

# Combined Urea Complexation and Argentated Silica Gel Column Chromatography for Concentration and Separation of PUFAs from Tuna Oil: Based on Improved DPA Level

Hongyan Mu<sup>1,2</sup> · Jun Jin<sup>1</sup> · Dan Xie<sup>1,3</sup> · Xiaoqiang Zou<sup>1</sup> · Xiaosan Wang<sup>1</sup> · Xingguo Wang<sup>1</sup> · Qingzhe Jin<sup>1</sup>

Received: 2 October 2015 / Revised: 8 April 2016 / Accepted: 27 April 2016 / Published online: 27 May 2016  
© AOCs 2016

**Abstract** Eicosapentaenoic acid (EPA, 20:5n-3), docosapentaenoic acid (DPA) isomers (22:5n-6 and 22:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) derived from tuna oil were concentrated by three stages of urea fractionation at various crystallization temperatures and different fatty acid/urea ratios. Thereafter, polyunsaturated fatty acids concentrate containing comparatively enriched DPA levels was purified by argentated silica gel column chromatography. A product containing  $22.2 \pm 0.6$  % EPA,  $4.6 \pm 0.0$  % DPAn-6,  $5.9 \pm 0.1$  % DPAn-3 and  $42.3 \pm 1.2$  % DHA was obtained at 1:1.6 fatty acid/urea ratio (w/w) by crystallization at  $-8$  °C for 16 h,  $-20$  °C for 8 h, and  $-8$  °C for 16 h. A DPA isomer concentrate containing  $26.1 \pm 0.5$  % DPAn-6 and  $22.3 \pm 0.4$  % DPAn-3 was achieved by argentated silica gel chromatography in the 6 % acetone/*n*-hexane solvent fraction (v/v), and the recovery of both fatty acids was  $66.1 \pm 3.2$  and  $70.7 \pm 2.2$  %, respectively. Furthermore,  $91.9 \pm 2.5$  % EPA and  $99.5 \pm 2.1$  % DHA with recoveries of  $47.8 \pm 2.0$  and  $56.7 \pm 3.3$  %, respectively, were obtained in various fractions.

**Keywords** Tuna oil · Urea inclusion · Eicosapentaenoic acid · Docosapentaenoic acid · Docosahexaenoic acid · Argentated silica gel column chromatography

## Introduction

Because the low incidence of cardiovascular disease in the Greenland Eskimos was discovered to be associated with their high intake of marine products, the positive role of polyunsaturated acids (PUFAs) on human wellbeing has received increased attention, particularly long-chain PUFAs derived from marine oils such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The health benefits of these two fatty acids have been intensively investigated [1–4]. However, docosapentaenoic acid (DPA) has received little review. DPA contains two isomers, namely DPAn-3 and DPAn-6, which are widely present in marine sources. DPAn-3 is mainly found in fish oils and seal oils, whereas some micro-algae oils contain substantial amounts of DPAn-6 [5]. DPA isomers are intermediates of the metabolism and biosynthesis of PUFAs, and research has indicated that DPA may have pharmaceutical effects different from those of EPA and DHA. The Kuopio ischemic heart disease risk factor study [6] suggested that the proportions of serum DPA and DHA imparted a significantly reduced risk of acute coronary events, whereas there was no association between the proportion of EPA and the risk of acute coronary events. DPA may be more effective than EPA or DHA in some therapeutic applications, such as keeping artery walls soft and plaque-free and helping to heal damaged blood vessels [7, 8].

The health benefits of DPA have not been extensively studied due to its much lower concentration and similar molecular structure shared with EPA and DHA. Therefore,

✉ Qingzhe Jin  
jqzwx12@163.com

<sup>1</sup> State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Synergetic Innovation Center of Food Safety and Nutrition, Jiangnan University, 1800 Lihu Road, Wuxi 214122, Jiangsu, People's Republic of China

<sup>2</sup> College of Food Science and Engineering, Qingdao Agricultural University, 700 Changcheng Road, Qingdao 266109, Shandong, People's Republic of China

<sup>3</sup> Zhong Hai Ocean (wuxi) Marine Equipment Engineering Co., Ltd., Jiangnan University National University Science Park, 100 Jinxi Road, Wuxi 214125, Jiangsu, People's Republic of China

**Table 1** Major fatty acid composition of tuna oil and free fatty acids recovered from saponification of tuna oil

Fatty acid	Tuna oil (%)	Free fatty acids (%)
C14:0	4.13	4.09
C16:0	18.92	18.79
C16:1	4.96	5.1
C18:0	5.32	5.33
C18:1n-9	13.77	13.85
C18:1n-7	2.58	2.61
C18:2	2.2	1.98
C18:3n-6	0.48	0.48
C18:3n-3	0.5	0.59
C20:1	0.59	0.56
C20:2	1.34	1.28
C20:3	0.62	0.69
C20:4n-6	2.18	2.29
C20:5	7.32	7.27
C22:5n-6	1.75	1.73
C24:1	0.7	0.72
C22:5n-3	1.34	1.26
C22:6	27.33	27.75
ΣSFA	28.37	28.18
ΣMUFA	22.6	22.84
ΣPUFA	45.02	45.32
Other FA	4.01	3.66

Free fatty acids: fatty acids obtained by saponification of tuna oil

SFA saturated fatty acid, MUFA monounsaturated fatty acid, PUFA polyunsaturated fatty acids, FA fatty acid

it is worthwhile to obtain highly concentrated fatty acids, which may be fundamental for exploring and extending their nutritional, epidemiological and pharmaceutical discoveries. Urea complexation is an efficient way to concentrate fatty acids with different degrees of unsaturation. The tetragonal conformation of urea in the natural state is converted to the hexagonal crystalline structure by forming a spiral-shaped channel with an average diameter of approximately 5.5–5.8 Å, thus making it possible to entrap smaller-sized linear chain-length molecules [9–11]. With this technique, saturated and monounsaturated fatty acids can be easily removed as urea complexation compounds while leaving PUFAs in the non-urea complexation fraction (NUCF). However, if highly purified PUFAs are desired, it is indispensable to combine urea complexation methods with other techniques, such as molecular distillation [12], argentated silica gel chromatography [13] and preparative HPLC [14]. Argentated silica gel column chromatography is an appropriate technique for isolating fatty acids methyl or ethyl esters. The principle is that various numbers of double-bonded fatty acids can interact with aqueous

silver ions through  $\pi$ - $\pi$  complexation by forming reversible polar complexes. Stronger complexation occurred with greater numbers of double bonds [15]. Therefore, the separation of fatty acids differing in number, position and geometric configuration of double bonds could be carried out by stripping with various solvents [16]. Argentated silica gel column chromatography has been used to obtain high-purity EPA [17], DHA [18],  $\alpha$ -linolenic acid [19] and stearidonic acid [19] from a variety of fatty acid esters.

