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Positional distribution of Δ5-olefinic acids in triacylglycerols from *Torreya grandis* seed oil: Isolation and purification of sciadonic acid

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ABSTRACT

In recent years, in concern for human health, increasing attention was focused on sciadonic acid (SA), a unique Δ5-olefinic acid. *Torreya grandis* (*T. grandis*), a large, evergreen coniferous tree, whose seed yields rare and is known for a high level of SA. Triacylglycerols (TAGs) from the seeds of four different *T. grandis* varieties (Yuanfei, Xifei, Xiangyafei, and Zhimafei), were analyzed with respect to fatty acid profiles and positional distributions. The results indicated that SA represented the majority of the Δ5-olefinic acids in *T. grandis* seed oil. Irrespective of the variety, SA was naturally abundant at the sn-3 position of TAGs where they accounted for approximately 7.52%–12.00%. Based on this specific characteristic of Δ5-olefinic acids, *T. grandis* seed oil was subjected to a two-step enrichment process involving specific lipase-catalyzed ethanolysis and urea complexing. The content of SA in the fatty acid ethyl ester was increased to 80.14% from an initial value of 9.95%. These results suggested that the developed enrichment strategy was simple and effective in concentrating SA, and had the potential for commercial application.

1. Introduction

Natural plant oil fats are complex mixtures consisting primarily of triacylglycerols (TAGs) with the numbers of TAG units being ≥ 50 (Acheampong et al., 2011). Triacylglycerols are formed almost invariably by glycerol and long chain fatty acids, including saturated fatty acids (SFAs) and unsaturated fatty acids (UFAs), which position on the glycerol backbone (sn-1/3 and sn-2). Most common plant oils contain polyunsaturated fatty acids (PUFAs), frequently with 18 carbon atoms and a single methylene interrupted double bonds (Lísa et al., 2007). However, there exist unusual fatty acids such as unsaturated polymethylene interrupted fatty acids (UPIFAs), which can be found in some taxonomical groups. In recent years, increasing attention has been focused on Δ5-UPIFAs, which have the first site of unsaturation at the fifth carbon atom, and which are characteristic of seeds of *Gymnosperms,* and differ from the structure of other PUFAs (Xie et al., 2016). The Δ5-UPIFAs include cis-5,9-octadecadienoic acid (taxoleic acid), cis-5,11-octadecadienoic acid (ephedrenic acid), cis-5,9,12-octadecatrienoic acid (pinolenic acid), trans-5, cis-9, cis-13-octadecatrienoic acid (columbinic acid), cis-5,9,12,15-octadecatetraenoic acid (coniferonic acid), cis-5,11,14-eicosatrienoic acid (sciadonic acid), and cis-5,11,14,17-eicosatetraenoic acid (juniperonic acid) (Endo et al., 2009).

Torreya, a large, evergreen coniferous tree, is a primitive member of the *Gymnospermous* yew family (*Taxaceae*). *Torreya grandis (T. grandis)* is a common ornamental plant in China (Wua et al., 2018). Due to the influence of the environment and cultivation management, *T. grandis* is morphologically variable. In China, they are distributing widely in Zhejiang, Anhui, and Jiangxi provinces (Shi et al., 2018), and include different varieties including Mufei, Xifei, Xiangyafei, and Zhimafei, with various seed shapes and qualities. *Torreya. grandis* is known for the presence of Δ5-UPIFAs, especially rich in sciadonic acid (SA), which represented 8.30%–16.40% of the oil.

The health benefits of Δ5-UPIFAs have been widely reported, which involve decreasing the blood pressure and lowering TAG levels in serum and liver of rats (Sugano et al., 1994; Asset et al., 1999; Endo et al., 2006). Furthermore, Δ5-UPIFAs such as SA and pinolenic acid also have anti-inflammatory effects by suppressing production of proinflammatory mediators like prostaglandin E_2 or competition with arachidonic acid (Chuang et al., 2009; Chen et al., 2015). Additionally, due to their preventative effects on angiosclerosis and obesity and in certain types of cancers and neurological disorders, Δ5-UPIFAs are important in human dietary modification to prevent several chronic diseases. In view of the biological importance of Δ5-UPIFAs, there is a need to develop a procedure for the enrichment of Δ5-UPIFAs as a novel nutraceutical.

The isolation of Δ5-UPIFAs has been performed by using repeated argentated thin layer chromatography (TLC) or argentated liquid

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chromatography in combination with solid phase extraction on florisil (Berger et al., 2002). In addition, iodolaconization is also effective in separating and purifying the pinolenic acid from distilled tall oil (Hase et al., 1992). For chemical-catalyzed transesterification, sodium hydroxide and potassium hydroxide are commonly employed as catalysts. Although chemical-catalyzed transesterification method is the most popular process, it requires strict feedstock specification, incurs additional separation costs, and produces waste water (Ting et al., 2008; Maleki et al., 2013). Using immobilized enzymes in transesterification reactions may eliminate these problems. Enzymatic methods involving lipases have been reported for the enrichment of various biologically active fatty acids, including the SA from *Biota Orientalis* seed oil (Marcel et al., 1995), pinolenic acid from pine nut oil (No et al., 2015), and docosapentaenoic acid from algae oil (Wang et al., 2018). It has been reported that lipase can catalyze the esterification of free fatty acids with ethanol to concentrate Δ5-UPIFAs from pine nut oil (Kim et al., 2018). This enzymatic method proceeds under mild reaction conditions and consumes less energy. Interestingly, the desired fatty acid was enriched in an unesterified fatty acid fraction. However, these enriched effects were all based on the fatty acid selectivity of lipases. ^{13}C NMR spectroscopy combined with partial chemical deacylation with Grignard reagent analysis suggested that the Δ5-olefinic acids in conifer seed oils were mainly esterified at the sn-3 position of the triglyceride (Wolff et al., 1997). This position characteristic may facilitate the enzymatic preparation of Δ5-olefinic acids.

