Composition and oxidative stability of silflower (Silphium integrifolium) seed oil and its potential as a new source of squalene

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Abstract

Silphium integrifolium Michx. (silflower), a perennial plant, is of great interest as a potential new oilseed crop due to its long, strong, deep, extensive root systems, which can prevent erosion, capture dissolved nitrogen, and out-compete weeds eliminating the need for frequent irrigation and herbicide uses. In this study, oil was extracted from unhulled silflower seeds, and its composition and oxidative stability were evaluated. The oil content in unhulled silflower seeds was 15.2 % (w/w), and its fatty acid composition was similar to that of sunflower oil. The level of total polar compounds (TPC) in the oil was 12.3 % (w/w), and the content of total phenolics was 1.12 mg gallic acid equivalent (GAE)/g oil. Noteworthily, 4.89 % squalene was isolated from silflower oil indicating its potential application as an alternative source of squalene. Silflower oil had lower oxidative stability as indicated by the oxidative stability index (OSI) at 110 °C and thermogravimetric analysis (TGA), presumably due to its high level of chlorophyll (1002.8 mg/Kg). Even after a typical refining process involving degumming, alkali refining, and bleaching with Fuller's earth, silflower oil contained 725.5 mg/kg chlorophyll, and its oxidative stability was not improved. Further treatments with bleaching agents including bentonite, sepiolite, and Tonsil® lowered the chlorophyll level to 4.2, 474.5, and 38.5 mg/kg, respectively, and some aspects of oxidative stability were improved and better than those of refined sunflower oil. This study presents the potential of silflower oil as new edible oil and a great plant source of squalene.

INTRODUCTION

Perennial crops are known to have many advantages over annual counterparts such as deeper rooting depth, better drought tolerance, and less tillage required (Crews & DeHaan, 2015; Glover et al., 2010). They can reduce soil erosion, increase nutrition retention, increase carbon sequestration, and enhance agricultural sustainability (Crews & DeHaan, 2015; Schiffner et al., 2020). Recently, *Silphium integrifolium*Michx. (silflower), a perennial plant in the sunflower family native to the central United States, has drawn great interest as a potential oilseed crop, and studies have been conducted to domesticate crops and to understand the properties of crops, seeds, and seed oil (Evangelista et al., 2023; Price et al., 2022; Van Tassel et al., 2014; Van Tassel et al., 2017).

Silflower seeds are known to be high in protein (33.53%), fat (22.05%), and fiber (22.07%) contents (Kowalski & Wierciński, 2004), which are similar to sunflower (Helianthus annus L.) seeds. Fatty acid composition of the silflower seed oil with linoleic (62.3-63.0%) and oleic (18.7-19.6%) acids as the major fatty acids is close to that of sunflower oil (Evangelista et al., 2023; Kowalski & Wierciński, 2004). Although dehulling is challenging for silflower seed, studies found that its high-protein meal had potential for food and industrial applications (Evangelista et al., 2023).

Since silflower has many advantages as a crop and its oil has a great potential in the food industry, more studies are needed to utilize it as an oilseed crop. Especially, no study has been conducted on the oxidative stability of the seed oil although oxidative stability is one important property of edible oils. Silflower oil has high contents of unsaturated fatty acids, which are beneficial to human health but susceptible to oxidation. Oxidative stability of oil not only depends on the fatty acid composition, but also the inherent antioxidants in oil (Madhujith & Sivakanthan, 2019). Therefore, it is also important to study compounds other than triacylglycerols in an oil and their effects on the oxidative stability of the oil.

In this study, oil extracted from unhulled silflower seeds was analyzed for total phenolics, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay, total polar compounds (TPC), free fatty acids (FFA), tocopherols, and chlorophyll, and its oxidative stability was compared with the oil extracted from unhulled sunflower seeds. Oxidative stability index (OSI) and thermal stability measured by thermogravimetry analysis (TGA) were used to evaluate the oxidative stability of oils. Since the oxidative stability of oil can be affected by refining processes, which change the levels of antioxidants such as tocopherols and prooxidants such as free fatty acid and chlorophyll, different refining processes were evaluated to improve the oxidative stability and appearance of silflower oil.

MATERIAL AND METHODS

Materials

Unhulled silflower seeds were provided by The Land Institute (Salina, KS, USA). Unhulled sunflower seeds were purchased from Nuts.com (Cranford, NJ, USA). Gallic acid, Folin-Ciocalteu reagent, sodium carbonate, ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)), Trolox® (6-hydroxy-2,5,7,8-tetramethlchroman-2-carboxylic acid), ammonium thiocyanate, barium chloride, iron (II) sulfate heptahydrate, 2,2,4-trimethylpentane (isooctane), citric acid, Fuller's earth, and sepiolite were purchased from Sigma–Aldrich (St. Louis, MO, USA). Bentonite was purchased from Spectrum Chemical Mfg. Corp. (New Brunswick, NJ, USA), and Tonsil® was kindly provided by Clariant (Munich, Germany). Silica gel 60 (70-230 mesh) and thin layer chromatography (TLC) plates (MK6F silica gel 60 Å) were purchased from EMD Chemical Inc. (Darmstadt, Germany) and Whatman (Clifton, NJ, USA), respectively. Hexane, ethyl acetate, acetone, THF, methanol, ethanol, potassium hydroxide, sodium hydroxide, and hydrochloric acid solution were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

Oil extraction

Unhulled silflower seeds (700 g) were ground, and hexane (2.5 L) was added. The mixture was heated at 55 °C in an oven for 6 h, filtered, and the solvent was evaporated with a rotary evaporator. The defatted meal was extracted again with hexane (2.5 L) at 55 °C for 3 h. The combined oil was dissolved in 200 ml hexane, stored in a freezer (-20 °C) overnight, and filtered. Solvent was removed with a rotary evaporator and then a vacuum pump. The exactly the same conditions were used to extract oil from unhulled sunflower seeds. The oils were stored under argon until analyses. Oil contents are reported as the average values of duplicate extractions.

