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Production and characterisation of high-quality silkworm pupal oil for omega-3 fatty acid supplementation

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Abstract

The present work aimed to produce edible oil that is low-cost and rich in ω-3 fatty acids (mainly α-linolenic acid; ALA). Silkworm pupae are industrial wastes which could be a good choice as the raw material for silkworm pupal oil (SPO) is. Solvent extraction (SE), supercritical fluid extraction (SFE), and aqueous enzymatic extraction (AE) were compared (oil yields of 28.34, 26.75, and 23.11%, respectively), and crude SPOs showed similar fatty acid compositions. Crude SPO extracted by SE contained a total tocopherol of 289.56 µg/g, which was higher than that of AE. SE was recommended due to its low cost, high oil yield, and high preservation of bioactive compounds, and a further refinement process was adopted only for crude SPO from SE. The fatty acid composition of the refined SPO was not different from that of the crude SPO, which contained 37.51% ω-3 fatty acids. A low acid value (AV, 0.65 mg KOH/g) and peroxide value (PV, 5.79 meq O_2/kg), with almost no detectable toxicants indicated that SPO would be a high-quality edible oil for ω-3 fatty acid supplementation.

Introduction

As a main source of energy, dietary lipids play an important role in the human diet. However, imbalanced consumption of saturated and unsaturated fatty acids leads to detrimental effects on human health (Subash-Babu and Alshatwi, 2018). Recently, the excessive intake of saturated fatty acids has aroused widespread concern, with unsaturated fatty acids recommended to ameliorate these conditions. For unsaturated fatty acids, a balanced ratio of ω-6 to ω-3 is important, especially for metabolic homeostasis (Wang *et al*., 2016). Currently, the ω-3 to ω-6 fatty acids ratio has reached nearly 1:15-20 (even 1:25 in the United States) (Trebaticka *et al*., 2020), and a deficiency in ω-3 and excessive ω-6 have become obvious features in recent decades. Previous reports have shown that a diet with high ω-6 / ω-3 might promote the production of inflammatory mediators, and contribute to some related diseases (Melo *et al*., 2017).

The main reason for this imbalance is that the edible oils consumed in the human diet mainly contain ω-6 fatty acids such as rapeseed, soybean, and sunflower oils, but the case of ω-3 is different. The best dietary source of ω-3 fatty acids is oily fish, which could be beneficial to cardiovascular diseases (Eltweri *et al*., 2017). However, the intake of oily fish in most Western countries is still limited, mainly due to taste, fish supply, and the potential for heavy metal contamination (Walker *et al*., 2013). Linseed and perilla oils are the main plant oils that contain high levels of ω-3 fatty acids (mainly α-linolenic acid; ALA); however, these oils are expensive, and their applications are still limited. Therefore, the production of cheap edible oil with a high level of ω-3 fatty acids is vitally important for ω-3 supplementation in daily life. Silkworm pupal oil (SPO) is a good choice for this.

Crude SPO is a type of valuable oil extracted from silkworm pupae. Silkworm pupae are the main by-product of mulberry silkworms (*Bombyx mori* L.) which are only used as fertiliser and feed, and currently regarded as industrial wastes (Hu *et al*., 2017). High levels of ALA (almost 40%) exist in crude SPO. ALA is an essential ω-3 fatty acid that is needed for human development (Hu *et al*., 2017), and can be converted to docosahexaenoic acid (DHA) and

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eicosapentaenoic acid (EPA) in the human body. Crude SPO is very special, and most animal lipids contain a high level of saturated fatty acids and a low level of unsaturated fatty acids. Apart from the high ALA content, crude SPO also contains high levels of bioactive compounds (*e.g.*, tocopherol and sterol) which are beneficial molecules, and could also improve the oxidation stability (Ravinder *et al*., 2016). The consumption of SPO could provide several health benefits such as lower concentrations of serum total cholesterol (TC), reduced accumulation of visceral fat, and a reduced probability of cardiovascular disease, mainly due to the high content of unsaturated fatty acids (ALA and oleic acid) and β-sitosterol (Zou *et al*., 2016). Therefore, crude SPO would be a good source for the production of cheap, valuable, and ω-3 fatty acid (ALA)-rich edible oil. However, there is no systematic research comparing suitable extraction methods for crude SPO. Additionally, crude SPO was reported to contain free fatty acids (FFAs) and other undesirable components (mainly pigments and phospholipids), and the refinement process is necessary to remove these compounds (Ravinder *et al.*, 2015). Different processing methods might significantly influence the nutritional value of the final product.

The objective of the present work was therefore to produce a low-cost edible oil for ω-3 fatty acid (ALA) supplementation, where silkworm pupae were chosen as raw materials. A suitable extraction method for crude SPO was selected by comparing several frequently used extraction methods. The oil yield, acylglycerol profile, acid value (AV), peroxide value (PV), iodine value (IV), and bioactive compound contents of crude SPO were evaluated as parameters to select the most suitable extraction method. A refinement process was further performed to improve the quality. Finally, the acylglycerol profile, fatty acid composition, oxidation status, unsaturation level, and toxicity evaluation of the refined SPO were characterised to reveal the quality of the final product.