Tuna oil, a major source of PUFAs, contains approximately 6 % EPA, 25 % DHA and about 3 % DPA isomers [20], which is higher than is found in other fish oils. Here, we obtained PUFA concentrates with an increased level of DPA isomer in two main steps: (1) enrichment of PUFAs from tuna oil by urea complexation at various fractionation temperatures and with different free fatty acids (FFA)-to-urea ratios with the goal of obtaining improved content of DPA isomer and (2) separation of the individual fatty acids methyl esters by argentated silica gel chromatography.

## Materials and Methods

### Materials and Chemicals

Refined tuna oil was generously provided by NovoSana (Taicang, Jiangsu, China), which was stored in a sealed aluminum pot at  $-60$  °C before use. FFA of tuna oil was prepared according to the method described by Wanasundara et al. [21] with minor modifications. Tuna oil (100 g) was treated with 200 ppm of *tertiary* butylhydroquinone to prevent the oil from oxidizing and was saponified with potassium hydroxide (23 g), 95 % (v/v) aqueous ethanol (240 mL) and distilled water (60 mL) by stirring at 60 °C for 1.5 h. The un-saponified matter was extracted into 100 mL of *n*-hexane and discarded; then the saponified substance, namely the aqueous phase, was acidified with 6 N HCl to pH 1.0, and the FFA was extracted into *n*-hexane ( $2 \times 100$  mL). Finally, the FFA was recovered by evaporating the solvent at 40 °C. The fatty acid composition of tuna oil and FFA are shown in Table 1. PUFAs accounted for more than 45 % of the total fatty acids, in which the overall proportion of EPA, DPAn-3, DPAn-6 and DHA was greater than 37 %.

### PUFAs Enrichment by Urea Complexation Technique

Urea complexation was performed according to the following procedure. The FFA received by saponification of tuna oil was mixed with different amounts of urea in urea-saturated 95 % (v/v) aqueous ethanol and stirred at 60 °C with  $N_2$  fluxing until a clear homogenous solution was obtained.

Initially, the urea-FFA-ethanol mixture was allowed to cool at room temperature, and then the mixture was cooled to the required temperature ( $-40$ ,  $-20$ ,  $-8$ ,  $-4$ ,  $0$  or  $4$  °C) for 20 h for further crystallization. The urea adducts were separated as the urea complexation fraction (UCF, solid phase) and non-urea complexation fraction (NUCF, liquid phase) by filtration on a Buchner funnel under suction. Ethanol of the NUCF was evaporated, and an adequate amount of hot water was added to remove the urea residue. The upper layer was then extracted with *n*-hexane, and the solvent was removed at 45 °C by a vacuum rotary evaporator after drying over anhydrous sodium sulfate. The fatty acids from the UCF were recovered in the same manner. The products were kept under a blanket of N<sub>2</sub> at  $-20$  °C until analysis. All the urea complexation reactions were performed in triplicate. The recovery of the fatty acids was estimated as the weight proportion of the amount of fatty acids in a fraction to those in the starting material (FFA).

### Argentated Column Chromatographic Fractionation of PUFAs Concentrate Methyl Esters

Ten grams of AgNO<sub>3</sub> powder was weighed into 70 mL of 70 % (v/v) aqueous ethanol, and stirring was maintained until the AgNO<sub>3</sub> was completely dissolved. One hundred grams of silica gel (100–200 mesh) for column chromatography was slurried in 200 mL of 95 % (v/v) aqueous ethanol for 10 min, and then the AgNO<sub>3</sub> solution was added and agitated for another 10 min. Ethanol was removed by a rotary evaporator at 50 °C, and the Ag<sup>+</sup>-silica gel slurry was activated by heating at 120 °C under vacuum for 3 h and then stored in a desiccator by wrapping it with aluminum foil. Forty grams of the Ag<sup>+</sup>-silica gel was slurried in an adequate amount of *n*-hexane and transferred to a glass column (2 cm × 60 cm), which had previously been plugged with glass wool on the bottom and wrapped with dark paper on the outside. Sea sand was placed on the top of the column to retain solids. A slight flow of solvent by gravity was allowed during packing. The stationary phase impregnated in *n*-hexane was settled for 2 h. Then, 400 mg of PUFA methyl ester was dissolved in 16 mL *n*-hexane and applied onto the column and eluted by gravity with *n*-hexane/acetone eluents containing increasing amounts of acetone. The flow rate was maintained at 1 mL/min. A series of fractions were collected corresponding to the increment of acetone proportion. The fractions were then repeatedly washed with an adequate amount of NaCl aqueous solution (5 %, w/v) for three times. Evaporating the solvents at 45 °C after the upper organic layer was collected and dried with anhydrous sodium sulfate. The recovery of the components was estimated by direct weighing, then the fractions were dissolved in 1 mL of *n*-hexane for GC analysis.

### Derivatization of Fatty Acids

The FFA was derived as fatty acid methyl esters according to the method described in [20] with minor modifications. FFA (50 mg) was treated with 2 mL of BF<sub>3</sub> (13–15 % in methanol) and heated at 70 °C for 50 min. Then, 2 mL of *n*-hexane and 3 mL of saturated aqueous sodium chloride were added to the cooled solution and vortexed for 30 s. The solution was allowed to settle until two layers of phases appeared. The upper layer was collected and dehydrated over anhydrous sodium sulfate before GC analysis.

### Fatty Acid Analysis

The fatty acid composition was analyzed on a gas chromatography (GC) (Agilent Model 7820A) equipped with a flame ionization detector and a TRACE TR-FAME fused silica capillary column (60 m × 0.32 mm × 2.5 μm; Thermo, USA). Ultra-high-purity nitrogen (99.99 %) was used as the carrier gas at a constant flow rate of 25 mL/min. The split ratio was 100:1. The temperatures of the injector and the detector were 220 and 250 °C, respectively. The oven temperature was initially held at 60 °C for 3 min, followed by a 5 °C/min increase up to 175 °C for 15 min, then increased to 220 °C at 2 °C/min and held isothermally for 10 min.

Identification of FAME were based on the retention times of a Supelco 37 Component FAME mix and the fatty acid proportion was quantified by normalization of the relative area of the chromatogram.