In the present study, the hypotheses that Δ5-UPIFAs are almost exclusively esterified to the sn-3 position of TAG was tested with four *T. grandis* varieties of seed oils. This distribution characteristic was used to concentrate SA. Seed oil from *T. grandis* (Xifei variety) was transesterified to enrich SA by a 1,3-specific lipase, followed by optimization of the process. The obtained SA-enriched fatty acid ethyl ester (FAEE) fractions were subjected to urea complexation and argentated column chromatography to prepare SA of high purity.

2. Materials and methods

2.1. Materials and chemicals

Four different varieties of *T. grandis* seeds (Yuanfei, Xifei, Xiangyafei, and Zhimafei) were from Zhejiang A & F University (Hangzhou, Zhejiang, China). Bile salts, calcium chloride, diethyl ether, 37% HCl, boric acid, anhydrous Na_2SO_4, n-hexane, acetic acid, formic acid, pyridine, choline chloride, ammonia, $CaCl_2$, 95% aqueous ethanol, methanol, urea, phosphorus oxychloride, ethyl magnesium bromide (EMB), sodium ethoxide, and silver nitrated, silica gel (200–300 mesh) were purchased from Aladdin Industrial Corporation (Shanghai, China). Phospholipase A_2 and porcine pancreatic lipase were obtained from Sigma-Aldrich (St. Louis, MO, USA). Lipozyme TLIM (lipase from *Thermomyces lanuginosus*, immobilized on silica granulation) was from Novozymes (Beijing, China). All reagents were of analytical grade.

2.2. Analytical method for position distribution of fatty acids on TAG

Pancreatic lipase is used to specifically hydrolyze the fatty acids esterified to the primary positions of TAG yielding 2-monoacylglycerol (MAG), the fatty acid composition of which accurately reflects that of sn-2 in the original TAG. After converting 1,2- plus 2,3-diacylglycerols (DAGs) obtained from partial degradation of TAG with Grignard reagent to phosphatidylcholines (PC), it was reacted with phospholipase A_2, which hydrolyzed the ester bond stereospecifically, reflecting the fatty acid composition of sn-1 in the original TAG. The detailed scheme of positional distribution of fatty acid was shown in Fig. 1.

2.2.1. Preparation of 2-MAG from TAG with porcine pancreatic lipase

Preparation of 2-MAG from TAG was carried out according to the method of Sahin et al. (2005). Purified TAG (200 mg) was dissolved in 4 mL of Tris buffer (pH 8.0) with addition of 1 mL of bile salts solution (0.1%) and 0.4 mL of calcium chloride solution (22%). The mixture was incubated at 40 °C in a water bath for 1 min, purified porcine pancreatic lipase (22.6 mg) was added to initiate the hydrolysis reaction, and the sample was kept for 1 min. Then, 6 M HCl (4 mL) was added to stop the reaction and addition of diethyl ether (5 mL) was used to extract the partial glyceride obtained. After 1 min of vigorous agitation, the reaction mixture was centrifuged at 5000 × *g* for 15 min. Diethyl ether layer was evaporated under a stream of N_2 and the residue was applied to a TLC plate. The reaction products were fractionated with n-hexane/diethyl ether/acetic acid (50:50:1, v/v/v), visualized by iodine vapors, and the band corresponding to 2-MAG was scraped into centrifuge tubes for further analysis.

Purified TAG was obtained by a preparative TLC plate fractionated by n-hexane/diethyl ether/acetic acid (75:25:1, v/v/v). Neutral TAG and polar lipid were fractioned and visualized by iodine vapors and the band corresponding to TAG was scraped into centrifuge tubes and extracted with n-hexane.

2.2.2. Partial degradation of TAG with Grignard reagent to prepare 1,2- plus 2,3-DAG

Partial degradation of TAG with the Grignard reagent followed previously reported methods (Becker et al., 1993; Wolff et al., 1997). Purified TAG (20 mg) was added to a centrifuge tube with N_2 flushing, which contained 1.4 mL diethyl ether, then 0.1 mL EMB was added. After 30 s of vigorous agitation, the reaction was stopped by addition of 4 mL of acidic buffer (mixture of 1 volume of 37% HCl and 36 volumes of a 0.4 M boric acid solution). The partially degraded glyceride mixture was extracted with diethyl ether (5 mL). The aqueous phase was discarded, the diethyl ether phase was washed with a 0.4 M boric acid solution (2 mL), and dried by excessive anhydrous Na_2SO_4. After filtering, the filtrate was evaporated to dryness under a stream of N_2 and then applied to a TLC plate. The reaction products were fractionated with n-hexane/diethyl ether/formic acid (70:30:1, v/v/v), visualized by iodine vapors, and the bands corresponding to 1,2- plus 2,3-DAG were scraped into centrifuge tubes.

2.3. Preparation of phosphatidylcholines from 1,2- plus 2,3-DAG

The procedure was performed as previously described by Myher and Kuksis (1979), with slight modifications. The 1,2- plus 2,3-DAG recovered from the TLC plate was dissolved in 0.65 mL of chilled solution consisting of chloroform/pyridine/phosphorus oxychloride (47.5:47.5:5, v/v/v). The solution was mixed and allowed to stand for 1 h at 0 °C and for 1 h at 25 °C. The solution was then transferred to a similar tube containing dry powdered choline chloride (200 mg) and a small magnetic stirring bar. The mixture was vigorously stirred for 15 h at 30 °C. After addition of 20 μL of water, the sample was kept for 30 min more. Most of the chloroform and pyridine was removed by nitrogen. The products were extracted with 12 mL of chloroform/methanol/water/acetic acid (58:39:18:1, v/v/v/v), and partitioned with 4 M ammonia (4 mL). The aqueous phase was re-extracted with 2 mL of chloroform/methanol/water/acetic acid (58:39:18:1, v/v/v/v). The organic phase was combined, dried with anhydrous Na_2SO_4, and evaporated to dryness under a stream of N_2. The residue was then applied to a TLC plate. The reaction products were fractionated with chloroform/methanol/water (70:20:2, v/v/v). The band corresponding to PC was then scraped.