Materials and methods

Materials and reagents

Raw silkworm pupae were provided by the Sericulture and Agri-food Research Institute, Guangdong Academy of Agricultural Sciences (Guangdong, China). Seropeptidase (optimal pH: 7.0; optimal temperature: 50°C; enzyme activity: 45 U/mg) was purchased from Wuxi Weisai Technology Co., Ltd. (Jiangsu, China). Formic acid, 2-propanol, and *n*-hexane were purchased from Kermel Chemical Reagent Co., Ltd. (Tianjin, China), and of HPLC grade. Fatty acid methyl ester (FAME) standards (37) were purchased from Sigma-Aldrich (Shanghai, China), and of HPLC grade. *n*-Heptane, tetrahydrofuran methanolic boron trifluoride, phosphoric acid, and sodium hydroxide were purchased from Sigma-Aldrich (Shanghai, China), and of analytical grade. All other reagents were of HPLC grade.

Oil extraction

The extraction of edible oil was performed using various methods including solvent extraction (SE) (Rui *et al*., 2009), supercritical fluid extraction (SFE) (Pan *et al*., 2012), and aqueous enzymatic extraction (AE) (Jia *et al*., 2014). The oil yield was calculated using Eq. 1:

$$\text{Oil yield (\%)} = \frac{\text{mass of extracted oil}}{\text{mass of dried material}} \times 100\% \qquad \text{(Eq. 1)}$$

Solvent extraction

Silkworm pupae were dried in an oven at 60°C for 24 h until the moisture content was less than 5%. The dried pupae were finely ground and sieved using a 250-μm sieve. The obtained powder (100 g) was added to the sample bottle with 800 mL of *n*-hexane. The sample bottle was then placed in a shaker (an oil bath equipped with a thermostat) at 120 rpm and 60°C for 8 h. Suction filtration was then conducted to obtain the filtrate, and *n*-hexane was removed via a rotary vacuum evaporator (100 rpm, 80°C; RE-2000A, Shanghai Yarong Biochemical Instrument Factory, Shanghai, China). Finally, a certain amount of anhydrous sodium sulphate was added to the obtained oil to remove the water and obtain crude SPO after centrifugation (6,744 *g*, 5 min). The optimal conditions were selected in the pre-test experiment.

Supercritical fluid extraction

Silkworm pupae were dried in an oven at 60°C for 24 h until the moisture content was less than 5%. The dried pupae were finely ground and sieved using a 250-μm sieve. The SFE process was performed according to Pan *et al*. (2012) with slight modifications. The extraction pressure, temperature, time, and CO_2 flow rate were 324.5 bar, 39.6°C, 2.15

h, and 19.3 L/h, respectively. The optimal conditions were selected in the pre-test experiment.

Aqueous enzymatic extraction

The implementation of AE was according to Jia *et al.* (2014) with slight modifications. The silkworm pupae (100 g) were ground and mixed with water (900 mL) and seropeptidase (2849.4 U/g). The extraction process was performed in a shaker (an oil bath equipped with a thermostat) at 120 rpm and 60°C for 3 h. The oil phase was then collected after suction filtration. *n*-Hexane was removed via a rotary vacuum evaporator (100 rpm, 80°C). Certain amounts of anhydrous sodium sulphate were added to the obtained oil to remove the water and obtain the crude SPO after centrifugation (6,744 *g*, 5 min). The optimal conditions were selected in the pre-test experiment.

Refining process

Only crude SPO obtained from SE underwent the refining process.

Degumming

Water and acid degumming were used to remove phospholipids. The degumming process was performed according to Paisan *et al.* (2017) with slight modifications. Due to thermal degradation of bioactive compounds, a low temperature was used.

Crude SPO (50 g) was added to a three-necked round-bottom flask (250 mL). The oil was heated to 60°C, and kept at this constant temperature. Deionised water (40%) was then added, and the reaction was maintained for 2 h in a shaker (an oil bath equipped with a thermostat) at 250 rpm and 60°C. Centrifugation (2,580 *g*, 5 min) was performed to remove the hydratable phospholipids.

Phosphoric acid (1%) was added to the oil phase to remove non-hydratable phospholipids (Yao *et al*., 2020). The reaction stayed in a shaker (an oil bath equipped with a thermostat) for 2 h at 250 rpm and 60°C. The mixture was washed with deionised water (60°C) several times to remove hydratable phospholipids and residual phosphoric acid. Centrifugation (2,580 *g*, 5 min) was then performed to remove water, and degummed SPO was obtained. The optimal conditions were selected in the pre-test experiment.