### Statistical Analyses

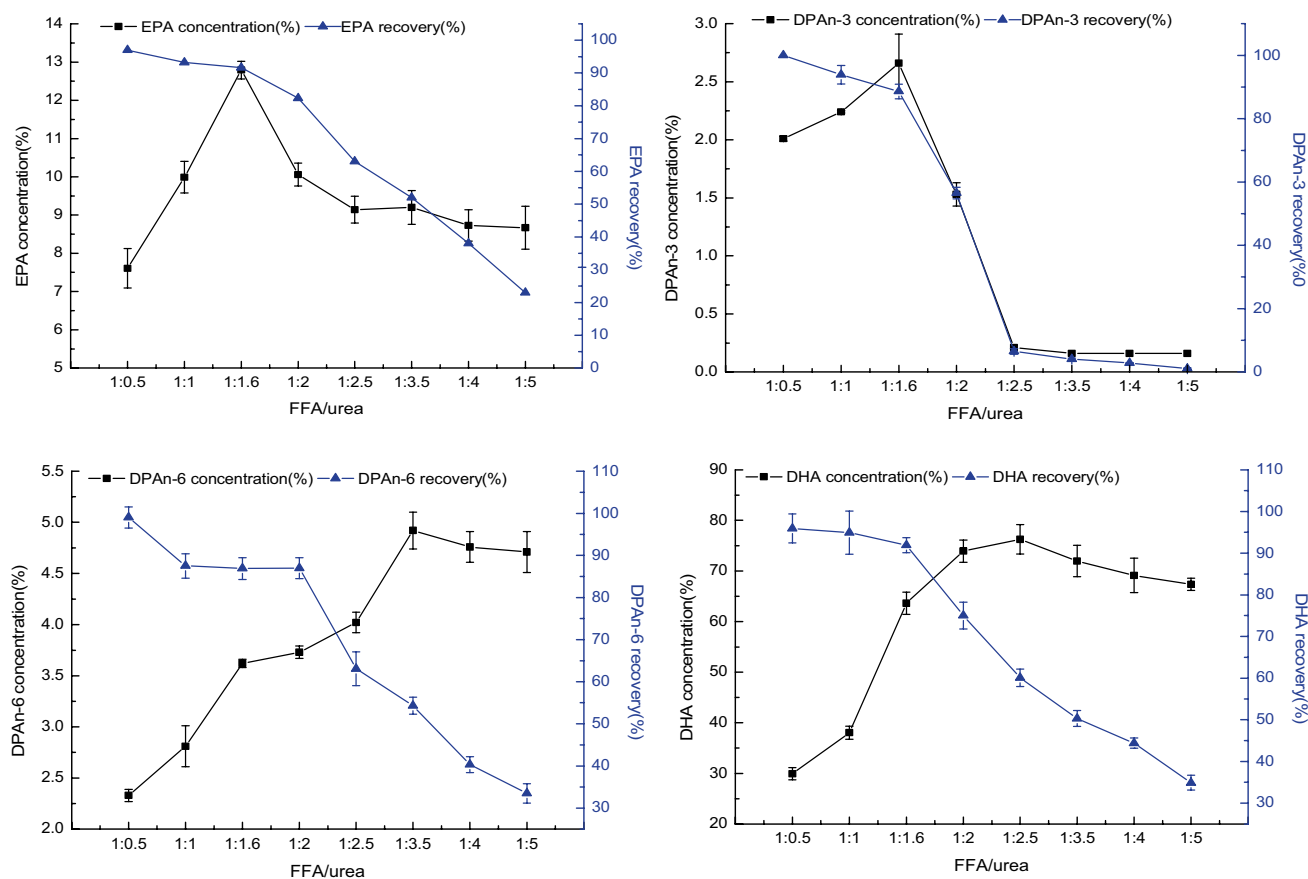
The percentage of individual fatty acid methyl esters from tuna oil was expressed as mean ± standard deviation of three different trials, and one-way analysis of variance (ANOVA) was performed at a  $p < 0.05$  level using Origin 8.0 software package (Northampton, MA, USA) to determine the significant differences.

## Results and Discussion

### Urea Complexation Reaction

#### *Effect of FFA/Urea Ratios*

Increasing the amount of urea caused more fatty acids to be entrapped in the UCF, resulting in an improved concentration of four PUFAs in the NUCF (Fig. 1). The maximum levels of EPA and DPAn-3 ( $12.8 \pm 0.2$  and  $2.7 \pm 0.0$  %, respectively) were obtained at 1:1.6 FFA/urea ratio (w/w), the recovery was  $91.6 \pm 3.4$  and  $88.6 \pm 2.3$  %,



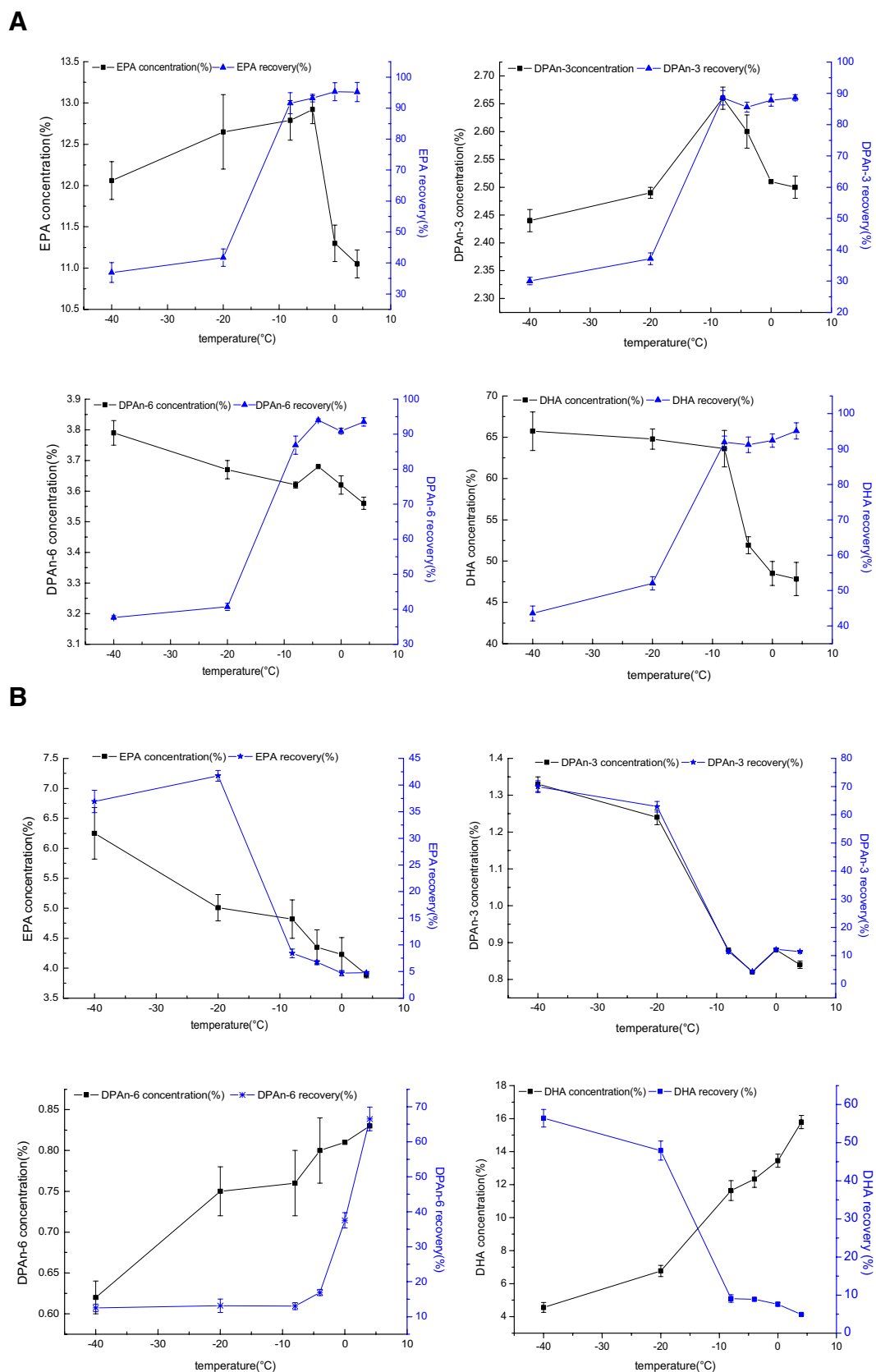
**Fig. 1** The concentration and recovery of EPA, DPAn-3, DPAn-6, and DHA as a function of FFA/urea ratios upon crystallization at  $-8\text{ }^{\circ}\text{C}$