Determination of total polar compounds (TPC) in oil

The previously reported procedure (Hwang et al., 2022) was followed to determine TPC with some modifications. Oil sample (1.0 g) was dissolved in hexane (2 ml) and added into a glass column filled with a silica gel (about 12 g, 70-230 mesh) slurry in hexane. Ethyl acetate in hexane (3% in volume, 200 ml) was used to elute the non-polar fraction and then ethyl acetate (100 ml) for the polar fraction. After the separation of two fractions was confirmed with thin-layer chromatography (TLC, silica), the solvent in the polar fraction was removed by a rotary evaporator. The polar fraction was dissolved in a small amount of acetone, transferred to a 100 ml round bottom flask, which was kept in a desiccator and weighed beforehand, subjected to a rotary evaporator and then a vacuum pump, and weighed. TPC analysis was conducted in triplicate and reported as the average weight percentage of the polar fraction in the starting oil.

Total phenolics

Oils (0.5 g) were extracted with methanol (5 ml) twice, dried by gently blowing nitrogen, and resuspended in 1 ml methanol. Then, the content of total phenolics was determined following the previously reported method (Singleton & Rossi, 1965) using PerkinElmer Lambda 35 UV/Vis spectrophotometer (Perkin Elmer, Waltham, MA, USA) at 765 nm. The calibration curve was obtained with 0, 50, 100, 150, 250, 500, and 100 mg/L gallic acid solutions, which were prepared by dissolving gallic acid (0.050 g) in 1 ml ethanol, bringing to 100 ml with deionized water, and diluting with water to the desired concentrations. Total phenolics is reported as an average gallic acid equivalent (GAE, mg/g) value of four values (two experimental replicates × two analytical replicates).

Fatty acid composition and squalene analysis

First, oil samples were converted to fatty acid methyl esters (FAME) by dissolving oil samples (about 10 mg) in 1.4 ml hexane followed by adding 2 N methanolic KOH (200 µL) and shaking for 2 min with a Vortex mixer (Ichihara et al., 1996). After two layers were separated, the top layer was transferred to a vial, and then analyzed with a Shimadzu 2010 gas chromatograph (Shimadzu, Columbia, MD) with a flame ionization detector (FID). The column was a Phenomenex (Torrance, CA) ZBFAME capillary column (30 m × 0.25 mm ID × 0.20 µm film). The GC conditions were the same as reported by Winkler-Moser et al. (2023). Commercial FAME and squalene standards were used to identify peaks in the GC chromatograms. The average values of relative peak areas measured in duplicate are reported.

Isolation of squalene

A column was prepared with 210 g of silica gel and hexane. Silflower oil (7.0 g) was loaded into the column and eluted with a gradient solvent from 0 to 2% (v/v) ethyl acetate in hexane. Squalene was eluted before triglycerides. The solvent was removed by a rotary evaporator and then a vacuum pump, and squalene was weighed to obtain the yield. Isolation of squalene was conducted in duplicate. The molecular structure was confirmed with ^{1}H NMR.

Analysis of tocopherols

A Shimadzu Prominence-I LC-2030A high performance liquid chromatograph (HPLC), equipped with a photodiode array detector (scanning 200-500 nm, set wavelength for data collection and quantitation at 295 nm) and fluorescence detector (excitation 292 nm, emission at 344 nm) in series was used to analyze tocopherols in oils as described by (Winkler-Moser et al., 2023). Tocopherols were identified by retention time of commercial standards, and peak confirmation with a photodiode array detector scanning between 200-500 nm. Quantitation was conducted with external standard curves using the fluorescence detector. Analysis was conducted in duplicate.

Free fatty acid (FFA)

Free fatty acids were analyzed using free fatty acid analysis test kits by the CDR FoodLab® Jr. (CDR, Florence, Italy). Analysis was done in duplicate, and results are presented as % oleic acid.

Chlorophyll analysis

A previously reported procedure was followed to determine the amount of chlorophyll (Pokorny et al., 1995). Results were expressed as mg/kg pheophytin a, which is the major form in oils. Analysis was conducted in duplicate.

Differential scanning calorimetry (DSC)

DSC experiments were conducted following the previously reported procedure (Mokbli et al., 2018) with a Model Q2000 DSC (TA Instruments, New Castle, DE, USA). Samples (about 10 mg) were weighed into Tzero aluminum pans and sealed with aluminum lids (TA Instruments, New Castle, DE, USA), and melting and crystallization curves were obtained under nitrogen at a flow rate of 60 mL/min. The program was: Equilibrate at 100 °C, isothermal for 1 min, ramp at 10 °C/min to -70 °C, isothermal for 1 min, and ramp

at 10 °C/min to 100 °C. TA Universal V4.5A software was used to analyze data. Samples were analyzed in triplicate.

2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay

Oils (0.5 g) were extracted with 5 ml methanol twice, and the methanol solution was dried by gently blowing nitrogen. The residue was resuspended in 1 ml methanol. The ABTS decolorization assay was determined as described by Re et al. (1999). The Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) standard curve at the 5 min time point was used to determine the Trolox equivalent (TE), and the results are reported as the µmol TE per g of extracted oil. Analysis was done in triplicate.

Oxidative stability index (OSI)

The OSI values were determined following the AOCS Official Method Cd 12b-92 (AOCS, 2011) using a Rancimat 743 (Metrohm, Riverview, FL, USA) at 110 °C. Each sample was analyzed in triplicate.

Thermogravimetric analysis (TGA)

The previously reported method was used to determine the thermal stability of oil (Mokbli et al., 2018) with a Model Q50 TGA (TA instruments, New Castle, DE, USA). Oil samples (about 9 mg) were weighed in platinum sample pans, which were loaded by an autosampler. Samples were heated at the rate of 10 °C/min up to 600 °C under air at the flow rate of 60 ml/min. TA Universal V4.5A software was used to analyze data. Samples were analyzed in triplicate. The thermal stability was determined by the onset temperature of oil decomposition following the previous method (Kalam et al., 2017).

