 1–6 h. After that, there was an increase of 0.70% (for 12 h), for 0.74% (for 18 h) and 0.46% (for 24 h), the extraction yield did not significantly increase with a further increase of time. At the early extraction stage, oil can more easily diffuse into the solvent, resulting in the rapid increase of extraction yield [32]. As time increases, oil in the solid phase is more difficult to be extracted. Therefore, considering both extraction efficiency and time cost, an extraction time of 18 h was selected as the extraction condition for subsequent experiments.

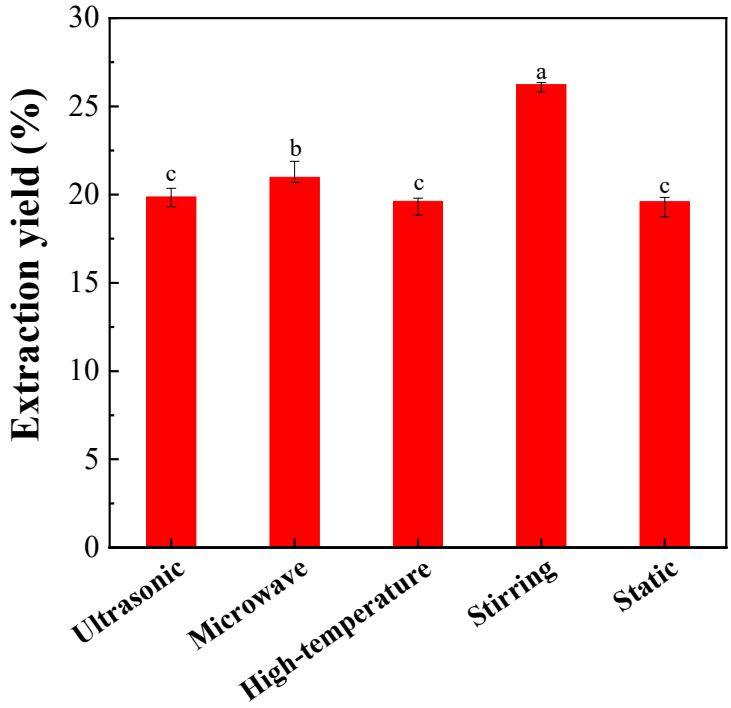

Figure 2. Extraction yields of KSO using n-butanol as the extractant and different intensification methods. The extraction conditions were as follows: 3:1 liquid–solid ratio, 6 h extraction time, 4/3 phase ratio (top/bottom phase), and 25 wt% ammonium sulfate. Different letters represent the statistically significant difference.

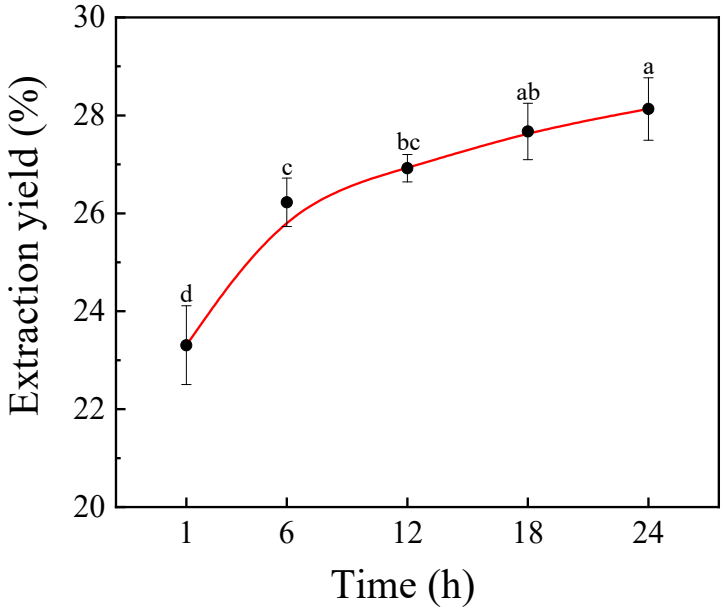

Figure 3. Extraction yields of KSO at different time lengths. The extraction conditions were as follows: n-butanol used as the extractant with stirring assisted extraction, 3:1 liquid-solid ratio, 4/3 phase ratio, and 25 wt% ammonium sulfate. Different letters represent the statistically significant difference.