respectively. Satisfactory concentration and recovery of DPAn-6 ( $3.6 \pm 0.0$ ,  $86.9 \pm 2.6\%$ ) and DHA ( $63.6 \pm 2.2$ ,  $91.9 \pm 1.8\%$ ) were respectively obtained at this ratio. The respective concentrations of DPAn-6 and DHA in the NUCF were the highest ( $4.0 \pm 0.1$  and  $76.2 \pm 2.4\%$ ) when the amount of urea was 2.5-fold that of FFA, whereas the recovery of PUFAs declined remarkably, suggesting a sufficient complexation of urea with fatty acids. The DPAn-3 level in the NUCF was sharply decreased ( $0.2 \pm 0.0\%$  concentration,  $6.5 \pm 0.9\%$  recovery) at this ratio, suggesting that DPAn-3 was almost completely complexed with the urea. The amount of EPA was also greatly decreased, indicating that EPA and DPAn-3 are more prone to form urea adducts with increased urea amounts. The different crystallization performance for EPA and DPAn-3 compared to that for DPAn-6 and DHA as a function of the various FFA/urea ratios showed that the molecular configuration is a significant parameter with which to differentiate PUFAs.

#### Effect of Crystallization Temperature

Another experiment was carried out by analyzing several crystallization temperatures. The concentration and

recovery of the four PUFAs in the NUCF and UCF as a function of fractionation temperature are illustrated in Fig. 2a, b. Because the process is accompanied by exothermic activities, crystallization at lower temperatures facilitates highly enriched PUFAs in the NUCF. Both the overall concentrations of EPA, DPAn-3, DPAn-6 and DHA and their individual concentrations in the NUCF showed a tendency to increase with the decrease of temperature, but the distribution of the four fatty acids in the NUCF and UCF varied at very low temperatures. EPA was more readily to find its way into the UCF when the crystallization temperature decreased below  $-20\text{ }^{\circ}\text{C}$ , as it presented a recovery of  $41.7 \pm 2.8\%$  in the NUCF upon crystallization at  $-20\text{ }^{\circ}\text{C}$ , which agreed with the findings of Ratnayake et al. [22], who reported that the amount of EPA in the urea adduct compound increased sharply below  $-18\text{ }^{\circ}\text{C}$ . The amount of DPAn-3 in the UCF also increased with decreasing temperature, with a recovery of  $62.9 \pm 1.9\%$  obtained in the UCF at  $-20\text{ }^{\circ}\text{C}$ . Nevertheless, DPAn-6 and DHA showed different crystallization performances. Both compounds were almost exclusively concentrated in the NUCF at lower temperatures. The maximum concentrations of these two fatty acids in the NUCF were found to be  $3.8 \pm 0.0\%$  and



**Fig. 2** Influence of crystallizing temperature on the concentration of PUFAs with a 1:1.6 FFA/urea from fatty acids of tuna oil. **a** The concentration and recovery of EPA, DPAn-6, DPAn-3, and DHA in the

non-urea complexation fraction; **b** the concentration and recovery of EPA, DPAn-6, DPAn-3, and DHA in the urea complexation fraction

65.7 ± 2.3 % respectively, by crystallization at −40 °C. Our results were in accord with that of Wanasundara et al. [21], who obtained the highest DPA content in the NUCF when crystallization occurred at −18 °C with a urea/FFA ratio of 2:1. However, the yields of both were significantly decreased (37.7 ± 0.6 % for DPAn-6 and 43.6 ± 2.1 % for DHA). It is recognized that urea complexation depends upon the configuration of fatty acids molecules rather than pure physical properties such as melting point or solubility; therefore, at lower temperatures, it is necessary to consider the changes that brought about in the aliphatic chain of one or more double bonds, conjugated or non-conjugated, and different geometrical configurations [23]. Ma et al. [24] compared the enrichment of Δ9c, 11t- and Δ10t, 12c-18:2 isomers by urea complexation and suggested that the *trans*-configuration provides a more linear geometry of its methyl ends compared to the *cis*-isomer, thus making it possible to form urea complexes. However, this is inadequate to explain the crystallization priority of fatty acids isomers that are differentiated only by the location of double bonds. The different crystallization performance of DPAn-6 from that of DPAn-3 might be ascribed to the fact that DPAn-6 contributes a longer distance from the first double bond to the methyl ends than DPAn-3. In terms of DPAn-3, the side of the chain close to the terminal methyl group is essentially ethylenic, whereas the other side is essentially saturated. The opposite is seen in DPAn-6, where the side of the chain close to the methyl group is saturated, and the other side is unsaturated. Moreover, the nearer distance from the first double bond to the carboxyl head might provide a more “gauche” configuration of DPAn-6, making it difficult to be complexed.