Color analysis

A Labscan XE colorimeter (HunterLab, Inc., Reston, VA.) with the UV filter at nominal position, calibrated with standard white and black plates was used to analyze the color of oil samples. Oil was resented in a sample cup with a black ring and a white disk covered with a black sample cover. Three readings of each sample were recorded. The mean values of the color coordinates L* (lightness - darkness), a* (redness - greenness), and b* (yellowness -blueness) are reported.

Refining of oils

Crude silflower and sunflower oils were refined by degumming (Diosady et al., 1982; Ghazani et al., 2013), alkali refining (Suliman et al., 2013), and bleaching with Fuller's earth (Soldo et al., 2019). In brief, crude oil (200 g) was heated to 70 °C under argon, a 64 % aqueous citric acid solution (1.4 grams) was added to oil, stirred for 30 min, distilled water (4.0 g) was added, and stirred for another 30 min. After cooling to room temperature, the gums were removed via centrifugation at 7500 rpm for 10 min, and oil was washed with deionized water (25 ml). Degummed oil was mixed with 2% sodium hydroxide solution (16 g) at 40 °C with slow agitation for 30 min. After cooling to room temperature, soap stock was separated by centrifuging at 7500 rpm for 10 min. The oil was dissolved in hexane (100 ml), washed with deionized water (100 ml) three times using a separatory funnel, and dried over $MgSO_4$, filtered, and the solvent was removed using a rotary evaporator and then a vacuum pump. Fuller's earth (8.0 g) was added to the oil and stirred at 40 °C for 40 min under argon. Then, it was cooled to room temperature and filtered through a thin layer of celite (Strieder et al., 2017).

Further refining

Silflower oil was further refined to reduce the chlorophyll level with three different bleaching clays, bentonite, sepiolite, and Tonsil® following the previous method (Kwaśnica et al., 2022; Strieder et al., 2017). In brief, oil (40.0 g) was dried with a vacuum pump for 30 min at 100 °C, 2.0 g of bleaching clay was added, heated at 100 °C with stirring for 20 min under argon, and filtered through a pre-layer of celite. Tonsil® was used as received while bentonite and sepiolite were activated by following the previously reported methods (Saneei et al., 2015; Sarioğlan et al., 2010; Srasra et al., 1989).

Peroxide value (PV)

PV was determined following a previously reported method (Shantha & Decker, 1994). Absorbance was measured at 510 nm using a PerkinElmer Lambda 35 UV/Vis spectrophotometer (Perkin Elmer, Waltham, MA, USA). Average values of two measurements are reported. The PV is given by the following formula:

$$\mathrm{PV} = \frac{(A_s - A_b) * m}{55.84 * m_o * 2}$$

As: the absorbance of the sample, Ab: the absorbance of the blank,m: the slope of the standard curve, m_o: mass of the sample.

Conjugated diene value (CDV)

The AOCS official method (Ti 1a-64) was used to determine CDV (AOCS, 2017). In brief, approximately 10 mg oil samples were dissolved in 10 ml isooctane, and the solution (1.0 ml) was diluted with 9 ml isooctane. The solution was added in a quartz cuvette, and the absorbance was measured with the same spectrophotometer used for PV at 233 nm. The average values of two measurements are reported. The CDV was calculated by the following equation:

$$CDV = 0.84 \times [\left(\frac{A_s}{b \times c}\right) - k_o]$$

A_s: observed absorbance at 233 nm of the sample,b: cuvette length in cm, c: concentration of the sample in g/L, K_o: absorptivity by acid or ester groups.

Statistical analysis

All the experiments were conducted in duplicate or triplicate. One-way analysis of variance (ANOVA) was performed with JMP 15 program (SAS Institute, Cary, NC, USA). Tukey-Kramer Honestly Significant Difference test with statistical significance at $P < 0.05$ was used to compare means.

RESULTS AND DISCUSSION

Properties of silflower oil extracted with hexane

Unhulled silflower seeds were extracted with hexane at 55 °C, and for side-by-side comparisons, unhulled sunflower seeds were purchased and extracted under the same conditions. Table 1 shows the oil contents and properties of silflower oil in comparison with sunflower oil. The oil content in unhulled silflower seeds (15.2%, w/w) was slightly lower than that in unhulled sunflower oil (18.0%, w/w). TPC in silflower oil was significantly higher (12.3%, w/w) than sunflower oil (4.1%, w/w). It also had a higher content of total phenolics (1.12 mg GAE/g) than sunflower oil (0.46 mg GAE/g). In addition to the major components, triacylglycerols, plant oils contain polar lipids such as phospholipids and galactolipids, monoacylglycerols, diacylglycerols, free fatty acids, sterols and sterol derivatives (for example, phytosterols), tocopherols, carotenoids, chlorophylls, other phytochemicals (Zambelli et al., 2015). Apparently, silflower oil contained more of these compounds than sunflower oil. High total phenolics of silflower oil may be beneficial to its oxidative stability since many phenolic compounds are known to have antioxidant activity. It is also possible that silflower oil may contain large amounts of biologically active compounds that are beneficial to human health, which should be further studied. The ABTS assay is a widely used method to assess the radical scavenging ability of antioxidants in oil (Ilyasov et al., 2020; Re et al., 1999). The ABTS assay of the methanol extract of crude silflower oil was slightly, but not statistically significantly lower than that of crude sunflower oil despite having higher total phenolics content.

The fatty acid composition of silflower oil (Table 1) analyzed in this study is similar to those in previous studies (Evangelista et al., 2023; Kowalski & Wierciński, 2004) and that in sunflower oil indicating that this oil has potential as a new edible oil. As shown in Table 1, the linoleic acid (C18:2, c9,12) and oleic acid (C18:1, c9) contents in silflower oil (65.53 and 19.86%, respectively) were similar to those in sunflower

oil (65.48% and 22.49%, respectively). Some minor fatty acid contents were different in the two oils. For example, contents of myristic acid (C14:0) and palmitic acid (C16:0) were higher in silflower oil while those of stearic acid (C18:0) and behenic acid (C22:0) were lower. Total contents of unsaturated fatty acids were similar in the two oils with 86.2% in silflower oil and 88.5% in sunflower oil.