2.4. Specific hydrolysis of sn-2 fatty acid in phosphatidylcholines with phospholipase A_2

The PC was extracted with 5 mL of chloroform/methanol (1:1, v/v) and vortexed for 5 min, twice. After evaporation under a stream of N_2, diethyl ether (6 mL) containing 1.2 mL of 0.5 M Tris-HCl (pH = 7.5)

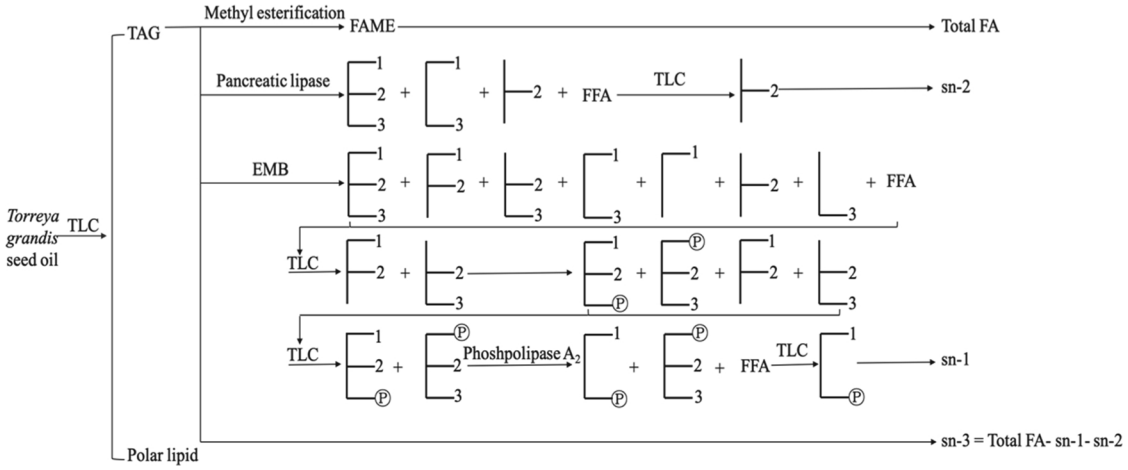

Fig. 1. The detailed scheme of positional distribution of fatty acid. TLC, thin layer chromatography; TAG, triacylglycerol; FAME, fatty acid methyl ester; FA, fatty acid; FFA, free fatty acid; EMB, ethyl magnesium bromide.

and 2 mM $CaCl_2$ together with 20 μL of phospholipase A_2 was added (Carballeira et al., 1994). The reaction mixture was thoroughly mixed and incubated at 37 °C for 10 min, then added to 0.4 mL of 1 M HCl to stop the reaction. The products were extracted with 12 mL of chloroform/methanol (2:1, v/v), and the aqueous phase was re-extracted with 12 mL of chloroform. The organic phase was mixed and evaporated to dryness under a stream of N_2. The residue was applied to a TLC plate and developed by chloroform/methanol/acetic acid (55:45:4, v/v/v). The fractions were visualized under iodine vapor and the band corresponding to lysophosphatidylcholine (lysoPC) was scraped for further analysis (Amate et al., 1999).

2.5. Fatty acid analysis

Fatty acids from lysoPC, 2-MAG, and TAG were converted into fatty acid methyl ester (FAME) according to the method reported by Wolff et al. (1998). The silica gel powder was dispersed into 5 mL of BF_3/methanol solution (12%, w/v) and the reaction was initiated with heating in a boiling water bath for 1 h. Two milliliter of n-hexane was added and then partitioned with saturated NaCl solution. The n-hexane layer containing the FAME was recovered and evaporated to dryness under a gentle stream of N_2, and the residue was quantified by gas chromatography/mass spectrometry on an Agilent 7890A chromatography (Agilent Technologies, Santa Clara, CA, USA) fitted with an HP-88 capillary column (60 m × 0.25 mm i.d.). The injection volume was 1 μL, and the temperatures of injector and flame ionization detector were set at 250 °C and 260 °C, respectively. The oven temperature was held for 1 min at 120 °C and then ramped to 175 °C at 10 °C/min, held for 10 min; ramped to 210 °C at 5 °C/min, held for 5 min; ramped to 230 °C at 5 °C/min, and finally held for 5 min for a total analysis time of 37.5 min. The methyl esters of Δ5-olefinic acids were identified by mass spectrometry combined with comparing their retention times with those of the known standards.

2.6. Determination of TAG structures

The positional distribution of fatty acids in TAG was deduced from the fatty acid compositions according to the following formulas:

$$\text{sn-3} = \text{total FA} - \text{sn-1} - \text{sn-2}$$

The sn-2-MAG was generated with porcine pancreatic lipase, whereas sn-1-lysoPC was obtained after deacylation of TAG with Grignard reagent and was then transferred to PC, with stereospecific hydrolysis with phospholipase A_2.

2.7. Concentration of Δ5-olefinic acids

2.7.1. Lipase-catalyzed ethanolysis

The lipase-catalyzed ethanolysis of *T. grandis* (Xifei variety) seed oil with 95% aqueous ethanol was conducted. A mixture of *T. grandis* seed oil (12 g), 95% aqueous ethanol (48 g), and Lipozyme TLIM (3 g) was stirred at 250 rpm in a glass culture flask at the desired temperature controlled by a water circulator for a desired time. The sample was withdrawn at various time intervals and applied to a TLC plate, and fractionated by petroleum ether/diethyl ether (95:5, v/v). The band corresponding to FAEE was scraped for fatty acid profile analysis.

2.7.2. Urea complexation of FAEE

The FAEE obtained from raw oil by sodium ethoxide-catalyzed transesterification and enzymatic ethanolysis was used as starting material for urea complexing (Vázqueza et al., 2017). For chemically-catalyzed transesterification, 100 g oil was heated to 55 °C and then 25 mL ethanol containing 1.5 g sodium ethoxide was added to the reactor and reacted for 2 h. The phase separation was carried out in a separatory funnel at room temperature. The supernate was then washed with distilled water and vaporized to dryness to obtaining FAEE.