#### Effect of Successive Urea Complexations

The NUCF obtained by crystallizing the fatty acids of tuna oil with a 1:1.6 FFA/urea ratio (w/w) at −8 °C was designated as NUCF<sub>1st</sub>, in which the concentrations of EPA, DPAn-3, DPAn-6 and DHA were 12.8 ± 0.2, 3.6 ± 0.0, 2.7 ± 0.0 and 63.6 ± 2.2 %, respectively. This fitted well with successive urea complexation to achieve highly improved DPAn-3 and DPAn-6. The second-stage urea complexation was performed with the PUFA concentrates upon crystallization at −20 °C with the same proportion of urea and ethanol for 16 h. Then, the urea complexation fractions (UCF<sub>2nd</sub>) recovered from the complexation reaction were crystallized at −8 °C for 16 h with the same ratio of FFA/urea (w/w) for a third stage of urea complexation. The results of the three successive urea complexations are summarized in Table 2.

Compared with the original material, EPA, DPAn-6, DPAn-3 and DHA were all significantly purified in the NUCF<sub>1st</sub>, the recoveries of the four PUFAs being

**Table 2** The concentration and recovery of EPA, DPAn-3, DPAn-6, and DHA profiles affected by successive urea complexation processes

FFA	First urea complexation <sup>a</sup>		Second urea complexation <sup>b</sup>		Third urea complexation <sup>c</sup>	
	NUCF <sub>1st</sub> (%)	Recovery (%)	UCF <sub>2nd</sub> (%)	Recovery (%)	NUCF <sub>3rd</sub> (%)	Recovery (%)
EPA	12.79 ± 0.23 b	91.59 ± 3.41	13.36 ± 1.01 b	53.48 ± 2.67	22.22 ± 0.57 a	30.24 ± 1.55
DPAn-6	3.62 ± 0.04 c	88.6 ± 2.31	1.84 ± 0.02 d	34.72 ± 1.25	4.58 ± 0.04 b	30.04 ± 2.32
DPAn-3	2.66 ± 0.02 b	86.88 ± 2.60	5.31 ± 0.06 a	63.63 ± 2.03	5.93 ± 0.13 a	45.14 ± 0.86
DHA	63.62 ± 2.21 a	91.91 ± 1.81	19.81 ± 0.27 c	15.06 ± 0.76	42.31 ± 1.22 b	10.78 ± 1.21

Results are expressed as mean ± SD, n = 3. Values followed by different letters in each line differ significantly (p < 0.05)

NUCF non-urea complexation fraction, UCF urea complexation fraction

<sup>a</sup> The first stage urea complexation performed with a 1:1.6 FFA/urea ratio (w/w) upon crystallization at −8 °C; NUCF<sub>1st</sub> the non-urea complexation fraction obtained from the first stage urea complexation

<sup>b</sup> The second stage urea complexation performed with the non-urea complexation fraction obtained from the first stage urea complexation process by crystallization at −20 °C at a 1:1.6 (FFA: urea, w/w) ratio; UCF<sub>2nd</sub> the urea complexation fraction obtained from the second stage urea complexation; NUCF<sub>2nd</sub> the non-urea complexation fraction obtained from the second stage urea complexation

<sup>c</sup> The third stage urea complexation with the UCF<sub>2nd</sub> and NUCF<sub>2nd</sub> performed at −8 °C; NUCF<sub>3rd</sub> the non-urea complexation fraction obtained by crystallization of the UCF<sub>2nd</sub> with urea; NUCF<sub>3rd</sub> the non-urea complexation fraction obtained by crystallization of the NUCF<sub>2nd</sub>

91.6 ± 3.4, 88.6 ± 2.3, 86.9 ± 2.6 and 91.9 ± 1.81 %, respectively, indicating that crystallization at −8 °C was appropriate for the enrichment of PUFAs. Considering the same or a higher temperature made less sense for further PUFAs concentration, so −20 °C was chosen in the second-stage crystallization for the formation of the urea–FFA complexation compound. There appeared to be significant differences in the PUFAs concentrates in the liquid (NUCF<sub>2nd</sub>) and solid fractions (UCF<sub>2nd</sub>) of this stage. The concentrations of EPA and DPAn-3 in the UCF<sub>2nd</sub> (13.4 ± 1.1 and 5.3 ± 0.1 %) were significantly higher than those in the NUCF<sub>2nd</sub> (8.5 ± 0.1 and 0.8 ± 0.1 %), respectively. Furthermore, the two fatty acids were preferentially distributed in the UCF<sub>2nd</sub> with the recovery of 53.5 ± 2.7 and 63.6 ± 2.0 %, respectively. Contrastingly, DPAn-6 and DHA were prone to be enriched in the NUCF<sub>2nd</sub> with the recovery of 53.1 ± 1.0 and 71.7 ± 2.6 %, respectively, and the concentrations of these two fatty acids in the NUCF<sub>2nd</sub> were significantly higher than those in the UCF<sub>2nd</sub>. The discriminating complexing performance of DPAn-3 from DPAn-6 indicated that the complexation of the DPA isomer with urea was altered at a much lower crystallization temperature. Traitler et al. [25] successively separated α-linolenic acid from γ-linolenic acid by urea complexation, but stearic acid was co-fractionated with γ-linolenic acid, suggesting that the double bond at the Δ6 position for γ-linolenic acid and stearic acid probably explained the specificity of the two fatty acids. It is assumed that the first double bond from the carboxyl head present at different positions for the two DPA isomers might contribute to the discrimination in the urea complexation. Moreover, according to the result from Traitler et al. [25] and this research, it can be concluded that the complexing force of n-3 fatty acids with urea is stronger than that of their n-6 isomers. However, whether the distance from the first double bond to the carboxyl group is a determining factor remains to be verified.

The third-stage urea complexation process was performed with UCF<sub>2nd</sub> from the second-stage urea complexation procedure, and the liquid and solid fractions obtained were referred to as the urea complexation fraction of UCF<sub>2nd</sub> (UCF<sub>3rd</sub>) and the non-urea complexation fraction of NUCF<sub>2nd</sub> (NUCF<sub>3rd</sub>), respectively. The overall concentration of DPA isomers was greatly improved in the NUCF<sub>3rd</sub>, which increased from 2.99 % of the original material to 10.5 ± 0.2 % (4.6 ± 0.0 % for DPAn-6 and 5.9 ± 0.1 % for DPAn-3).

### Separation of PUFAs by Argentated Silica Gel Column Chromatography

The NUCF<sub>3rd</sub> fraction obtained by three successive urea complexation processes was methylated, then argentated

silica gel column chromatography was used to separate PUFA methyl esters. The technique represented a series of separation by π–π complexation, which was the subgroup of chemical complexation where the mixture was contacted with second phase containing complexing agent [26]. The complex formed by π bond was comparatively more stable than those formed by van der Waals forces, although liable enough to be broken by using simple engineering operations [27]. Various solvents could form stronger π complexation with Ag<sup>+</sup>, which contributed to replacing double-bond fatty acids from the sorbent; moreover, the polarity of solvents also influenced the interaction of Ag<sup>+</sup> and C–C double bond. Based on a series of trials in our laboratory, *n*-hexane with gradually increasing proportions of acetone (1, 2, 5, 6, 7, 8, 10 and 15 %, v/v) were selected as the stripping solvents. The volume of each eluent and the weights of the fractions during the separation are shown in Table 3. After silica gel column chromatography, 393.9 mg of the fatty acid methyl esters was obtained, which indicated that the volume of solvents was adequate for stripping nearly all samples.