Deacidification

Alkali refining is the most traditional deacidification method and was performed based on a previously reported paper (Shi *et al*., 2018) with slight modifications. Degummed SPO (30 g) was weighed and slowly mixed with a sodium hydroxide solution (20° Be) in a three-necked round bottom flask (250 mL). The mixture was then heated in a shaker (an oil bath equipped with a thermostat) at 200 rpm and 60°C for 30 min. After the addition of *n*-hexane (80 mL), the mixture was stirred for 10 min (50 rpm) and allowed to settle for 20 min to separate the phases. The supernatant SPO was collected and washed with hot water, and then the deacidified SPO was obtained after evaporation. The optimal conditions were selected in the pre-test experiment.

Bleaching

Deacidified SPO (20 g) was mixed with bleaching earth (4%), and the reaction was performed in a shaker (an oil bath equipped with a thermostat) at 200 rpm and 60°C for 30 min. The final product (refined SPO) was obtained after suction filtration with filter paper. The optimal conditions were selected in the pre-test experiment.

Acylglycerol profile analysis

The acylglycerol profile of the sample was determined via HPLC (Waters Corporation, Milford, MA, USA) with a refractive index detector (Waters Corporation, Milford, MA, USA) according to Li *et al.* (2016). Briefly, 1 mL of the mobile phase (*n*-hexane, 2-propanol, and formic acid; 18:1:0.003, v/v/v) was added to a 2 mL vial with 40 µL of sample. A certain amount of anhydrous sodium sulphate was added to remove the water, and then centrifugation (6,744 *g*, 3 min) was performed. The supernatant was used for HPLC injection after filtration through a 0.45 µm PVDF membrane.

Fatty acid composition analysis

The fatty acid composition analysis was performed according to Hu *et al.* (2017) with slight modifications using GC coupled with a flame ionisation detector (FID) (Agilent 7890A, Agilent Technologies, CA, USA). The sample was first converted to FAME. Approximately, 1 g of sample was pipetted into a 50 mL flask, which was placed in a water bath (60°C, 30 min) after 6 mL of methanolic sodium hydroxide solution was added (2%). Methanolic boron trifluoride solution (3 mL, 15%) was then added and reacted for 5 min at 60°C. *n*-

Hexane (5 mL) and a saturated sodium chloride solution (10 mL) were introduced to the mixed solution. The upper layer was collected, and a certain amount of anhydrous sodium sulphate was added to remove the water. The supernatant obtained after centrifugation (6,744 *g*, 3 min) was used for GC injection.

Analysis of the oxidation status and unsaturation level

The AV, PV, and IV were determined according to AOCS official methods Ca 5a-40, Cd 8b-90, and Cd 1e-01 respectively (AOCS, 2009).

The tocopherol content was determined following the method described by Wang *et al*. (2019) using HPLC equipped with a silica column (4.6 × 250 mm i.d., 5 µm particle size). *n*-Heptane containing 3.85% tetrahydrofuran was used as the mobile phase, where the flow rate was 1 mL/min. The temperature of the column oven was 30°C.

The sterol content was determined following the method described by Wang *et al*. (2019) using gas chromatography-mass spectrometry (GC-MS) equipped with an Agilent DB-5MS UI column (30 m × 0.25 mm, 0.25 µm film thickness). Full-scan mode (*m/z* 30 - 550) was recommended for all measurements. The temperature was raised to the injection temperature (280°C) with a ramp of 15°C/min, and the temperature was kept for 25 min. The carrier gas was helium, and the flow rate was 25 mL/min.

Toxicity evaluation

A toxicity evaluation was only performed in the refined SPO. The determination of 3-monochloropropane-1,2-diol esters (3-MCPDE) was performed according to Abd Razak *et al*. (2019) using GC-MS. Briefly, 3-MCPDE was converted to free forms, and FAMEs were extracted. Derivatives of 3-MCPD were used to evaluate the 3-MCPDE content.

The determination of dibenzo-*p*-dioxins (PBDDs) and polybrominated dibenzofurans (PBDFs) was performed according to Falandysz *et al*. (2020) using HRGC-HRMS (Waters Autospec Ultima Instrument, Malaysia). Polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs) were determined following a method reported by Hayward and Traag (2020) with a TRACE 1300 GC interfaced to a Q-Exactive™ (Orbitrap™) mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). Additionally, the program lasted for 39 min with the temperature kept at 120°C for 2 min, increased from 120 to 200°C (20°C/min), increased from 200 to 240°C (5°C/min), kept at 240°C for 12 min, increased from 240 to 280°C (10°C/min), and kept at 280°C for 10 min.

The determination of heavy metals including arsenic (As), cadmium (Cd), and lead (Pb) was performed according to Szyczewski *et al*. (2016). A fast sequential atomic absorption spectrometer SpectrAA 20 FS (Varian, Australia) was recommended for the determination.

Statistical analysis

The experiment was conducted in triplicate. The data were expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) was used to analyse the results. Duncan's new multiple range test was performed to obtain the significant difference ($p < 0.05$). The results were analysed using SPSS software 2018 (IBM Corp., Chicago, USA).