It should be noted that silflower oil contained 4.34% (the peak area in GC) squalene. Since this finding is very important for the application of silflower oil as an alternative source of squalene, the isolation of squalene was conducted using column chromatography on silica gel. Squalene was isolated from silflower oil in the yield of 4.89% (w/w), and its structure was confirmed with ^{1}H NMR in comparison with previous results (Shi et al., 2019). Squalene has antioxidant, anticancer, immune stimulating, and emollient activities, and its use in nutraceutical, cosmetic, pharmaceutical, and vaccine industries is growing (Rani et al., 2018). Shark liver oil is the most abundant source of squalene (up to 79%) and has been the most common source (Lozano-Grande et al., 2018). However, due to the international concern on overfishing as well as potential risks of polychlorinated biphenyls (PCBs), heavy metals, and other contaminants in the oil, consumers and industries are seeking alternative sources. Currently, the best plant sources of squalene are olive oil (0.15-0.75%) and amaranth oil (6-8%) (Gutiérrez-Luna et al., 2022; Lozano-Grande et al., 2018). Therefore, the high content of squalene in silflower oil indicates the potential application of this oil as a promising alternative source of squalene.

Analysis of all the tocopherols including α-, β-, γ, and δ-tocopherols was attempted, but their signals were overlapped with other unknown signals, and only the level of α-tocopherol could confidently be determined in this study. The level of α-tocopherol was lower in silflower oil (305.5 μg/g) than in sunflower oil (660.1 μg/g). Free fatty acid content in silflower oil (1.00%, w/w) was higher than in sunflower oil (0.47%, w/w). It contained a very large amount of chlorophyll (1002.8 mg/kg).

Melting and crystallization properties

Figure 1 and Table 2 show melting properties of silflower and sunflower oils. Melting and crystallization properties of silflower oil were similar to those of sunflower oil reflecting the similar fatty acid composition. Silflower oil had broader and wider melting peaks than sunflower oil, which might be caused by its higher TPC (Huang & Sathivel, 2010). The predominant type of triacylglycerol of sunflower oil is LLL (L = linoleic acid) since the major fatty acid in sunflower oil is linoleic acid (Tan & Che Man, 2000). Therefore, the major melting and crystallization peaks of silflower and sunflower oils are likely attributed to the melting and crystallization of LLL.

Oxidative and thermal stabilities

Oxidative stability index (OSI) measured according to AOCS standard method Cd 12b-92 is one of the most widely used methods to determine susceptibility to oxidation of edible oils (Márquez-Ruiz et al., 2008). TGA determining the thermal stabilities of oils has been used to predict the onset of oil oxidation during heating (Borugadda & Goud, 2014; Gao & Birch, 2016; Kalam et al., 2017; Mokbli et al., 2018). Thermal stability of oil can be determined by the temperature where 5 or 90% mass loss occurs (Mokbli et al., 2018) or by the onset temperature of weight loss, which is defined as the temperature where oil starts to decompose (Kalam et al., 2017). These two methods were used to evaluate oxidative stabilities of silflower oil in this study. Table 3 shows that the OSI at 110 °C of silflower oil (2.42 h) and the onset temperature of degradation of silflower oil (282.5 °C) were significantly lower than those of sunflower oil (4.35 h and 298. 2 °C). Apparently, these results indicated that oil extracted from unhulled silflower seed with hexane had a lower oxidative stability than that from unhulled sunflower seed.

The two major factors determining the oxidative stability of oil are the fatty acid composition and antioxidants present in the oil (Gao & Birch, 2016). The amount and activity of antioxidants in oil may be more important factors than the fatty acid composition for the different oxidative stabilities of these two oils because their fatty acid compositions were very similar as shown in Table 1. Silflower oil contained higher total phenolics than sunflower oil, but lower α-tocopherol (Table 1). Therefore, the tocopherol level might

have a significant effect on the oxidative stability of these oils. In addition, another factor to consider for the oxidative stability of these oils is the chlorophyll level because chlorophyll is known to have prooxidant activity in edible oils (Usuki et al., 1984), and silflower oil contained much higher chlorophyll than sunflower oil. The level of chlorophyll can be controlled by some ways such as harvest time, ripening stage, and storage conditions (Yilmaz & Gökmen, 2016) and also by oil refining processes. In this study, a few refining processes were evaluated to decrease the level of chlorophyll and then, oxidative stabilities of refined oils were evaluated.

Refined oils

First, a published general refining process was applied to both oils, which involved degumming with citric acid (Diosady et al., 1982; Ghazani et al., 2013), alkali refining with 2% sodium hydroxide solution (Suliman et al., 2013), and then bleaching with Fuller's earth (Soldo et al., 2019). Table 4 shows the composition and oxidative stability of refined silflower and sunflower oils. While chlorophyll in sunflower oil was completely removed by this refining process, the chlorophyll level in silflower oil was still high (725.5 mg/kg). TPC slightly decreased while total phenolics did not change for both oils after the refining process. Radical scavenging ability determined by ABTS assay of silflower oil decreased after refining while that of sunflower was not significantly changed (Tables 1 and 4).

Silflower oil lost a very slight amount of squalene. Nergiz and Celikkale (2011) also observed a very slight loss (0.002-0.1%, w/w, in oil) of squalene during a neutralization/physical refining followed by bleaching. The FFA levels in both oils were significantly lowered after the refining process, and it was not detected in refined sunflower oil. It is interesting that ∝-tocopherol in silflower oil increased. In general, tocopherols and other phytochemicals decompose during refining. However, some studies (Pestana et al., 2008; Rossi et al., 2001; Van Hoed et al., 2006) reported increased tocopherols after refining processes including bleaching treatments with acid clays, degumming, and deodorization.