The elution order of different fatty acids from the column is mainly attributed to the unsaturation extent and chain length, which determined the strength of π–π complexation between Ag<sup>+</sup> and C–C double bonds. Saturated fatty acids, which are more nonpolar and associate weakly with the stationary phase, are eluted first, followed by less unsaturated fatty acids, and the most polar PUFAs are eluted last [18]. It has been reported that acetone/*n*-hexane, diethyl ether/petroleum and acetonitrile/*n*-hexane are partially successful in purifying PUFAs [18, 28]. The fatty acid profiles of various elution fractions obtained from the argentated

**Table 3** Fractions eluted by argentated silica gel column chromatography

Fraction (%) <sup>a</sup>	Volume (mL)	Weight (mg)
1 F	20	–
2 F	20	106.9
5 F	15	110.4
6 F	15	9
7 F1	15	17.5
7 F2	20	10
8 F	18	16.9
10 F1	20	10.5
10 F2	10	49.5
15 F	15	63.2
Total	168	393.9

<sup>a</sup> 1 % acetone/*n*-hexane (1:99, v/v), 2 % acetone/*n*-hexane (2:98, v/v), 5 % acetone/*n*-hexane (5:95, v/v), 6 % acetone/*n*-hexane (6:94, v/v), 7 % acetone/*n*-hexane (7:93, v/v), 8 % (8:92, v/v), 10 % acetone/*n*-hexane (10:90, v/v), 15 % acetone/*n*-hexane (15:85, v/v)

**Table 4** Percent composition and recovery of fatty acids from PUFAs concentrate methyl esters eluted with various proportion of acetone/*n*-hexane solvent (v/v) by argentated silica gel column chromatography

FFA methyl ester	PUFAs	Eluent: % acetone in hexane (v/v)		5F		6F		7F1	
		2F	Recovery	%	Recovery	%	Recovery	%	Recovery
16:00	4.74	43.15 ± 0.21	57.14 ± 1.02	31.48 ± 0.05	31.56 ± 0.96	5.62 ± 0.25	0.67 ± 0.02		
16:01	1.82	12.62 ± 0.11	66.13 ± 2.03	8.80 ± 0.12	24.56 ± 3.21	0.56 ± 0.02	0.24 ± 0.01		
18:1n-9	1.82	20.9 ± 0.22	87.94 ± 0.45	13.04 ± 0.34		4.47 ± 0.02	0.17 ± 0.01		
18:1n-7	1.11	18.54 ± 0.01	67.43 ± 0.99	14.72 ± 0.02	50.09 ± 2.57	0.31 ± 0.01	0.23 ± 0.05		
18:02	3.64			6.14 ± 0.21	85.60 ± 0.47	3.21 ± 0.03	3.65 ± 0.36		
18:3n-6	0.5			1.40 ± 0.01	80.69 ± 1.28	2.13 ± 0.02	10.00 ± 0.45	0.42 ± 0.01	3.83 ± 0.44
18:3n-3	0.89			1.72 ± 0.02	80.72 ± 2.47	2.45 ± 0.11	9.35 ± 0.24	0.31 ± 0.02	2.30 ± 0.45
20:02	1.79			4.17 ± 0.42	90.5 ± 0.44	2.26 ± 0.13	3.97 ± 0.56	0.11 ± 0.0	0.37 ± 0.02
20:03	1.78			1.87 ± 0.22	75.09 ± 3.12	6.59 ± 0.23	21.52 ± 2.00	0.52 ± 0.03	3.30 ± 0.54
20:4n-6	6.69			6.55 ± 0.21	79.22 ± 2.11	11.94 ± 0.45	11.74 ± 0.53	3.12 ± 0.02	2.15 ± 0.07
20:5n-3	22.22			0.41 ± 0.01	6.05 ± 0.15	20.89 ± 0.23	7.71 ± 0.44	35.64 ± 1.01	21.47 ± 2.01
22:5n-6	4.58					20.28 ± 0.57		26.07 ± 0.46	66.07 ± 3.16
22:5n-3	5.93					14.69 ± 0.62		22.34 ± 0.35	70.70 ± 2.21
22:6n-3	42.31								

FFA methyl ester	Eluent: %acetone in hexane(v/v)		8F		10F1		10F2		15F	
	7F2	Recovery	%	Recovery	%	Recovery	%	Recovery	%	Recovery
16:00										
16:01										
18:1n-9										
18:1n-7										
18:02										
18:3n-6	0.13 ± 0.0		0.68 ± 0.03							
18:3n-3	0.15 ± 0.0		0.64 ± 0.02							
20:02										



**Table 4** continued

FFA methyl ester	Eluent: %acetone in hexane(v/v)											
	7F2		8F		10F1		10F2		15F			
	%	Recovery	%	Recovery	%	Recovery	%	Recovery	%	Recovery	%	Recovery
20:03	0.32 ± 0.02	1.16 ± 0.43										
20:4n-6	0.79 ± 0.12	1.96 ± 0.23										
20:5n-3	74.19 ± 2.11	15.62 ± 1.24	91.93 ± 2.45	47.77 ± 2.03	50.41 ± 1.34	19.91 ± 0.66	24.19 ± 0.44	6.53 ± 0.21	0.42 ± 0.01	0.91 ± 0.01		
22:5n-6	3.56 ± 0.23	5.16 ± 0.98	0.31 ± 0.01	0.75 ± 0.0								
22:5n-3	1.74 ± 0.04	3.41 ± 0.45	0.15 ± 0.0	0.50 ± 0.02								
22:6n-3	0.23 ± 0.0	2.08 ± 0.46	1.33 ± 0.03	0.20 ± 0.0	40.22 ± 0.34	3.80 ± 0.33	70.33 ± 1.22	31.34 ± 3.12	99.45 ± 2.10	56.7 ± 3.27		

<sup>a</sup> Elution was under gravity flow and the flow rate was estimated for each fraction based on the fraction volume and collection period

<sup>b</sup> The solvent fractions were collected manually and the mass of each fraction was determined gravimetrically