Results and discussion

Comparison of different extraction methods
Oil yields and acylglycerol profiles of crude SPO

The oil yield of SE was 28.34 ± 0.46%, and significantly higher than that of SFE (26.75 ± 0.42%) and AE (23.11 ± 0.80%) (Figure 1, $p < 0.05$). Although SE requires a relatively long time to complete, this method still has a great application value due to its low-cost property and highest oil yield. SFE displayed a lower oil yield than SE ($p < 0.05$), and Lucas *et al.* (2002) showed similar results. The presence of high molecular weight non-polar compounds in silkworm pupae might have led to a low oil yield for SFE (Lucas *et al.*, 2002). SFE resulted in a higher oil yield than AE ($p < 0.05$); however, operational complexity and high cost prevented frequent utilisation (Hu *et al.*, 2017). During AE, there was no good separation between the oil and protein, which led to a low oil yield.

The acylglycerol profile of crude SPO extracted by three different methods is shown in Figure 1. The crude SPO extracted by SE consisted of 81.97 ± 1.26% TAG, 8.91 ± 0.23% FFA, 4.02 ± 0.36% phospholipids, 3.75 ± 0.28% 1,3-DAG (diacylglycerol), and 1.35 ± 0.09% 1,2-DAG. TAG was the major constituent, followed by FFAs and phospholipids. The crude SPO extracted by the other

two methods showed a similar acylglycerol profile, and the extraction methods did not influence the acylglycerol profile of crude SPO. The FFA contents reported herein were significantly higher than those reported by Shanker *et al.* (2006). The reason was the different processing methods or processing times. High FFA content increased the difficulty of consumption and storage; thus, the subsequent deacidification process was needed to improve the quality of crude SPO.

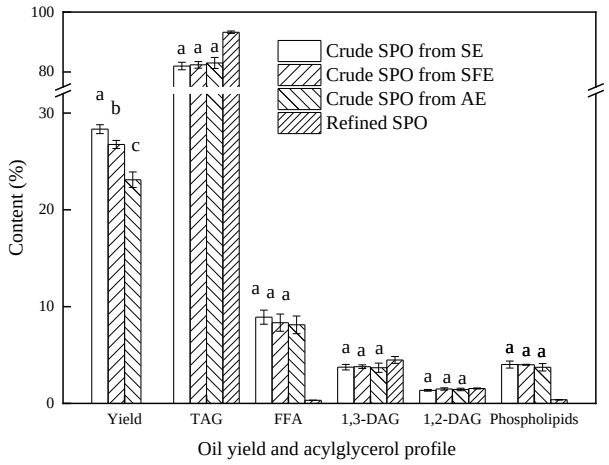

Figure 1. Oil yields and acylglycerol profiles of crude SPO obtained from solvent extraction, supercritical fluid extraction, and aqueous enzymatic extraction, and acylglycerol profile of refined SPO. SPO: silkworm pupal oil; SE: solvent extraction; SFE: supercritical fluid extraction; AE: aqueous enzymatic extraction; crude SPO from SE was further refined to refined SPO. Different lowercase letters indicate significance difference among the crude SPO extracted from different extraction methods ($p < 0.05$).

Ravinder *et al.* (2016) reported similar phospholipid contents (2.39 - 2.65%). Kotake-Nara *et al.* (2002) reported a higher phospholipid content (10%), where the difference was mainly due to geographical regions or feed. It was quite strange that the crude SPOs obtained from the three methods showed similar phospholipid contents. Normally, aqueous media facilitate the removal of hydratable phospholipids, thus the crude SPO obtained from AE should have yielded a lower phospholipid content (Hu *et al.*, 2020). However, the maturity, processing, transportation, and processing conditions of raw materials also have an impact on the activity of phospholipase D, thus leading to the transformation of hydratable phospholipids to non-hydratable phospholipids. In the present work, silkworm pupae were located in a wet environment for quite some time before processing, and this might have influenced the removal of hydratable phospholipids. These two reasons might have led to similar phospholipid contents, but the specific reason should nonetheless be further researched. The phospholipid content was quite high and would cause negative effects on the sensory and storage properties (Yao *et al.*, 2020); thus, a degumming process was needed.

Fatty acid composition

The fatty acid compositions of soybean oil and crude SPO extracted by SE, SFE, and AE are shown in Table 1. The crude SPO obtained from SE contained high levels of unsaturated fatty acids (71.16 ± 0.44%), especially polyunsaturated fatty acids (41.89 ± 0.78%), and low levels of saturated fatty acids (28.84 ± 0.44%). These values are close to those reported by Hu *et al.* (2017) and Wang *et al.* (2019). The major fatty acid was ALA (37.51 ± 0.49%), followed by oleic acid (28.05 ± 0.28%) and palmitic acid (24.35 ± 0.59%). The fatty acid composition agree with previous reports (Shanker *et al.*, 2006; Pan *et al.*, 2012; Gao *et al.*, 2016). The extraction method had no influence on the fatty acid composition, and the crude SPO obtained from different extraction methods showed similar fatty acid composition results ($p > 0.05$, Table 1).