OSI of silflower oil slightly lowered, and the thermal stability measured by TGA also decreased after refining. Changes in silflower oil by the refining process such as the loss of total phenolics might have negatively affected the oxidative stability. OSI and thermal stability of sunflower oil significantly decreased after refining, which might be attributed to the decreased tocopherol level.

Oil color is also an important for sensory property for the acceptability of edible oils (Rhazi et al., 2022). The yellowish color of crude sunflower oil was removed after refining (Fig. 2). Refined silflower oil still had a dark color reflecting the high level of chlorophyll even though it was somewhat lightened. Table 5 shows the color analysis results. Reflecting the lighter color of refined oils shown in Fig. 2, lightness (L*) increased from 73.56 to 80.49 for sunflower oil and 31.22 to 46.47 for silflower oils. Chlorophyll is the major component responsible for green color. Table 4 showed that a* value was higher for refined silflower oil compared to crude oil reflecting the decreased chlorophyll level. The greater negative a* value for refined sunflower oil indicates that some compounds that are responsible for red color were removed along with the small amount of chlorophyll. The positive b* values of both oils before and after refining indicate that they all had yellowish color. The low b* value of refined sunflower oil reflected its very light color as shown in Figure 2.

Additional bleaching processes

Since silflower oil had very high chlorophyll even after the general refining process, silflower oil was further refined to reduce the level of chlorophyll and evaluated for the oxidative stability. Treatments with acid-activated bentonite (Sarioğlan et al., 2010; Srasra et al., 1989), acid-activated sepiolite (Saneei et al., 2015), and Tonsil® (Sabah & Çelik, 2005) have been reported to be effective to remove chlorophyll, and therefore, these methods were employed in this study. Table 6 summarizes the properties of silflower oils that were refined by these three methods. Bentonite was the most effective in removing chlorophyll (4.2 mg/kg) followed by Tonsil® (38.5 mg/kg), and then sepiolite (474.5 mg/kg). Since oil samples had been exposed to oxidation during the multiple refining processes, peroxide value (PV) and conjugated diene value (CDV) were determined from this point to examine oil oxidation. PV and CDV decreased after the treatment, rather

than increased. PV was significantly lowered by bentonite and Tonsil® while it was only slightly decreased by sepiolite. CDV significantly decreased by all the three methods. Total phenolics value and TPC also decreased after further refining. Radical scavenging ability determined by ABTS assay increased for silflower oil that were treated with bentonite and Tonsil®. The ABTS assay slightly (not statistically significantly) decreased after the treatment with sepiolite. The level of α-tocopherol decreased after the treatments with sepiolite while the increased by bentonite and Tonsil®. Again, some studies found that the contents of tocopherols could increase after refining processes (Pestana et al., 2008; Rossi et al., 2001; Van Hoed et al., 2006) although the mechanism for an increase in tocopherols was not well known. The FFA level increased after treating with bentonite while it decreased by sepiolite and remined similar after the treatment with Tonsil®.

Oxidative stability indicated by OSI decreased by refining with bentonite and sepiolite while it slightly increased by Tonsil®. Thermal stability of silflower oil significantly increased after the treatments with all three bleaching agents. The thermal stability of silflower oil treated with Tonsil® was slightly higher than refined sunflower oil. However, OSI values of further refined silflower oils were lower than refined sunflower oil. Further studies are needed to understand the factors affecting the oxidative stability of silflower oil and to improve the properties of silflower oil.

The color of further bleached silflower oils shown in Figure 3 well reflected the amount of chlorophyll. Chlorophyll content had statistically significant ($p < 0.05$) correlation coefficients with L* ($r = -0.995$) and a* ($r = 0.995$) values of the further refined oils. Silflower oil refined with bentonite had the lightest color followed by that with Tonsil®, and then that with sepiolite, which was also shown in lightness (L^*) in Table 7. The negative a* values for oils treated with bentonite and Tonsil® indicates that other compounds responsible of red color were also removed along with chlorophyll.

CONCLUSIONS

Silflower oil was extracted from unhulled silflower seeds with hexane and compared with the oil extracted from unhulled sunflower seeds. The oil content in unhulled silflower seeds was 15.2 % (w/w), and its fatty acid composition was very similar to that of sunflower oil. Silflower oil had high contents of TPC (12.35%, w/w), total phenolics (1.12 mg GAE/g oil), and chlorophyll (1002.8 mg/kg). It should be noted that it had a high squalene level (4.89%, w/w, isolated yield) indicating its potential application as a new plant source of squalene. Crude silflower oil had lower radical scavenging activity and oxidative stability than crude sunflower oil as indicated by ABTS assay, OSI, and TGA. The chlorophyll level of silflower oil was lowered, but still high (725.5 mg/Kg) after refining by a general refining process involving degumming, alkali refining, and bleaching with Fuller's earth. The oxidative and thermal stabilities of silflower oil decreased after the refining process.

Further treatments with bentonite, sepiolite, and Tonsil® significantly lowered the chlorophyll level to 4.2, 474.5, and 38.5 mg/kg, respectively. Some aspects of oxidative stability improved by these bleaching agents. For example, the ABTS assay increased by treatments with bentonite and Tonsil®, the OSI slightly increased by Tonsil®, and the thermal stability determined by TGA increased by all three bleaching agents.

More studies should be conducted to understand the factors affecting the oxidative stability of silflower oil and to further improve the oil quality. Oils from unhulled and hulled seeds may have different properties, crop year and harvest time may affect the level of chlorophyll, and different extract methods may result in different oil properties. With the given seeds and the oil extracted under the experimental conditions used in this study, it could be concluded that silflower oil, which can be produced from the perennial plant with many advantages such as higher drought tolerance and less tillage required, has a great potential as a new edible oil. This study also found that silflower oil can be a great plant source of squalene.