<sup>c</sup> The recovery for each fatty acid methyl ester was calculated as: (the concentration of specific fatty acid methyl ester in each solvent fraction × the weight of each solvent fraction)/(the concentration of specific fatty acid in the PUFAs concentrate methyl ester × the weight of PUFAs concentrate methyl ester) × 100

silica gel column chromatography are shown in Table 4. No fatty acid ester was detected in the 20 mL of 1 % acetone/*n*-hexane fraction (1F, v/v). The fraction eluted by 2 % acetone in *n*-hexane (2F, v/v) contained substantial amounts of saturated and monounsaturated fatty acids, the recoveries of 16:0, 16:1, 18:1n-9 and 18:1n-7 being 57.1 ± 1.0, 66.1 ± 2.0, 87.9 ± 0.5 and 67.4 ± 1.0 %, respectively. With the increase of acetone proportion, the polarity of the mobile phase increased, which rendered more unsaturated fatty acids excluded from the stationary phase. The unsaturated fatty acids containing 2–4 double bonds were mostly eluted by 5 % acetone/*n*-hexane (5F, v/v), while EPA content was negligible. The solvent fractions eluted by 6 % acetone/*n*-hexane (6F, v/v) contained great number of fatty acids, but the concentration and recovery of saturated and monounsaturated fatty acids decreased significantly, DPAn-6 and DPAn-3 were mostly enriched within 6 % and the first 15 mL of 7 % acetone/*n*-hexane fractions (7F1, v/v). The highest contents of 26.1 ± 0.5 % for DPAn-6 and 22.3 ± 0.4 % DPAn-3 were achieved in 7F1, with the recovery of 66.1 ± 3.2 and 70.7 ± 2.2 %, respectively. The fraction was also enriched with 36.6 ± 1.1 % EPA, although with a lower recovery (21.5 ± 2.0 %), suggesting that the fatty acids with the same number of double bonds were co-eluted in the column chromatography, and the number of double bonds might have a more significant effect than chain length on the affinity of the complex with the stationary phase. The maximum purification and recovery of EPA (91.9 ± 2.5 and 47.8 ± 2.0 %, respectively) were found in the 8 % acetone/*n*-hexane fraction (8F, v/v). EPA was co-eluted with DHA in the subsequent solvent fractions; however, the amount gradually decreased. DHA was firstly eluted in the 7F2 fraction, the maximum purity of 99.5 ± 2.1 and 56.7 ± 3.3 % recovery were achieved in the 15 % acetone/*n*-hexane fraction (15F, v/v).

The health benefit of PUFAs has attracted much research on the separation of them from saturated fatty acids. Urea-ethanol complexation is commonly used for PUFAs enrichment, but there could be some potential carcinogenic substance of ethyl carbamate, which might prevent further application. Ethyl carbamate dissolved easily in water, but hardly in organic solvents [29]. The determination of ethyl carbamate by solid phase extraction preceded with nonpolar *n*-hexane elution for removing other residues except for ethyl carbamate indicates that successive washing by water might be adequate to eliminate the ethyl carbamate from the UCF and NUCF. Furthermore, the high polarity of the ethyl carbamate could make itself difficult to be stripped from the argentated silica gel column [18].

Based on the theory that π–π complexation could be formed by C–C double bonds with π electron acceptors, the mesoporous materials supported ionic liquid compounds coated with silver salts [30] or ionic liquid containing

aromatic rings without silver salts was employed as  $\pi$  complexing sorbents in extracting and enriching n-3 polyunsaturated compounds [31]. Regardless of the high price of ionic liquid, the short processing time, the possibility of recyclability and the higher extraction capacity could comparatively increase the cost efficiency. However, the extraction or desorption process also involved organic solvents such as diethyl ester and 1-hexene. The selectivity of the  $\pi$  complexing sorbent for separating PUFAs with various number of double bonds should be further investigated.

Research from the 1940s to the 1960s showed that urea complexation could protect guest molecules from oxidation and be used to fractionate FFA and derivatives [32]. Charkraborty et al. [33] reported that a marginal increase of lipid oxidation of sardine oil occurred during solvent extraction because of the antioxidant effects. Additionally, no significant changes were denoted in the POV, TBS and CDs during the urea complexation and argentated silica gel chromatography processes. In our research, the POV of the original tuna oil was 3.16 meq  $O_2/kg$ , while those of EPA, DPA and DHA methyl esters upon argentated silica gel chromatography were, respectively, 5.61, 6.10, 5.97 meq  $O_2/kg$ , which were all in satisfactory ranges. Conjugated diene and triene were assayed by measuring the absorbance at 234 nm and 268 nm, respectively, which indicated that the oxidation reaction was avoided during the process.

## Conclusion

This study has demonstrated a combined method of urea complexation and argentated silica gel chromatography for the enrichment and separation of PUFAs derived from tuna oil, with the goal of obtaining greatly improved levels of DPA isomer. The urea complexation reaction considerably raised the content of the PUFAs, with a product with  $10.5 \pm 0.2$  % DPA isomer,  $22.2 \pm 0.6$  % EPA and  $42.3 \pm 1.2$  % DHA achieved after investigating several fractionation temperatures and different FFA/urea ratios by three successive urea complexation processes; it allowed for further purification by argentated silica gel column chromatography. The fractions eluted with various ratios of acetone/n-hexane solutions (v/v) resulted in different fatty acids profiles. A purity of  $26.1 \pm 0.5$  % for DPAn-6 and  $22.3 \pm 0.4$  % for DPAn-3 was obtained in the first 15 mL of the 7 % acetone/n-hexane solvent (v/v), with the recovery of  $66.1 \pm 3.2$  and  $70.7 \pm 2.2$  %, respectively. Additionally,  $91.9 \pm 2.5$  % EPA and  $99.5 \pm 2.1$  % DHA were also subsequently obtained. It can be concluded that argentated silica gel chromatography appears to be a promising method for separation of PUFAs with similar molecular size and numbers of double bonds.

**Acknowledgment** This study is supported by the Jiangsu province “Collaborative Innovation Center for Food Safety and Quality Control” industry development program.