Urea, at concentrations of 10, 5, 3, 2, and 1.5 g were mixed with 20 mL of 95% aqueous ethanol in a 120 mL vial. The mixture was mixed at 70 °C until urea was completely dissolved. Then, FAEE (1 g) was added and agitated until the reaction mixture became a clear solution, which was subsequently cooled to room temperature and was transferred to an ice bath. Overnight, the obtained urea inclusion complexes were filtered. Then, 4 mL of hexane were added to the filtrate twice to effectively recover the FAEE. The extraction mixture was subsequently washed with 40% (v/v) of acidic water [1% H_2SO_4 (v/v) in distilled water] and 40% (v/v) distilled water. The hexane layer was passed through anhydrous Na_2SO_4. This non-urea complexing fraction was applied to a TLC plate and fractionated by petroleum ether/diethyl ether (95:5, v/v). The FAEE fraction was scraped for gas chromatography (GC) analysis.

2.7.3. Argentated column chromatography

The FAEE obtained from urea complexation was subjected to argentated column chromatography as described by Chen et al. (2012). Silver nitrate (4.8 g) was dissolved in 100 mL of 90% methanol (methanol/water, 9/1 by volume), then the silver nitrate solution was slowly mixed with 80 g of silica gel (200–300 mesh, Aladdin Industrial Corporation) in the dark for 30 min. The argentated silica gel was then dried for 1 h at 100 °C and activated for 1 h at 130 °C. A slurry of argentated silica gel in hexane was poured into a glass column

(60 cm × 3 cm) previously half-filled with hexane. Two gram FAEE was then dissolved in hexane and loaded into the column and was eluted with 500 mL of hexane/acetyl acetate (9:1, v/v) solution. The eluates were collected at fixed intervals and used for GC analysis.

2.7.4. Analysis of products

The TLC bands corresponding to FAEE from the lipase-catalyzed ethanolysis, urea complexation, and argentated column chromatography were scraped and extracted with 5 mL n-hexane and vortexed for 3 min. The supernate was evaporated to dryness under a gentle stream of N_2 and the residue was resolved in hexane and quantified by GC as described in Section 2.5. Due to structural similarities, their retention time could be different but the elution order was the same, hence, FAEE was deduced by comparison with known FAME standards.

3. Results

3.1. Fatty acid composition

Torreya grandis seed oil is the most important conifer seed oil, which is rich in SA that is not yet available commercially. Moreover, SA is believed to have favorable health benefits. GC analysis suggested that the fatty acid profile in *T. grandis* seed oil included palmitic acid ($C_{16:0}$), hexadecenoic acid ($C_{16:1}$ cis-9), heptadecanoic acid ($C_{17:0}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$ cis-9), linoleic acid ($C_{18:2}$ cis-9,12), octadecatrienic acid ($C_{18:3}$ cis-5,9,12), eicosenoic acid ($C_{20:1}$ cis-11), linolenic acid ($C_{18:3}$ cis-9,12,15), eicosenoic acid ($C_{20:2}$ cis-8,11 and $C_{20:2}$ cis-11,14), SA, and juniperonic acid ($C_{20:4}$ cis-5,11,14,17) (Table 1). The uncommon Δ5-UPIFAs were identified by the similarities of the corresponding mass spectra with those from a mass spectra database (NIST MS search 2.0). The results showed that octadecatrienic acid ($C_{18:3}$ cis-5,9,12, Fig. 2A), SA (Fig. 2B), and juniperonic acid ($C_{20:4}$ cis-5,11,14,17, Fig. 2C) constituted the main Δ5-UPIFAs. The fatty acid content of *T. grandis* seed oil varied between 0.04% and 45%. SFAs represented by palmitic acid and stearic acid were less than 9% in all varieties. The highest content of UFAs in *T. grandis* seed oil was linoleic acid, which varied from 32% to 45% and the content of oleic acid was also higher than 22%. As for the Δ5-UPIFAs, the content of SA was between 9% and 15%, specifically with a value of 14.50% for Mufei, 9.95% for Xifei, 9.42% for Xiangyafei, and 8.91% for Zhimafei. These results showed that linoleic acid, oleic acid, palmitic acid, and SA were the main fatty acids in *T. grandis* seed oil. When considering overall fatty acid compositions of *T. grandis* seed oil, it followed the order: PUFAs > monosaturated fatty acids (MUFAs) > SFAs. Li et al. also analyzed eight *T. grandis* seed oils from two varieties of *Fort. Ex. Lindl* and *var. Merrillii*. The results showed that the highest content of Δ5-UPIFAs in *T. grandis* seed oil was SA, which consisted of 9.18%–18.65% of total fatty acids (Shi et al., 2018). All these results suggested that *T. grandis* seed oil was an ideal oil resource for SA preparation.