3.1.4. Effect of Liquid-Solid Ratio

The liquid-solid ratios of 1:1 to 6:1 were compared. As shown in Figure 4, the extraction yield increased when the liquid-solid ratio increased from 1:1 to 4:1, but when the liquid-solid ratio was further increased, the extraction yield decreased. These results may be attributed to the hydraulic effect formed by the narrow and long tube at high liquid levels, which hindered the internal and external oil diffusion. Therefore, a liquid-solid ratio of 4:1 was chosen.

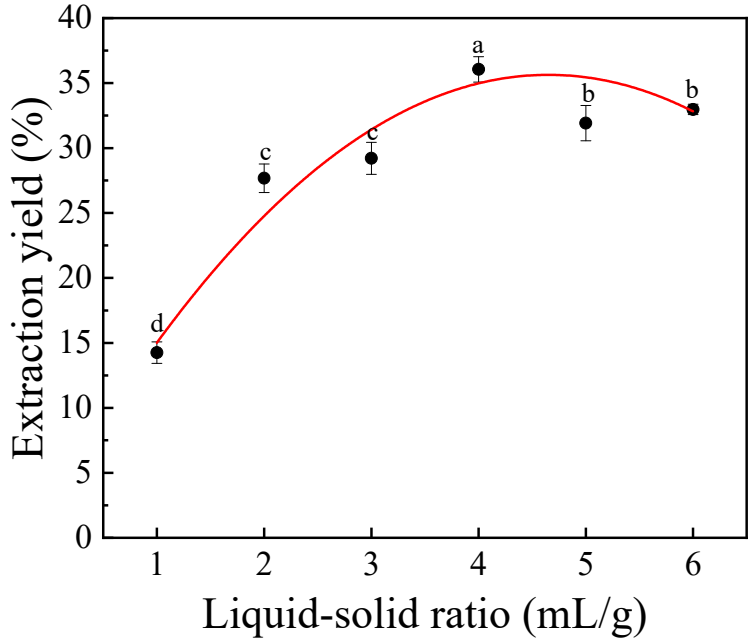

Figure 4. Extraction yields of KSO at different liquid-solid ratios. The extraction conditions were as follows: n-butanol used as the extractant with stirring assisted extraction, 18 h extraction time, 4/3 phase ratio, and 25 wt% ammonium sulfate. Different letters represent the statistically significant difference.

3.2. Factors Influencing the Purification of KSO Using TPP

3.2.1. Effect of Salt Type

As an essential component of TPP, salt plays an important role in the separation process, especially in floating proteins and enzymes through the salting-out effect. The addition of salt induces changes in the solubility of proteins and enzymes in the solvent, leading to their precipitation and separation from the desired KSO, thereby contributing to the overall separation effectiveness [33,34]. Six common types of salt have been compared in this study, including NaCl, Na_2CO_3, Na_2SO_4, K_2HPO_4, $C_6H_5O_7(NH_4)_3$, and $(NH_4)_2SO_4$. As shown in Figure 5, the salt type has a relatively small impact on KSO extraction. The TPP formed by $C_6H_5O_7(NH_4)_3$ results in the lowest extraction efficiency of KSO, as $C_6H_5O_7(NH_4)_3$ has lower phase forming ability than other salts. This is mainly caused by its anions. Conversely, ammonium sulfate has the optimal extraction yield. This is because ammonium sulfate is more suitable for salting out the proteins, enzymes, and other components. Therefore, ammonium sulfate was chosen for subsequent experiments.