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Author contribution statement

Dr. H.-S. Hwang conceived and designed the study, conducted experiments for oil extraction, TPC, TGA, DSC, and NMR, and wrote the draft of the manuscript, Dr. S. X. Liu co-conceived and co-designed the study. Dr. J. K. Winkler-Moser conducted the research on most analyses of oils, Dr. M. Singh conducted the color analysis, Dr. D. V. Tassel cultivated the crop and provided seeds, and all authors contributed to and approved the final draft of the manuscript.

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Figure Legends

Figure 1. Melting (a) and crystallization (b) of silflower and sunflower oils extracted with hexane.

Figure 2. Color of crude and refined sunflower and silflower oils

Figure 3. Color of further refined oils with bentonite (Sil-1), sepiolite (Sil-2), and Tonsil® (sil-3).

Table 1. Properties of unrefined oils extracted with hexane from unhulled seeds.

	Silflower oil	Sunflower oil
Oil content (%, w/w)	15.2 ± 0.60 b[1]	18.0 ± 1.10 a
TPC (%, w/w)	12.3 ± 0.34 a	4.1 ± 0.11 b
Total phenolics (mg GAE/g sample)	1.12 ± 0.01 a	0.46 ± 0.03 b
ABTS assay (inhibition after 5 min, µmol TE/g)	0.78 ± 0.08 a	0.86 ± 0.11 a
Fatty acids		
C14:0	2.67 ± 0.00 a	0.04 ± 0.00 b
C16:0	8.14 ± 0.00 a	5.21 ± 0.01 b
C16:1	nd[2]	0.08 ± 0.00
C17:0	nd	0.04 ± 0.06
C18:0	2.26 ± 0.00 b	4.55 ± 0.00 a
C18:1 c9	19.86 ± 0.00 b	22.49 ± 0.04 a
C18:1 c11	0.52 ± 0.00 a	0.39 ± 0.01 b
C18:2 c9,12	65.53 ± 0.01a	65.48 ± 0.01 a
C18:3 c9,12,15	0.13 ± 0.00 a	0.00 ± 0.00 b
C20:0	0.27 ± 0.00 b	0.31 ± 0.00 a
C20:1 c11	0.16 ± 0.00 a	0.11 ± 0.00 b
C22:0	0.13 ± 0.00 b	0.95 ± 0.00 a
C24:0	0.34 ± 0.00 b	0.36 ± 0.00 a
%Unsaturated fatty acids	86.20	88.55
Squalene, %	4.34 ± 0.02 (by GC) 4.89 ± 0.00 (isolated yield)	nd
α-Tocopherol (µg/g)	305.5 ± 1.6 b	660.1 ± 7.9 a
Free fatty acid (%)	1.00 ± 0.01 b	0.47 ± 0.01 a
Chlorophyll as pheophytin a (mg/Kg)	1002.8 ± 48.3 a	2.7 ± 0.3 b

[1]Means not sharing the same letter(s) are significantly different by Tukey-Kramer HSD test ($P < 0.05$) in comparison between oils.

[2]nd: not detected

GAE: Gallic acid equivalent; TE: Trolox equivalent

Table 2. Melting and crystallization profiles of silflower and sunflower oils extracted with hexane.

DSC Melting (°C)	Silflower oil	Sunflower oil
Tm1	-27.95 ± 0.19 a[1]	-28.42 ± 0.25 a
Tm2	-39.87 ± 0.65 b	-36.91 ± 0.15 a
Tm3	-58.25 ± 0.24 a	-62.93 ± 0.67 b
DSC crystallization (°C)		
Tc1	-9.62 ± 0.04	Shown as a small shoulder
Tc2	-22.09 ± 0.79 b	-13.67 ± 0.14 a
Tc3	-48.89 ± 0.39 b	-41.82 ± 0.54 a

[1]Means not sharing the same letter(s) are significantly different by Tukey-Kramer HSD test ($P < 0.05$) in comparison between oils.

Table 3. Oxidative stability index (OSI) at 110 °C and thermal stability by thermal gravimetry analysis (TGA) of crude silflower and sunflower oils extracted with hexane.

Oxidative and thermal stabilities	Silflower oil	Sunflower oil	Sunflower oil
OSI at 110 °C, hours	2.42 ± 0.03 b[1]	4.35 ± 0.08 a	4.35 ± 0.08 a
Thermal stability (onset temperature of degradation), °C	282.5 ± 1.5 b	298.2 ± 4.3 a	

[1]Means not sharing the same letter(s) are significantly different by Tukey-Kramer HSD test ($P < 0.05$) in comparison between oils.

Table 4. Properties of refined silflower and sunflower oils.

Properties	Refined silflower oil	Refined sunflower oil
Oil analysis		
Chlorophyll as pheophytin a (mg/Kg)	725.5 ± 2.4	0
Total polar compounds (TPC, %, w/w)	11.35 ± 0.01 a[1]	3.73 ± 0.02 b
ABTS assay (inhibition after 5 min), µmol TE/g	0.58 ± 0.02 b	0.84 ± 0.04 a
Total phenolics (mg GAE/g sample)	1.10 ± 0.40 a	0.47 ± 0.13 b
Squalene	4.19 ± 0.02	0
α-Tocopherols (µg/g)	333.4 ± 44.8 a	203.9 ± 2.0 b
Free fatty acid (%)	0.13 ± 0.01 a	0 b
Oxidative and thermal stabilities		
Oxidative Stability Index (OSI) at 110 °C, hours	2.10 ± 0.01 b	2.69 ± 0.04 a
Thermal stability (onset temperature of degradation), °C	277.8 ± 1.3 a	287.2 ± 3.3 a

[1]Means not sharing the same letter(s) are significantly different by Tukey-Kramer HSD test ($P < 0.05$) in

comparison between oils.

GAE: gallic acid equivalent; TGA: thermal gravimetry analysis, ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); TE: Trolox equivalent; OSI: oxidative stability index

Table 5. Color analysis of refined silflower and sunflower oils compared to crude oils.