Ravinder *et al.* (2016) reported that the ALA content in crude SPO extracted from tapioca leaf-fed eri silkworm (*Samia cynthia ricini*) pupae was much higher than that in castor leaf-fed silkworm pupae. Pan *et al.* (2012) determined the fatty acid composition of crude oak SPO (the oil extracted from oak silkworm (*Antheraea pernyi* pupae) and crude mulberry SPO (the oil extracted from mulberry silkworm pupae), and reported that the ALA contents were 34.27 and 38.02%, respectively. They also demonstrated that the palmitoleic acid content was 4.77% in crude oak SPO, which was significantly higher than that in crude mulberry SPO (0.60%). The species and feed of silkworms significantly influence the fatty acid contents of their crude SPO (Shanker *et al.*, 2006).

The ALA and oleic acid contents in crude SPO were significantly higher than those in soybean oil (6.04 ± 0.44 and 23.50 ± 0.55%, respectively, $p < 0.05$), which is the most widely consumed edible oil and a main source of essential fatty acids for the human body (Messina *et al.*, 2021). High contents of

ALA and oleic acid could be regarded as having good nutritional value. Oleic acid could reduce cholesterol, and prevent body fat accumulation (Zhao *et al.*, 2015), while ALA is a precursor of DHA and EPA that could improve body health, promote the development of intelligence, and reduce the occurrence of chronic diseases related to obesity (Baba *et al.*, 1999). ALA and linoleic acid are essential, and must be ingested through foods and cannot be produced by the human body. Natural oils rich in oleic acid are abundant, such as soybean, sunflower, and peanut oils, but the case of ALA is different. Therefore, SPO could be a good source for providing ω-3 fatty acids for the human body. Additionally, crude SPO is special in animal fats, and normally contains a low content of unsaturated fatty acids, especially ALA.

Table 1. Fatty acid composition of soybean oil and crude SPO obtained from solvent extraction, supercritical fluid extraction, and aqueous enzymatic extraction.

Fatty acid	Soybean oil (%)	Crude SPO obtained from SE (%)	Crude SPO obtained from SFE (%)	Crude SPO obtained from AE (%)	Refined SPO (%)
C14:0	0.08 ± 0.01	0.16 ± 0.01	0.18 ± 0.01	0.18 ± 0.03	0.17 ± 0.04
C16:0	11.42 ± 0.41	24.35 ± 0.59	24.33 ± 0.77	24.02 ± 1.84	24.40 ± 0.86
C16:1	0.09 ± 0.01	1.21 ± 0.11	1.31 ± 0.11	1.32 ± 0.07	1.33 ± 0.18
C18:0	4.38 ± 0.32	4.05 ± 0.16	4.25 ± 0.31	4.33 ± 0.28	4.19 ± 0.31
C18:1	23.50 ± 0.55	28.05 ± 0.28	28.13 ± 0.70	28.16 ± 0.32	28.05 ± 0.62
C18:2	54.49 ± 0.42	4.38 ± 0.31	4.28 ± 0.31	4.53 ± 0.36	4.33 ± 0.23
α-C18:3	6.04 ± 0.44	37.51 ± 0.49	37.21 ± 0.89	37.14 ± 1.25	37.25 ± 1.08
C20:0	ND	0.28 ± 0.02	0.31 ± 0.03	0.32 ± 0.03	0.28 ± 0.02
SFAs	15.88 ± 0.17	28.84 ± 0.44	29.07 ± 0.47	28.86 ± 1.60	29.04 ± 0.74
UFAs	84.12 ± 0.17	71.16 ± 0.44	70.93 ± 0.47	71.15 ± 1.60	70.96 ± 0.74
PUFAs	60.53 ± 0.61	41.89 ± 0.78	41.49 ± 1.20	41.67 ± 1.45	41.58 ± 1.18

Values are mean ± SD. ND: not detected; SPO: silkworm pupal oil; SE: solvent extraction; SFE: supercritical fluid extraction; AE: aqueous enzymatic extraction; C14:0: myristic acid; C16:0: palmitic; C16:1: palmitoleic acid; C18:0: stearic acid; C18:1: oleic acid; C18:2: linoleic acid; α-C18:3: α-linolenic acid; C20:0: arachidic acid; SFAs: saturated fatty acids; UFAs: unsaturated fatty acids; PUFAs: polyunsaturated fatty acids; crude SPO from SE was further refined to refined SPO.