3.2.2. Effect of Ammonium Sulfate Concentration

The ammonium sulfate concentration observably affects the extraction efficiency of TPP, so its concentration range of 20–35 wt% was studied in this study. As shown in Figure 6, the maximum extraction yield was obtained at 28.5 wt% ammonium sulfate concentration. When the salt concentration is lower, it is difficult to form three-phase [35]. The salting out effect strengthens with the increase of salt concentration, resulting in an increase in extraction yield. However, when the ammonium sulfate concentration is further increased, the extraction yield decreases, which can be attributed to that the stronger salting out effect influences the dissolving capacity of n-butanol. Therefore, the ammonium sulfate concentration of 28.5 wt% was chosen.

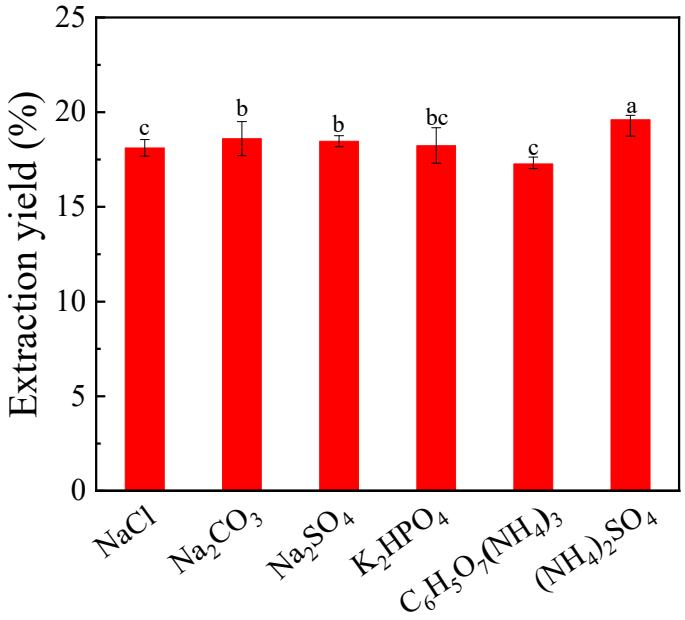

Figure 5. Extraction yields of KSO using different types of salt. The extraction conditions were as follows: n-butanol used as the extractant with stirring assisted extraction, 18 h extraction time, 3:1 liquid–solid ratio, 4/3 phase ratio, and 25 wt% salt concentration. Different letters represent the statistically significant difference.

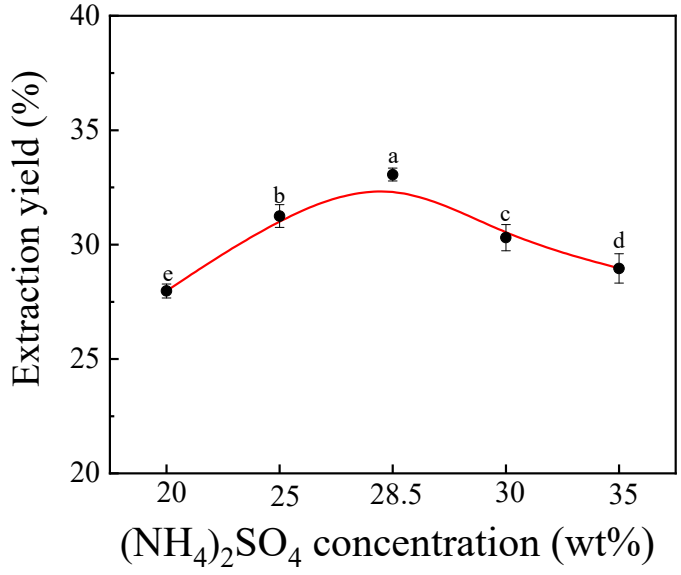

Figure 6. Extraction yields of KSO at different ammonium sulfate concentrations. The extraction conditions were as follows: n-butanol was used as the extractant with stirring assisted extraction, 18 h extraction time, 4:1 liquid–solid ratio, and 4/3 phase ratio. Different letters represent the statistically significant difference.