Sample	L^*	a^*	b^*
Sunflower oil	73.56 ± 0.53 b[1]	-1.26 ± 0.10 c	65.69 ± 1.36 b
Refined sunflower oil	80.49 ± 0.36 a	-4.35 ± 0.13 d	11.75 ± 0.44 d
Silflower oil	31.22 ± 0.45 d	8.93 ± 0.02 b	51.35 ± 0.51 c
Refined silflower oil	46.47 ± 0.15 c	11.11 ± 0.15 a	75.79 ± 0.31 a

[1]Means not sharing the same letter(s) are significantly different by Tukey-Kramer HSD test ($P < 0.05$) in comparison between oils. L^* : Lightness (0 = dark, 100 = light), a^* : green to red (Positive is red and negative is green), b^* : yellow to blue (Positive is yellow and negative is blue).

Table 6. Properties of further refined oils with bentonite (Sil-1), sepiolite (Sil-2), and Tonsil® (Sil-3)

	Refined silflower oil	Further refined oils	Further refined oils	Further refined oils
		Sil-1	Sil-2	Sil-3
Oil analysis				
Chlorophyll as pheophytin a (mg/kg)	725.5 ± 2.4 a[1]	4.2 ± 0.1 d	474.5 ± 0.1 b	38.5 ± 3.7 c
Peroxide value (meq/kg)	26.00 ± 1.56 a	1.40 ± 0.16 b	22.78 ± 0.86 a	4.32 ± 0.10 b
Conjugated diene value (mmol/L)	0.61 ± 0.01 a	0.26 ± 0.01 b	0.37 ± 0.03 b	0.33 ± 0.06 b
Total phenolics (mg GAE/g sample)	1.10 ± 0.40 a	0.53 ± 0.18 b	0.59 ± 0.17 b	0.79 ± 0.07 ab
ABTS assay, after 5 min, µmol TE/g	0.58 ± 0.02 c[1]	0.92 ± 0.01 a	0.55 ± 0.02 c	0.70 ± 0.06 b
Total polar compounds (TPC, %, w/w)	11.35 ± 0.01 a	10.06 ± 0.12 b	10.01 ± 0.10 bc	9.66 ± 0.21 c
α-Tocopherols (µg/g)	333.4 ± 44.8 bc	489.2 ± 22.3 a	242.2 ± 1.5 c	367.2 ± 1.2 b
Free fatty acid (%)	0.13 ± 0.01 b	0.25 ± 0.01 a	0.09 ± 0.00 b	0.12 ± 0.01 b
Oxidative and thermal stabilities	*Oxidative and thermal stabilities*	*Oxidative and thermal stabilities*		
Oxidative Stability Index (OSI), 110 °C (h)	2.10 ± 0.01 ab	1.69 ± 0.02 bc	1.42 ± 0.04 c	2.27 ± 0.28 a
Thermal stability (onset temperature of degradation), °C	277.8 ± 1.3 b	289.5 ± 9.1 ab	287.6 ± 1.7 ab	295.6 ± 0.6 a

[1]Means not sharing the same letter(s) are significantly different by Tukey-Kramer HSD test ($P < 0.05$) in comparison between oils.

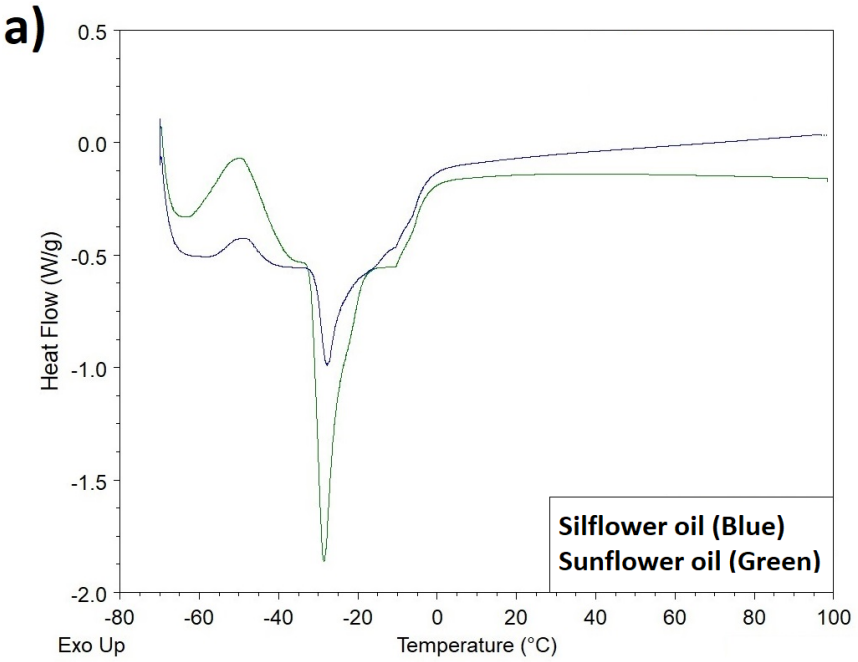
GAE: gallic acid equivalent; TGA: thermal gravimetry analysis, ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); TE: Trolox equivalent; OSI: oxidative stability index

Table 7. Color analysis of further refined oils with bentonite (Sil-1), sepiolite (Sil-2), and Tonsil® (Sil-3).

Sample	L^*	a^*	b^*
Refined silflower oil	46.47 ± 0.15 d[1]	11.11 ± 0.15 a	75.79 ± 0.31 b
Sil-1	74.88 ± 1.27 a	-6.41 ± 0.11 c	49.54 ± 1.75 c
Sil-2	53.54 ± 0.51 c	11.22 ± 0.27 a	81.23 ± 0.80 a
Sil-3	71.47 ± 0.44 b	-5.34 ± 0.08 b	46.02 ± 0.74 d

[1]Means not sharing the same letter(s) are significantly different by Tukey-Kramer HSD test ($P < 0.05$) in comparison between oils. L^* : Lightness (0= dark, 100=light), a^* : green to red (Positive is red and negative is green), b^* : yellow to blue (Positive is yellow and negative is blue).