3.2. Positional distribution of fatty acids in TAGs

Torreya grandis from the *Cephalotaxaceae* family and *Taxaceae Torreya* genus is characterized by a relatively high level of SA and small amounts of both octadecatrienic acid ($C_{18:3}$ cis-5,9,12) and juniperonic acid ($C_{20:4}$ cis-5,11,14,17). The fatty acid distribution on the carbon skeleton of glycerol for four oils from different *T. grandis* varieties have been analyzed and were shown in Tables 2–5. Sciadonic acid found at the sn-3 position was far more abundant than at the sn-1 and sn-2 positions. It should be noted that SFAs, like palmitic acid and stearic acid, were unevenly distributed, and primarily occupied the sn-1 position rather than the sn-3 and sn-2 positions. Unsaturated fatty acids like oleic acid and linoleic acid were highly represented at the sn-2 position, which agreed with other vegetable oils (Kim et al., 2007; Yoshida et al., 2007). Blaise et al., 1997 also analyzed positional distributions of Δ5-olefinic acids in TAGs from conifer seed oils determined by partial chemical cleavage, and concluded that the almost exclusive location of Δ5-acids in the external positions of TAGs appeared to be a general feature of conifer seed oils (Blaise et al., 1997). Later, Wolff et al. found that the most abundant Δ5-UPIFA was toxoleic acid ($C_{18:2}$ cis-5,9) for *Taxus baccata* (11%) and *Sciadopytis verticillata* (47.1%); pinolenic acid ($C_{18:3}$ cis-5,9,12) for *T. baccata* (28.8%), *Pinus koraiensis* (15.5%) and *P. pinas*ter (7.3%); SA for *S. verticillata* (13.8%); and juniperonic acid ($C_{20:4}$ cis-5,11,14,17) for *Juniperus communis* (17.6%), and concluded that the Δ5-olefinic acids were naturally abundant at the sn-3 position of TAGs, irrespective of the varieties, chain lengths, and number of ethylenic bonds (Wolff et al., 1997). These results indicated that enzymatic hydrolysis using 1,3-specific lipase may be effective in enriching the SA from *T. grandis* seed oil. In addition, considering the wide resources of *T. grandis* seeds and accepted content of SA, Xifei seed oil was used for subsequent concentration experiments of SA.

3.3. 1,3-Specific lipase-catalyzed transesterification

The lipase species is a crucial factor in terms of controlling the product purity and yield. It is reported that Lipozyme TLIM is more 1,3-site-specific compared with Lipozyme RMIM and Novozyme 435 (Irimescu et al., 2001). The time courses of ethanolysis of *T. grandis* (Xifei variety) seed oil at 37 °C and 50 °C catalyzed by Lipozyme TLIM are shown as Fig. 3. Generally, Lipozyme TLIM showed higher catalysis specificity at 37 °C. Although SA recovery (percentage of the amount of

Table 1
Fatty acid compositions of triacylglycerols from four different *Torreya grandis* seed oils (Yuanfei, Xifei, Xiangyafei, and Zhimafei) (results expressed as weight percentages).

Fatty acid	*Torreya grandis* varieties			
	Mufei	Zhimafei	Xiangyafei	Xifei
16:0	9.01 ± 0.21	8.44 ± 0.99	9.22 ± 1.08	8.94 ± 1.31
9-16:1	0.13 ± 0.02	0.09 ± 0.03	0.04 ± 0.01	0.06 ± 0.01
17:0	0.11 ± 0.01	0.08 ± 0.02	0.10 ± 0.00	0.07 ± 0.02
18:0	2.88 ± 0.03	3.02 ± 0.32	3.56 ± 0.27	3.24 ± 0.29
9-18:1	22.24 ± 0.04	32.77 ± 4.61	39.36 ± 0.14	32.59 ± 0.04
9,12-18:2	44.91 ± 0.44	38.63 ± 3.54	31.95 ± 2.54	39.21 ± 2.83
5,9,12-18:3	0.41 ± 0.41	0.15 ± 0.02	0.28 ± 0.11	0.09 ± 0.05
11-20:1	0.12 ± 0.06	0.45 ± 0.10	0.46 ± 0.00	0.35 ± 0.11
9,12,15-18:3	0.65 ± 0.47	0.75 ± 0.12	0.68 ± 0.17	0.52 ± 0.07
8,11-20:2	0.79 ± 0.04	0.59 ± 0.27	0.74 ± 0.21	0.65 ± 0.18
11,14-20:2	2.72 ± 0.10	4.52 ± 1.53	3.95 ± 1.37	3.87 ± 1.53
5,11,14-20:3	14.50 ± 0.13	8.91 ± 0.13	9.42 ± 0.53	9.95 ± 0.62
5,11,14,17-20:4	0.44 ± 0.01	0.17 ± 0.02	0.15 ± 0.02	0.09 ± 0.06

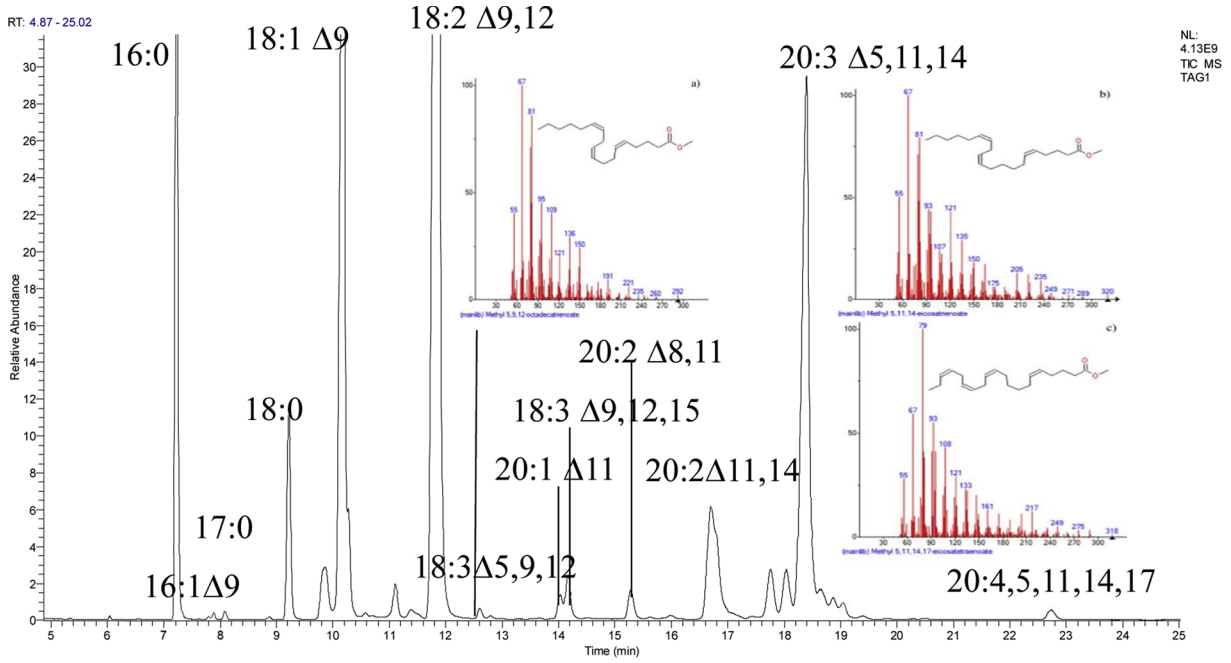

Fig. 2. Fatty acids profile of *Torreya grandis* seed oils by gas chromatography.