3.2.3. Effect of Phase Ratio

In the TPP system, the phase ratio (n-butanol top phase/ammonium sulfate bottom phase) was studied. As shown in Figure 7, the maximum extraction yield was obtained at 1/1 phase ratio. When the phase ratio is 4/1, there is no TPP formation. The middle phase rich in protein combines with the salt to form a homogeneous gel phase. Oil is stuck in the gel, resulting in low extraction yield. When the phase volume of salt phase further exceeds the n-butanol phase, the extraction yield decreases. This can be possibly attributed to the insufficient dissolution of oil in the middle phase. Therefore, a phase ratio (n-butanol phase/ammonium sulfate phase of 1/1) was chosen.

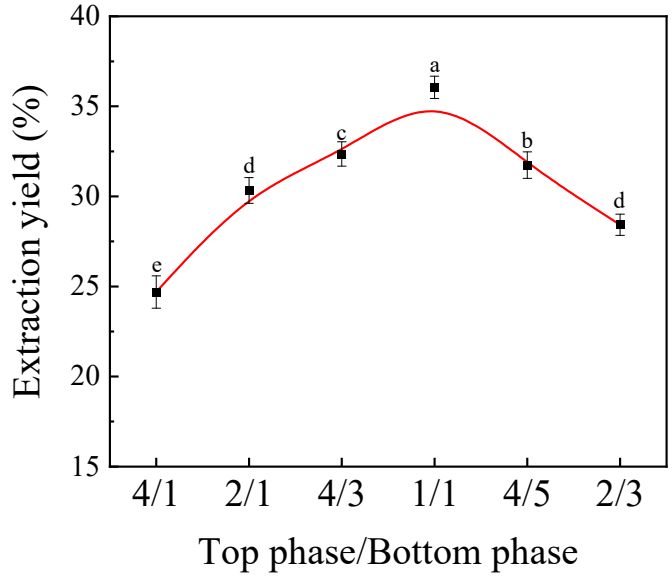

Figure 7. Extraction yields of KSO at different phase ratios. The extraction conditions were as follows: n-butanol used as the extractant with stirring assisted extraction, 18 h extraction time, 4:1 liquid–solid ratio, and 28.5 wt% ammonium sulfate. Different letters represent the statistically significant difference.

3.3. Repeated Extraction of KSO from Kiwifruit Seeds

To maximize the recovery of KSO, repeated extraction was performed using n-butanol. The total oil content was 45.51% when using low field nuclear magnetic resonance for kiwifruit seeds used in this study. The kiwifruit seeds were repeatedly extracted using TPP. As shown in Figure 8, the total extraction yield was 43.70% after five runs of extraction, which was 96.02% of the total KSO content. The first extraction resulted in an extraction yield of 36.06% oil (79.23% of the total oil content), while the second extraction was 5.47% oil. The further repeated extraction resulted in only a low recovery of the oil. Therefore, only one run of extraction was performed for the samples.