Figure 1. Melting (a) and crystallization (b) of silflower and sunflower oils extracted with hexane.



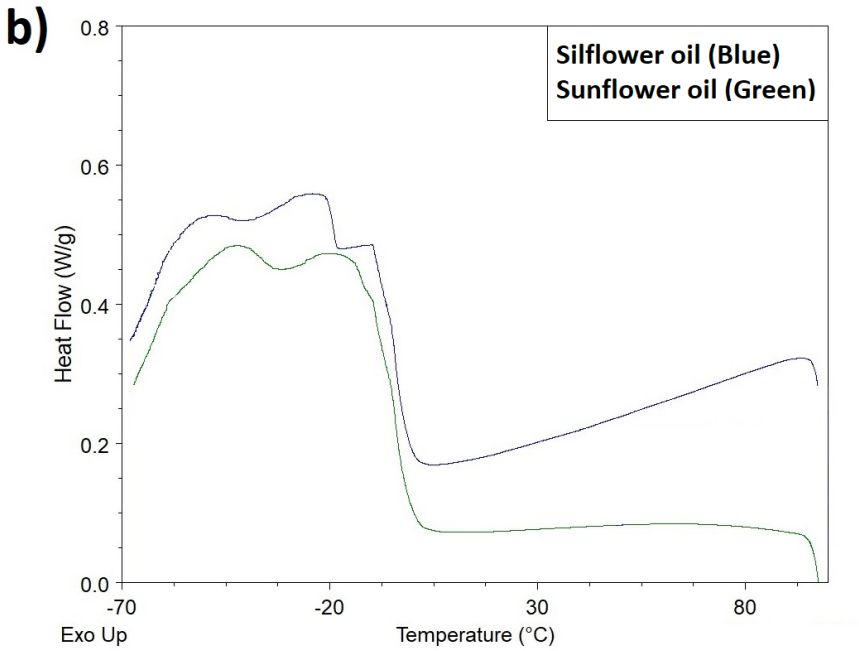


Figure 2. Color of crude and refined sunflower and silflower oils

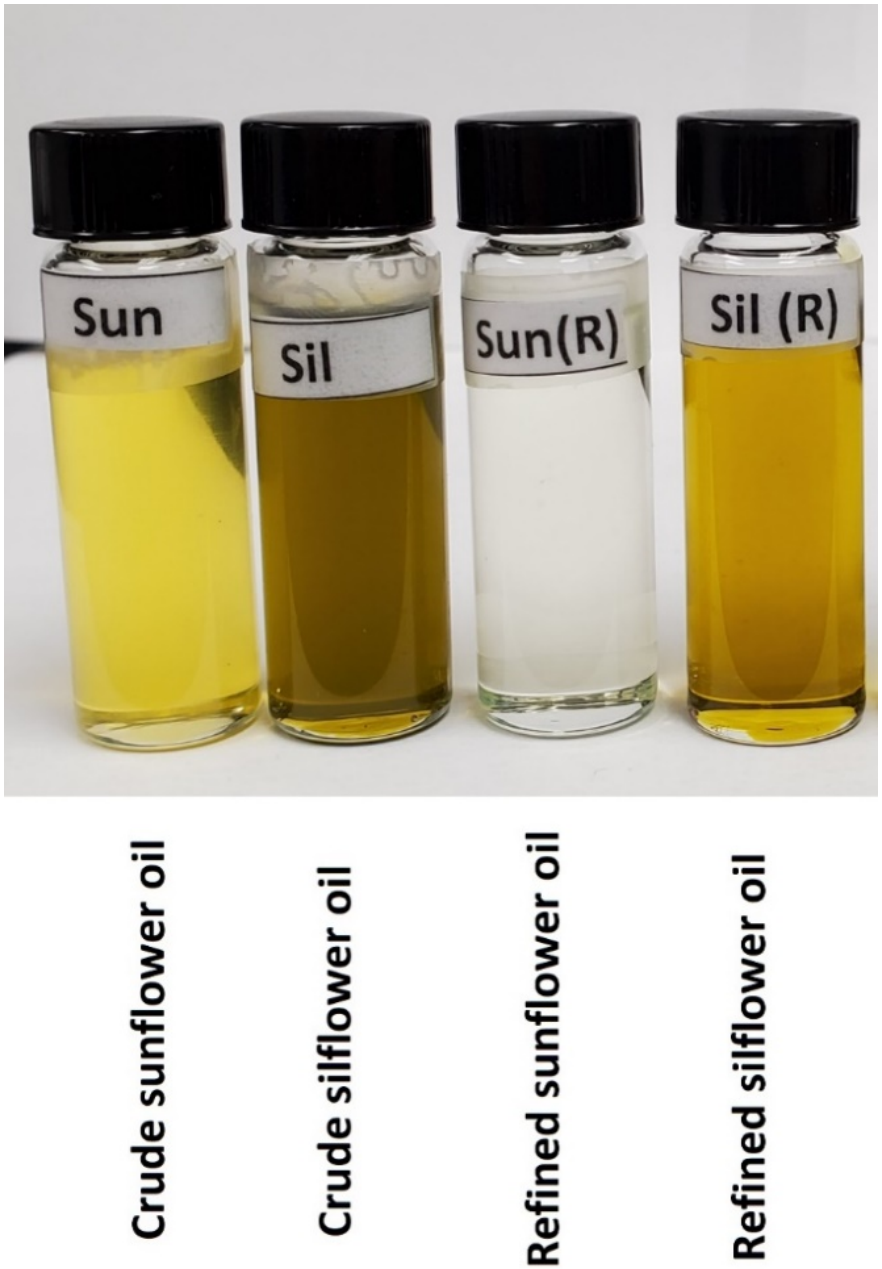


Figure 3. Color of further refined oils with bentonite (Sil-1), sepiolite (Sil-2), and Tonsil® (sil-3).

Refined silflower oil

Sil-1

Sil-2

Sil-3