Table 2
The distribution of fatty acids at the sn-1, sn-2, and sn-3 positions of triacylglycerols from *Torreya grandis* (Xifei variety) seed oil (results expressed as percentage of specific fatty acid at corresponding positions compared with the total fatty acid of triacylglycerols).

Fatty acid	Triacylglycerols	sn-1	sn-2	sn-3
16:0	8.94 ± 1.31	5.95 ± 0.13	0.37 ± 0.14	2.63 ± 0.27
9-16:1	0.06 ± 0.01	–	–	0.06 ± 0.00
17:0	0.07 ± 0.02	–	–	0.07 ± 0.00
18:0	3.24 ± 0.29	2.28 ± 0.00	0.09 ± 0.01	0.87 ± 0.02
9-18:1	32.59 ± 0.04	13.23 ± 0.37	18.20 ± 1.51	1.17 ± 1.89
9,12-18:2	39.21 ± 2.83	9.42 ± 0.23	12.89 ± 1.52	16.89 ± 1.75
5,9,12-18:3	0.09 ± 0.05	–	–	0.09 ± 0.00
11-20:1	0.35 ± 0.11	0.13 ± 0.18	0.03 ± 0.04	0.19 ± 0.22
9,12,15-18:3	0.52 ± 0.07	0.33 ± 0.10	0.02 ± 0.02	0.17 ± 0.13
8,11-20:2	0.65 ± 0.18	–	0.09 ± 0.13	0.56 ± 0.13
11,14-20:2	3.87 ± 1.53	0.44 ± 0.03	0.43 ± 0.61	3.00 ± 0.64
5,11,14-20:3	9.95 ± 0.62	0.83 ± 0.02	0.54 ± 0.02	8.58 ± 0.04
5,11,14,17-20:4	0.09 ± 0.06	0.62 ± 0.23	0.16 ± 0.23	−0.70 ± 0.46

Table 3
Distribution of fatty acids at the sn-1, sn-2, and sn-3 positions of triacylglycerols from *Torreya grandis* (Xiangyafei variety) seed oil (results expressed as the percentage of a specific fatty acid at the corresponding position compared with the total fatty acids of triacylglycerols).

Fatty acid	Triacylglycerols	sn-1	sn-2	sn-3
16:0	9.22 ± 1.08	9.05 ± 8.69	0.37 ± 0.05	−0.19 ± 8.74
9-16:1	0.04 ± 0.01	–	–	0.04 ± 0.00
17:0	0.10 ± 0.00	–	–	0.10 ± 0.00
18:0	3.56 ± 0.27	2.95 ± 2.63	0.04 ± 0.03	0.57 ± 2.65
9-18:1	39.36 ± 0.14	10.29 ± 0.64	20.12 ± 4.16	8.95 ± 4.80
9,12-18:2	31.95 ± 2.54	6.40 ± 9.05	9.33 ± 3.49	16.22 ± 12.54
5,9,12-18:3	0.28 ± 0.11	0.04 ± 0.05	–	0.24 ± 0.05
11-20:1	0.46 ± 0.00	0.09 ± 0.13	0.07 ± 0.01	0.30 ± 0.14
9,12,15-18:3	0.68 ± 0.17	1.66 ± 1.93	–	−0.98 ± 1.93
8,11-20:2	0.74 ± 0.21	0.10 ± 0.14	–	0.64 ± 0.14
11,14-20:2	3.95 ± 1.37	0.81 ± 1.14	2.79 ± 1.69	0.35 ± 2.83
5,11,14-20:3	9.42 ± 0.53	1.57 ± 1.62	0.33 ± 0.09	7.52 ± 1.71
5,11,14,17-20:4	0.15 ± 0.02	0.02 ± 0.03	0.06 ± 2.40	0.07 ± 2.44

SA in concentrated fractions accounted for the theoretical amount of SA in initial samples) increased as ethanolysis time increased, while the SA content (the weight percentage of SA in FAEE) decreased. Earlier, Lee et al. enriched pinolenic acid from pine nut oil via ethanolysis catalyzed by Novozym 435, and also found the pinolenic acid content of the FAEE decreased with increasing reaction time (Lee et al., 2011). At 50 °C, Lipozyme TLIM showed higher catalysis after 10 h of reaction, SA recovery of ~95% was obtained, while the content of SA only

Table 4
Distribution of fatty acids at the sn-1, sn-2, and sn-3 positions of triacylglycerols from *Torreya grandis* (Mufei variety) seed oil (results expressed as the percentage of a specific fatty acid at the corresponding position compared with the total fatty acids of triacylglycerols).