## References

- Phang M, Sinclair AJ, Lincz LF, Garg ML (2012) Gender-specific inhibition of platelet aggregation following omega-3 fatty acid supplementation. *Nutr Metab Cardiovasc* 22:109–114
- Khaw Kay-Tee, Friesen Marlin D, Riboli Elio (2012) Plasma phospholipid fatty acid concentration and incident coronary heart disease in men and women: the EPIC-Norfolk prospective study. *PLoS Med* 9:1–12
- Schuchardt Jan Philipp, Huss Michael, Stauss-Grabo Manuela, Hahn Andreas (2010) Significance of long-chain polyunsaturated fatty acids (PUFAs) for the development and behaviour of children. *Eur J Pediatr* 169:149–164
- Luc Djoussé J, Gaziano Michael, Buring Julie E, Lee I-M (2011) Dietary omega-3 fatty acids and fish consumption and risk of type 2 diabetes. *Am J Clin Nutr* 93:143–150
- Kaur Gunveen, DavidCameron-Smith Manohar Garg, Sinclair Andrew J (2011) Docosapentaenoic acid (22:5n-3): a review of its biological effects. *Progr Lipid Res* 50:28–34
- Lin W, Wu FW, Yue L, Du QG, Tian L, Wang ZX (2014) Combination of urea complexation and molecular distillation to purify DHA and EPA from sardine oil ethyl esters. *J Am Oil Chem Soc* 91:687–695
- Shahidi Fereidoon (2005) *Bailey’s industrial oil and fat products*, 6th edn. Wiley-Interscience, London, pp 273–274
- Ho C (2003) *Omega 3: the Seal connection*. Flanker Press, St. John’s, Nfld
- Linder Michel, Matouba Excellent, Fanni Jacques, Parmentier Michel (2002) Enrichment of salmon oil with n-3 PUFAs by lipolysis, filtration and enzymatic reesterification. *Eur J Lipid Sci Technol* 104:455–462
- Sajilata MG, Singhal RS, Kamat MY (2008) Fractionation of lipids and purification of  $\gamma$ -linolenic acid (GLA) from *Spirulina platensis*. *Food Chem* 109:580–586
- Eaton P, Vasanthan N, Shin ID, Tonelli AE (1996) Formation and characterization of polypropylene- urea complexation compounds. *Macromolecules* 29:2531–2536
- Zhang Guiyu, Liu Jing, Liu Yuanfa (2013) Concentration of Omega-3 polyunsaturated fatty acids from oil of schizochytrium-limacinum by molecular distillation: optimization of technological conditions. *Ind Eng Chem Res* 52:3918–3925
- Kleiner-Shuhler Leslie, Va’zquez L, Akoh CC (2011) Purification of stearidonic acid from modified soybean oil by argentated silica gel column chromatography. *J Am Oil Chem Soc* 88:1161–1171
- Robles Medina A, Giménez Giménez A, García Camacho F, Sánchez Pérez JA, Molina Grima EA, Gómez c (1995) Concentration and purification of stearidonic, eicosapentaenoic, and docosahexaenoic acids from cod liver oil and the marine micro-alga *isochrysisgalbana*. *J Am Oil Chem Soc* 72:575–583
- Rege SU, Padin J, Yang RT (1998) Olefin/paraffin separations by adsorption: p-complexation vs. kinetic separation. *AIChE J* 44:799–809
- Li M, Pham PJ, Wang T, Pittman CU, Li T (2009) Solid phase extraction and enrichment of essential fatty acid methyl esters from soy-derived biodiesel by novel  $\pi$ -complexing sorbents. *Bioresour Technol* 100:6385–6390
- Belarbi E-H, Molina E, Chisti Y (2000) A process for high yield and scaleable recovery of high purity eicosapentaenoic acid

- esters from microalgae and fish oil. *Enzyme Microb Technol* 26:516–529
18. Guil-Guerrero JL, Belarbi El-Hassan (2001) Purification process for cod liver oil polyunsaturated fatty acids. *J Am Oil Chem Soc* 78:477–484
  19. Ryu SN, Lee JD, Jeong BY, Hur HS (1997) Method for separating and purifying  $\alpha$ -linolenic acid from perilla oil. Assigned to the Republic of Korea. Represented by Rural Development Administration. US 5.672.726 Patent (512,829) Polyunsaturated Fatty Acids
  20. Tengku-Rozaina TM, Birch EJ (2014) Positional distribution of fatty acids on hoki and tuna oil triglycerides by pancreatic lipase and  $^{13}\text{C}$  NMR analysis. *Eur J Lipid Sci Technol* 116:272–281
  21. Wanasundara UN, Shahidi f (1999) Concentration of omega 3-polyunsaturated fatty acids of seal blubber oil by urea complexation: optimization of reaction conditions. *Food Chem* 65:41–49
  22. Ratnayake WMN, Olsson B, Matthews D, Ackman RG (1988) Preparation of omega-3 PUFA concentrates from fish oils via urea complexation. *Fat Sci. Technol* 10:381–386
  23. Stocchi A, Bonaga G (1975) Correlation between urea complexation compounds and conformational structure of unsaturated C-18 fatty acids methyl esters. *Chem Phys Lipids* 15:87–94
  24. Ma DWL, Field CJ, Clandinin MT (2002) Countercurrent approach to the enrichment of  $\Delta^9\text{c}$ ,  $11\text{t}$ -and  $\Delta^{10\text{t}}$ ,  $12\text{c}$ -18:2 isomer by urea complexation. *J Am Oil Chem Soc* 79:755–758
  25. Traitler H, Wille HJ, Studer A (1988) Fractionation of blackcurrant seed oil. *J Am Oil Chem Soc* 65:755–766
  26. King CJ (1987) Separation processes based on reversible chemical complexation. In: RW Rousseau (ed) *Handbook of separation process technology*, Chap. 15. Wiley, New York
  27. Yang RT, Kikkinides ES (1995) New sorbents for olefin/paraffin separations by adsorption via  $\pi$  complexation. *AIChE J* 41:509–517
  28. Özcimder M, Hammers WE (1980) Fractionation of fish oil fatty acid methyl esters by means of argentated and reversed-phase high performance liquid chromatography, and its utility in total fatty acid analysis. *J Chromatogr A* 187:307–317
  29. Zimmerli B, Schlatter J (1991) Ethyl carbamate: analytical methodology, occurrence, formation, biological activity and risk assessment. *Mutat Res/Fundam Molec Mech Mutagen* 259:325–350
  30. Li M, Pham PJ, Pittman CU, Li T (2009) SBA-15-supported ionic liquid compounds containing silver salts: novel mesoporous  $\pi$ -complexing sorbents for separating polyunsaturated fatty acid methyl esters. *Microporous Mesoporous* 117:436–443
  31. Ling-Zhi C, Zheng G, Zhiyong Y, Seong-Chea C, Xuebing X (2011) Extraction and enrichment of n-3 polyunsaturated fatty acids and ethyl esters through reversible  $\pi$ - $\pi$  complexation with aromatic rings containing ionic liquids. *J Agric Food Chem* 59:8961–8967
  32. Hayes DG, Bengtsson YC, Alstine JMV, Setterwall F (1998) Urea complexation for the rapid ecologically responsible fractionation of fa from seed oil. *J Am Oil Chem Soc* 75(10):1403–1409
  33. Chakraborty K, Raj RP (2007) Eicosapentaenoic acid enrichment from sardine oil by argentation chromatography. *J Agric Food Chem* 55(18):7586–7595