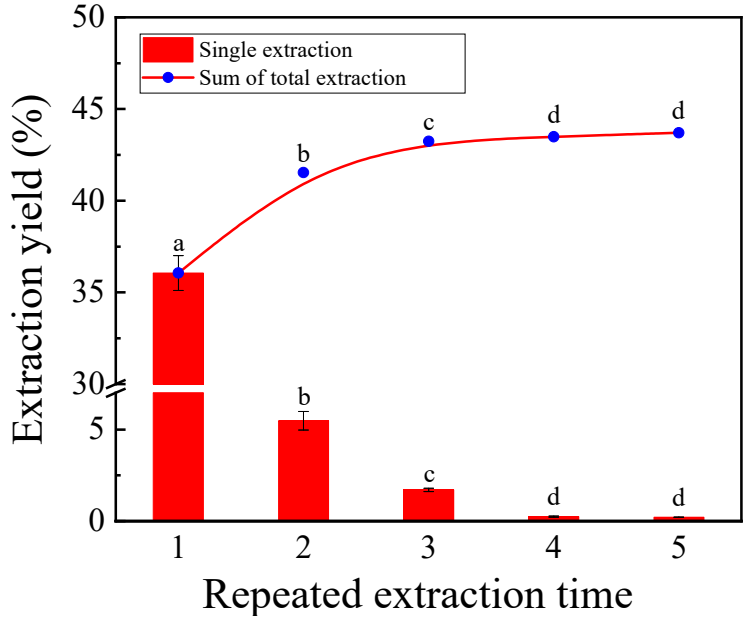

Figure 8. The repeat extraction of KSO. Different letters represent the statistically significant difference.

3.4. Analysis of KSO

GC-MS was used to analyze KSO, and the results are shown in Table 2. The content of α-linolenic acid reaches 51.26%, which is in agreement with previous reports [36]. α-Linolenic acid is a core substance for life and an important component for human brain cell development and histiocyte [37]. Therefore, α-linolenic acid is considered an essential nutrient required for human life. The content in mass of linoleic acid is 14.49%. Linoleic acid can regulate blood lipid levels and reduce cholesterol, which can effectively reduce the occurrence of hypercholesterolemia and reduce the risk of developing coronary heart disease [38]. The proportion of squalene reaches 15.07% for KSO obtained through this method. Squalene is a unique active nutrient, tricosahexaene, which has many physiological effects such as improving the activity of superoxide dismutase in the body, enhancing the immunity of the body, anti-fatigue, anti-aging, anti-tumor, et cetera. Smith et al. found through the A/J mouse experiment that dietary olive oil and squalene can effectively inhibit the occurrence of lung tumors induced by 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone (NNK) [39]. Hernández-Diaz et al. found that linoleic acid diet management significantly reduced blood pressure, blood sugar, cholesterol, and triglyceride levels in spontaneously hypertensive rats, as well as decreased the body fat index of adipose tissue in the heart, abdomen, and epididymis [40]. These effects were accompanied by tumor necrosis factor. The decrease in resistance in secretion suggests that linoleic acid may be an effective inhibitor of preadipocyte proliferation and differentiation. These results indicate that KSO can become a good supplementary nutrient for human health.

Table 2. Composition of KSO from TPP extraction.

Constituents	Content (%)
α-Linolenic acid	51.26 ± 1.04
Squalene	15.07 ± 1.11
Linoleic acid	14.49 ± 0.97
Conjugated linoleic acid	7.53 ± 0.46
Palmitic acid	5.18 ± 1.08
Oleic acid	4.39 ± 0.13
γ- linolenic acid	1.42 ± 0.39
Linolenic alcohol	0.66 ± 0.15

4. Conclusions

In this study, TPP formed by ammonium sulfate and n-butanol was used to extract KSO from kiwifruit seeds. The highest extraction yield of 36.06% was obtained for KSO under the following conditions: n-butanol as the extractant with stirring assisted extraction, 28.5 wt% ammonium sulfate, 18 h extraction time, 4:1 liquid–solid ratio, and 1/1 phase ratio. The one-run extraction recovered 79.23% of the total oil content. The composition of the KSO was obtained by GC-MS, indicating the highest content of 51.26% α-linolenic acid in the KSO obtained by this method. This method is simple, low-cost, and efficient, which can provide a guidance for agro-industrial residue utilization like kiwifruit seeds and other plant resources.

Author Contributions: Investigation, methodology, data curation, validation B.L. and Z.T.; writing-original draft preparation, B.L. and X.L.; resources, Q.L.; conceptualization, B.L. and Z.T.; supervision, Z.T. and X.L.; funding acquisition, Z.T.; project administration, B.L. and Z.T.; writing-review and editing, X.L. and Z.T. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships in this paper.

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