Fatty acid	Triacylglycerols	sn-1	sn-2	sn-3
16:0	9.01 ± 0.21	9.88 ± 6.64	0.63 ± 0.29	−1.50 ± 6.93
9-16:1	0.13 ± 0.02	–	–	0.13 ± 0.00
17:0	0.11 ± 0.01	–	–	0.11 ± 0.00
18:0	2.88 ± 0.03	5.71 ± 4.98	0.44 ± 0.48	−3.27 ± 5.46
9-18:1	22.24 ± 0.04	7.42 ± 6.94	13.58 ± 2.32	1.24 ± 9.27
9,12-18:2	44.91 ± 0.44	5.48 ± 4.85	15.95 ± 2.92	23.47 ± 7.76
5,9,12-18:3	0.41 ± 0.41	0.17 ± 0.24	0.03 ± 0.04	0.22 ± 0.27
11-20:1	0.12 ± 0.06	–	–	0.12 ± 0.00
9,12,15-18:3	0.65 ± 0.47	1.83 ± 2.59	–	−1.18 ± 2.59
8,11-20:2	0.79 ± 0.04	0.44 ± 0.62	0.05 ± 0.08	0.30 ± 0.69
11,14-20:2	2.72 ± 0.10	0.63 ± 0.89	1.23 ± 0.42	0.86 ± 1.32
5,11,14-20:3	14.50 ± 0.13	1.70 ± 0.38	0.80 ± 0.68	12.00 ± 1.06
5,11,14,17-20:4	0.44 ± 0.01	–	0.38 ± 0.53	0.06 ± 0.53

Table 5
Distribution of fatty acids at the sn-1, sn-2, and sn-3 positions of triacylglycerols from *Torreya grandis* (Zhimafei variety) seed oil (results expressed as the percentage of a specific fatty acid at the corresponding position compared with the total fatty acids of triacylglycerols).

Fatty acid	Triacylglycerols	sn-1	sn-2	sn-3
16:0	8.44 ± 0.99	9.64 ± 1.07	0.30 ± 0.15	−1.50 ± 1.21
9-16:1	0.09 ± 0.03	0.28 ± 0.56	0.01 ± 0.01	−0.20 ± 0.58
17:0	0.08 ± 0.02	–	–	0.08 ± 0.00
18:0	3.02 ± 0.32	5.25 ± 0.62	0.50 ± 0.88	−2.73 ± 1.50
9-18:1	32.77 ± 4.61	7.30 ± 0.89	15.15 ± 1.81	10.32 ± 2.69
9,12-18:2	38.63 ± 3.54	6.54 ± 0.78	13.64 ± 1.26	18.45 ± 2.04
5,9,12-18:3	0.15 ± 0.02	0.05 ± 0.09	0.05 ± 0.09	0.06 ± 0.19
11-20:1	0.45 ± 0.10	–	0.05 ± 0.05	0.40 ± 0.05
9,12,15-18:3	0.75 ± 0.12	0.64 ± 1.08	0.03 ± 0.03	0.08 ± 1.11
8,11-20:2	0.59 ± 0.27	–	0.03 ± 0.03	0.56 ± 0.03
11,14-20:2	4.52 ± 1.53	0.29 ± 0.30	0.77 ± 0.77	3.46 ± 1.07
5,11,14-20:3	8.91 ± 0.13	0.70 ± 0.27	0.43 ± 0.05	7.78 ± 0.32
5,11,14,17-20:4	0.17 ± 0.02	2.20 ± 4.40	2.36 ± 2.21	−4.39 ± 6.61

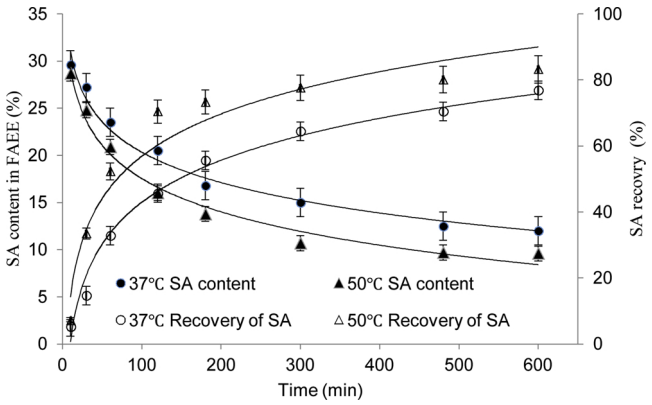

Fig. 3. Time courses of ethanolysis of *Torreya grandis* seed oil at 37 °C and 50 °C catalyzed by Lipozyme TLIM. SA, sciadonic acid; Lipozyme TLIM, lipase from *Thermomyces lanuginosus*, immobilized on silica granulation; FAEE, fatty acid ethyl ester.

corresponded to that of the initial material. Low SA content in FAEE obtained with Lipozyme TLIM at 50 °C may be a result of the high β/α site isomerization reaction. Zhao et al. also found that Novozym 435 showed less selectivity toward fatty acids at the sn-3 position under relatively higher temperatures (Zhao et al., 2012). The authors concentrated the pinolenic acid using a recirculating packed bed reactor, and resulted in a maximal pinolenic acid content of 36.1%. More recently, saponification and acidification coupled with lipase-catalyzed esterification was developed by Ting et al., and showed that pinolenic acid was enriched up to 43% from an initial amount of 13% (Ting et al., 2008). In this study, after 30–60 min ethanolysis by Lipozyme TLIM at 37 °C, the SA content increased to 27.2%–23.5% from 9.95%, and the SA recovery of 24.7%–37.9% was obtained. Prolonged reaction time may increase SA recovery but could cause fatty acids migrating from the sn-2 position, and thus decrease SA content. For industrial production, content and recovery of SA could be compromised, thus 30 min ethanolysis by Lipozyme TLIM at 37 °C was optimal for the SA concentration.

3.4. Urea complexation for FAEE from T. grandis seed oil

Seed oil of *T. grandis* has a large proportion of UFAs with a double bond number more than one, such as oleic acid (32.59%), linoleic acid (39.21%), and SA (9.95%). Due to this composition, long-chain lengths with a high number of double bonds make these structures less linear, and thus, less susceptible to getting into the urea channels to form adducts. The results obtained from various ratios of FAEE to urea were shown in Table 6. The initial SA level in the matrix was important in terms of SA enrichment. After complexation from raw FAEE, the highest SA purity of 61% was obtained in the enriched fraction, while when starting with enzymatically enriched FAEE, an SA purity of 80% was obtained. However, it should be noted that when the ratio of FAEE to urea was 0.2–0.3, the enriched performance was good, with higher SA content. Especially, with an FAEE to urea ratio of 0.3, the SA content and recovery could be compromised with values of 80% and 35.2%, respectively.