Oxidation status and unsaturation level

As shown in Table 2, the AV, PV, and IV of crude SPO extracted by SE were 16.13 ± 0.89 mg KOH/g, 5.76 ± 0.41 meq O_2/kg, and 121.23 ± 1.58 g/100 g, respectively. The extraction method did not influence the IV and AV of crude SPO, but significantly influenced the PV. A similar IV showed that the extraction methods did not influence the double bonds of the oil, and this was in accordance with the fatty acid composition analysis. The PV of crude SPO extracted by AE was 3.27 ± 0.24 meq O_2/kg, and significantly lower than that of crude SPO extracted from SE and SFE (5.81 ± 0.37 meq O_2/kg, $p < 0.05$). The drying step (60°C, 2 h) in SE and SFE, and the prolonged extraction duration of SE might have led to the obtained results (Hu *et al.*, 2017).

The PV of crude SPO extracted by the three methods reached the international standard limit (PV: 10 meq O_2/kg) (FAO/WHO, 2009) of edible oil, and Hu *et al.* (2017) reported similar results. However, the AV was beyond the standard (AV: 4 mg KOH/g) (FAO/WHO, 2009); thus, the subsequent deacidification process was needed to improve the quality of the crude SPO.

Bioactive substances

The total tocopherol contents of crude SPO extracted by SE, SFE, and AE were 289.56 ± 4.01, 305.65 ± 8.89, and 261.94 ± 4.80 μg/g, respectively. The crude SPO extracted by SFE displayed the highest total tocopherol content, followed by that extracted by SE and AE ($p < 0.05$). The low

Table 2. IV, AV, PV, and bioactive compounds of crude SPO extracted from solvent extraction, supercritical fluid extraction, and aqueous enzymatic extraction.

Parameter	Crude SPO obtained from SE (%)	Crude SPO obtained from SFE (%)	Crude SPO obtained from AE (%)
IV (g/100 g)	121.23 ± 1.58^{a}	123.60 ± 2.07^{a}	120.57 ± 0.56^{a}
AV (mg KOH/g)	16.13 ± 0.89^{a}	15.99 ± 1.00^{a}	16.21 ± 0.98^{a}
PV (meq O_2/kg)	5.76 ± 0.41^{a}	5.81 ± 0.37^{a}	3.27 ± 0.24^{b}
Total tocopherol (μg/g)	289.56 ± 4.01^{b}	305.65 ± 8.89^{a}	261.94 ± 4.80^{c}
α-tocopherol (μg/g)	175.29 ± 3.85^{ab}	182.45 ± 9.58^{a}	163.97 ± 4.92^{b}
β-tocopherol (μg/g)	46.07 ± 0.88^{a}	47.72 ± 2.34^{a}	38.74 ± 1.84^{b}
γ-tocopherol (μg/g)	33.29 ± 0.59^{a}	33.86 ± 2.48^{a}	29.49 ± 1.76^{b}
δ-tocopherol (μg/g)	35.24 ± 0.12^{b}	41.62 ± 1.98^{a}	29.74 ± 1.24^{c}
Total sterol (mg/100 g)	131.39 ± 2.87^{b}	144.62 ± 3.66^{a}	116.81 ± 3.37^{c}
Cholesterol (mg/100 g)	84.05 ± 1.37^{b}	89.38 ± 1.41^{a}	75.59 ± 3.12^{c}
β-sitosterol (mg/100 g)	34.91 ± 1.69^{b}	42.73 ± 3.57^{a}	30.35 ± 0.68^{b}
Campesterol (mg/100 g)	7.09 ± 0.11^{a}	7.37 ± 0.51^{a}	5.73 ± 0.30^{b}
Stigmasterol (mg/100 g)	5.34 ± 0.14^{b}	6.22 ± 0.60^{a}	5.13 ± 0.17^{b}

Values are mean ± SD. SPO: silkworm pupal oil; SE: solvent extraction; SFE: supercritical fluid extraction; AE: aqueous enzymatic extraction; AV: acid value; PV: peroxide value; IV: iodine value. Different lowercase superscripts indicate significance difference among the crude SPO extracted from different extraction methods ($p < 0.05$).

temperature, short extraction time, and low amount of solvent in SFE were attributed to the obtained result (Wang *et al*., 2011). Gómez-Coronado *et al.* (2004) demonstrated the higher antioxidant capacity of vegetable oil extracted by SFE, which was consistent with the results presented herein. The total tocopherol content of crude SPO extracted by SE was higher than that of crude SPO described by Kotake-Nara *et al.* (2002), which was also obtained by SE, and might have been due to the use of different organic solvents (Kotake-Nara *et al.*, 2002). Lucas *et al.* (2002) revealed that hexane extraction showed no tocopherol content, and the difference might have been caused by different extraction temperatures. Due to the thermal degradation of tocopherol, the high temperature (80°C) adopted would have caused major reduction in the tocopherol content (Asl *et al.*, 2020). The crude SPO obtained from AE exhibited the lowest content of total tocopherol ($p < 0.05$), which could have been caused by poor separation of oil and protein.

α-Tocopherol was the most abundant tocopherol in crude SPO (59.67 ± 1.47 to 62.59 ± 1.15%), which agrees with the results reported by Kotake-Nara *et al.* (2002). The high proportion of α-tocopherol could improve the oxidation stability and biological activity of crude SPO (Kotake-Nara *et al.*, 2002).

Sterols are precursors of hormones in the human body, and participate in the regulation of growth and metabolism. The sterol composition of crude SPO was therefore determined. As shown in Table 2, the total sterol contents of crude SPO extracted by SE, SFE, and AE were 131.39 ± 2.87, 144.62 ± 3.66, and 116.81 ± 3.37 mg/100 g, respectively. The extraction method significantly affected the sterol contents in crude SPO. SFE showed the highest total sterol content, and AE showed the lowest ($p < 0.05$). The poor separation of oil and protein in AE could have led to the lowest total sterol content. Plant sterols and cholesterol are easily oxidised when exposed to air, and oxidation can be enhanced by heating (Lampi *et al.*, 2002). Due to the high temperature used in SE, crude SPO obtained from SE showed a lower total sterol contents than crude SPO from SFE.

Cholesterol was the most abundant sterol (61.37 ± 1.48 to 64.70 ± 0.82%), followed by β-sitosterol (25.99 ± 0.48 to 29.30 ± 1.58%). The contents of campesterol and stigmasterol were

relatively low, with both less than 6% of the total sterol contents. A similar sterol composition of crude SPO was reported by Shanker *et al.* (2006). Similar to other animal fats, a high cholesterol content existed in crude SPO (Sridhara and Bhat, 1965). Although the excessive intake of cholesterol could have a negative effect on human health, the intake of moderate cholesterol is necessary. Cholesterol is distributed in various tissues throughout the human body, which greatly affects the physiological activities of the human body. β-sitosterol is an important bioactive compound that can inhibit the production of proinflammatory cytokines (Kim *et al.*, 2014). Crude SPO had a high content of β-sitosterol and few structural analogues. Therefore, high purity β-sitosterol could be easily obtained from crude SPO, and be used in the pharmaceutical field (Jenkins *et al.*, 2003).

As stated in the comparison of different extraction methods, SFE displayed an outstanding capacity in the aspect of preserving bioactive compounds. However, its operational difficulty and high cost limit its application. AE showed the lowest oil yield and bioactive compound contents, thus not suitable for the extraction of crude SPO. On the other hand, SE showed the highest oil yield and high preservation of bioactive compounds. SE could have excellent application due to it being low-cost and suitable for the extraction of crude SPO as demonstrated in the present work. Therefore, refining process was only performed on crude SPO from SE.

Characterisation of refined SPO

The acylglycerol profile, fatty acid composition, PV, AV, IV, bioactive compounds, and toxicant contents of the refined SPO were determined. No distinct differences were found in the fatty acid composition between the crude and refined SPO ($p > 0.05$), with the fatty acid composition of refined SPO shown in Table 1. The acylglycerol profile of the refined SPO is shown in Figure 1, and the results showed that the refining process greatly reduced the FFA (0.32 ± 0.01%) and phospholipid (0.37 ± 0.02%) contents. Although the relative contents of TAG, 1,3-DAG, and 1,2-DAG improved, but the refinement process did not influence the ratio of these three components. The refinement process improved the quality and storage stability of the refined SPO.

The PV, AV, IV, and bioactive compounds of refined SPO are shown in Table 3. The AV and PV of the refined SPO were 0.65 ± 0.01 mg KOH/g and 5.79 ± 0.23 meq O_2/kg, respectively, and met the international standards of AV (4 mg KOH/g) (FAO/WHO, 2009) and PV (10 meq O_2/kg) (FAO/WHO, 2009) for edible oil. A low AV and PV can be regarded as good quality edible oil (Hu *et al.*, 2017). The IV was 121.17 ± 1.49 g/100 g, and corresponded to the fatty acid composition. The total tocopherol and total sterol contents of the refined SPO were 123.72 ± 5.01 μg/g and 50.37 ± 1.63 mg/100 g, respectively, and refined SPO still exhibited good nutritional value.

Table 3. IV, AV, PV, and bioactive compounds of refined SPO.

Parameter	Refined SPO
IV (g/100 g)	121.17 ± 1.49
AV (mg KOH/g)	0.65 ± 0.01
PV (meq O_2/kg)	5.79 ± 0.23
Total tocopherol (μg/g)	123.72 ± 5.01
α-tocopherol (μg/g)	71.19 ± 1.35
β-tocopherol (μg/g)	18.21 ± 1.07
γ-tocopherol (μg/g)	16.69 ± 2.28
δ-tocopherol (μg/g)	17.63 ± 2.06
Total sterol (mg/100 g)	50.37 ± 1.63
Cholesterol (mg/100 g)	29.30 ± 1.69
β-sitosterol (mg/100 g)	17.53 ± 0.61
Campesterol (mg/100 g)	2.33 ± 0.27
Stigmasterol (mg/100 g)	1.22 ± 0.21

Values are mean ± SD. SPO: silkworm pupal oil; AV: acid value; PV: peroxide value; IV: iodine value; crude SPO from SE was further refined to refined SPO.