3.5. Fatty acid compositions of initial T. grandis seed oil and each enriched fraction

The fatty acid composition of the initial *T. grandis* seed oil, the enriched fraction of SA from the lipase-catalyzed ethanolysis, urea

Table 6
Fatty acids profiles for *Torreya grandis* (Xifei variety) seed oil and the free fatty acid ethyl ester (FAEE) fraction isolated after different steps of the purification process (results expressed as weight percentage).

Fatty acid	Original[a]	Enzymatic ethanolysis[b]	FAEE/urea ratio used in urea complexation					
			Torreya grandis (Xifei variety) seed oil as material[c]				Enzymatic enriched FAEE as material[d]	Argentated column chromatography[e]
			0.2	0.3	0.4	0.5	0.3	
16:0	8.94 ± 1.31	11.13 ± 0.05	/		0.30 ± 0.02	0.25 ± 0.01	/	/
9-16:1	0.06 ± 0.01	–	–	–	–	0.09 ± 0.02	–	–
17:0	0.07 ± 0.02	–	/	/	/	/	/	/
18:0	3.24 ± 0.29	2.04 ± 0.18	/	/	/	/	/	/
9-18:1	32.59 ± 0.04	20.42 ± 0.12	–	1.88 ± 0.47	1.70 ± 0.38	1.53 ± 0.27	0.27 ± 0.27	–
9,12-18:2	39.21 ± 2.83	35.48 ± 1.22	35.83 ± 2.26	37.13 ± 1.47	47.81 ± 2.51	62.11 ± 6.50	18.59 ± 6.50	0.90 ± 0.05
5,9,12-18:3	0.09 ± 0.05	–	–	–	0.77 ± 0.67	0.96 ± 0.96	0.96 ± 0.96	–
11-20:1	0.35 ± 0.11	–	–	–	0.55 ± 0.08	–	–	–
9,12,15-18:3	0.52 ± 0.07	–	–	–	–	1.15 ± 0.26	0.26 ± 0.26	–
8,11-20:2	0.65 ± 0.18	0.88 ± 0.74	–	–	0.41 ± 0.22	1.02 ± 0.37	–	–
11,14-20:2	3.87 ± 1.53	2.64 ± 0.18	–	–	0.88 ± 0.43	1.25 ± 0.46	–	–
5,11,14-20:3	9.95 ± 0.62	27.41 ± 0.83	64.17 ± 3.56	60.98 ± 2.79	45.74 ± 3.27	31.69 ± 3.27	80.14 ± 3.27	99.10 ± 0.05
5,11,14,17-20:4	0.09 ± 0.06	–	–	–	0.60 ± 0.75	0.72 ± 0.75	0.75 ± 0.75	–

[a]Original fatty acids profiles of *Torreya grandis* (Xifei variety) seed oil.
[b]Fatty acids profiles of FAEE fraction obtained by enzymatic (Lipozyme TLIM, 37 °C, 30min) ethanolysis.
[c]Fatty acids profiles of FAEE fraction obtained by urea complexation using original FAEE mixture as substrate.
dFatty acids profiles of FAEE fraction obtained by urea complexation using enzymatically concentrated FAEE as substrate (i.e., products from b).
eFatty acids profiles of FAEE fraction obtained by argentated column chromatography using urea complexation concentrated FAEE as substrate (i.e., products from d).
FAEE, fatty acid ethyl ester.

complexing, and the argentated column chromatograph were shown in Table 6. After specific ethanolysis by Lipozyme TLIM, the FAEE product mainly consisted of four major fatty acids, including linoleic acid, oleic acid, palmitic acid, and SA. Almost all of their levels were found to increase throughout lipase-catalyzed ethanolysis, except oleic acid. The mean composition of each fatty acid in the sn-1 and sn-3 positions could be calculated from the positional distribution procedure. For Δ5-UPIFAs, because they were naturally abundant at the sn-3 position and their content in sn-1 was relatively low, a one- to two-fold upward trend for SA content in FAEE was found, compared to the initial *T. grandis* seed oil after ethanolysis with lipozyme TLIM. The fatty acids released may not be similar to the mean composition of fatty acids originally present at the sn-1 and sn-3 positions of TAG, as lipase-catalyzed ethanolysis may not be complete and there will usually be contamination by fatty acids released from the sn-2 position after migration to the primary positions. Nevertheless, based on this primarily-enriched fraction, one-step urea complexation can efficiently remove most of the SFAs and MUFAs, and a high SA content of 80% in FAEE was obtained. This SA enriched fraction was easily concentrated to a purity of 99% with a recovery of 37% via argentated column chromatography. Overall, the strategy to combine the selective lipase-catalyzed transesterification and urea complexation methods was effective for enrichment of SA from *T. grandis* seed oil.

4. Conclusions

The current study demonstrated that *T. grandis* seed oil obtained from different varieties and geographical origins resulted in almost the same fatty acid composition and distribution of fatty acids in the sn-1, sn-2, and sn-3 positions of TAG. Linoleic acid and oleic acid were the dominating fatty acids with average percentages of 31% and 41%, respectively. In addition, about 10% of SA was also found in the *T. grandis* seed oil. When considering positional distribution of fatty acids in TAG, despite all difference about varieties, SFAs were distributed primarily at the sn-1 and sn-3 positions while oleic acid and linoleic acid were found in greater amounts at the sn-2 position, and except for linoleic acid, the disparity between other positions was not so obvious as oleic acid. SA and other Δ5-UPIFAs were almost exclusively esterified at the sn-3 position. This characteristic principally resulted from a 5-site double bond interrupted by polymethylene, and was independent of chain length and double bond numbers. SA was successfully enriched from an initial value of 9.95% to 80.10% by using a combined process including 1,3-specific lipase-catalyzed ethanolysis and urea complexing. These results indicated that this simple strategy can potentially supply high purity multifunctional SA on a commercial scale.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2019.111917.

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