The toxicant concentrations in the refined SPO are shown in Table 4. 3-Monochloropropane-1,2-diol (3-MCPD) is an important contaminant in foods. The presence of their fatty acid ester (3-MCPDE) in lipids has recently caused serious problems (Jędrkiewicz *et al.*, 2016). During digestion, 3-MCPDE is hydrolysed into 3-MCPD, which is classified as a "possible human carcinogen (Group 2B)" by the International Agency for Research on Cancer (IARC). Oily fish are the best dietary source of ω-3 fatty acids; however, various reports have shown the presence of high amounts of 3-MCPDE in fish oil. The report of Jędrkiewicz *et al.* (2016) revealed that the content of 3-MCPDE in fish oil was quite high, in the range of 1.5 - 5.5 mg/kg. Kuhlmann (2011) reported similar

results: the determined levels of 3-MCPDE in salmon oil were in the range of 0.7 - 13 mg/kg. As compared to fish oil, the content of 3-MCPDE in refined SPO was not detectable.

PCDDs, PCDFs, PCBs, PBDDs, and PBDFs are important environmental pollutants (Domingo and Bocio, 2007). It has been reported that environmental pollutants could increase the risk to human health due to bioaccumulation through the food chain, and PCDD/Fs could produce multiple toxic endpoints in humans (Hayward and Traag, 2020). Recently, a number of studies have focused on PCDD/Fs and PCBs in fish and other seafood, and the results have corroborated that the levels of PCDD/Fs and PCBs were high in fish originating from specific areas, which was mainly due to environmental pollution (Domingo and Bocio, 2007). Falandysz *et al.* (2020) reported that cod liver oils sourced from northern Atlantic waters (Iceland, Norway) and the Baltic sea (Poland) contained detectable levels of PBDFs. Shelepchikov *et al.* (2019) also showed similar results. It is noteworthy that environmental pollutants were not detectable in the refined SPO obtained in the present work.

In addition, the presence of heavy metals in fish oil is one of the reasons that some Western people do not consume fish oil (Walker *et al.*, 2013). In the refined SPO obtained in the present work, the heavy metal contents were relatively low and negligible. Above all, the final refined SPO had good quality and could be utilised as a good source of ω-3 fatty acid supplementation.

Table 4. Toxicants in refined SPO.

Toxicant	Concentration
3-MCPDE (mg/kg)	N.D. (LOD: 0.01 mg/kg; LOQ: 0.03 mg/kg)
PBDDs (ng/L)	N.D. (LOD: 3.1 - 4.5 ng/L; LOQ: 4.2 - 5.9 ng/L)
PBDFs (ng/L)	N.D. (LOD: 3.2 - 4.2 ng/L; LOQ: 3.8 - 6.2 ng/L)
PCBs (ng/L)	N.D. (LOD: 0.12 - 0.42 ng/L; LOQ: 0.36 - 1.26 ng/L)
PCDD/Fs (μg /L)	N.D. (LOD: 0.04 - 0.25 μg/L; LOQ: 0.19 - 0.37 μg/L)
Cd (mg/kg)	0.004 (LOD: 1.02 μg/kg; LOQ: 3.11 μg/kg)
Pb (mg/kg)	0.007 (LOD: 1.56 μg/kg; LOQ: 4.21 μg/kg)
As (mg/kg)	0.003 (LOD: 1.89 μg/kg; LOQ: 2.83 μg/kg)

Values are mean ± SD. ND: not detected; SPO: silkworm pupal oil; crude SPO from SE was further refined to refined SPO; LOD: limit of detection; LOQ: limit of quantitation; 3-MCPDE: 3-monochloropropane-1,2-diol esters; PBDDs: dibenzo-*p*-dioxins; PBDFs: polybrominated dibenzofurans; PCBs: polychlorinated biphenyls; PCDDs: polychlorinated dibenzo-*p*-dioxins; PCDFs: dibenzofurans; Cd: cadmium; Pb: lead; As: arsenic.

Conclusion

In the present work, three different extraction methods (SE, SFE, and AE) were performed, which could greatly influence the nutritional value of crude SPO. SE was recommended due to its highest oil yield (28.34 ± 0.46%), fairly high preservation of bioactive compounds (total tocopherol, 289.56 ± 4.01 μg/g; total sterol, 131.39 ± 2.87 mg/100 g), and low cost. High FFA (8.91 ± 0.23%) and phospholipids (4.02 ± 0.36%) contents could have a negative influence on the quality and storage stability. Therefore, a subsequent refining process (degumming, deacidification, and bleaching) was performed. The low AV (0.65 mg KOH/g) and PV (5.79 meq O_2/kg), and extremely low content of heavy metals indicated the high quality of the refined SPO. The ω-3 fatty acid content of the refined SPO showed no difference from that of the crude SPO, which was 37.51 ± 0.49%. The refined SPO could be used as an inexpensive edible oil for ω-3 fatty acid supplementation.

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