American Journal of Food Technology

ISSN 1557-4571

Academic Journals Inc.

www.academicjournals.com

OPEN ACCESS American Journal of Food Technology

ISSN 1557-4571
DOI: 10.3923/ajft.2016.76.83

Research Article

Concentration of Omega-3 Polyunsaturated Fatty Acids from Rana Egg Oil by Urea Complexation and Response Surface Methodology

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Abstract

The optimum enrichment parameters of main omega-3 polyunsaturated fatty acids (ω-3 PUFAs) from Rana egg oil were investigated. Firstly, it was optimized the saponification condition. Secondly, the PUFAs fractions from the first step were subjected to a urea complexation process using one-factor-at-a-time approach. Then, the enrichment time, temperature and urea to fatty acid ratio were mainly analyzed by response surface methodology. Consequently, it was gained the maximized relative contents of docosahexaenoic acid (DHA, 8.81%), docosapentenoic acid (DPA, 3.01%) and eicosapentaenoic acid (EPA, 8.31%) at an enrichment time of 16 h, an enrichment temperature of 0°C and a urea to fatty acid ratio of 4.5:1 (wt/wt). In addition, it was investigated the effect of enriched ω-3 PUFAs on cell viability and gained the inhibitory effect at dose of 2000 μg mL^{-1}. This data suggested that Rana egg oil could be utilized as alternative source of ω-3 PUFAs.

Key words: Rana egg oil, omega-3 polyunsaturated fatty acids, urea complexation, response surface methodology, cell viability

Received: January 20, 2016 **Accepted:** February 28, 2016 **Published:** April 15, 2016

Citation: Dan Li, Mengrou Ren, Jing Lu, Tong Wang, Yixuan Huang, Wei Jiang, Zhang Yan, Yuwen Fang, Xuming Deng and Shuang Guan, 2016. Concentration of omega-3 polyunsaturated fatty acids from Rana egg oil by urea complexation and response surface methodology. Am. J. Food Technol., 11: 76-83.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Rana grows in a region of Changbai mountain in Northeast China is a kind of precious amphibians, with edible bodies, high oviductus production rate and health beneficial functions. Rana egg oil, extracted from the egg is rich of a variety of polyunsaturated fatty acids (PUFAs), especially the omega-3 PUFAs (ω-3 PUFAs), which have many beneficial effects, such as mitigating the risk of cardiovascular disease and breast cancer (Kar, 2014; Liu and Ma, 2014). With the rising use as food supplement and infant formula, the market for ω-3 PUFAs is expending. Nowadays, the main source of ω-3 PUFAs is fish oil. However, this source is not sufficient due to the low concentration and marine pollutants (Liu *et al*., 2014). Thus, it is urgent to find a new source of ω-3 PUFAs. Rana egg is a waste of oviductus production in China and is low of environmental pollutants. Hence, Rana egg is a promising source of ω-3 PUFAs. The ω-3 PUFAs can be concentrated by many methods, such as lipase concentration (Wanasundara and Shahidi, 1998), silver ion complexation (Liu *et al*., 2006), freezing crystallization (Chen and Ju, 2002), molecular distillation (Martins *et al*., 2006), urea complexation (Lin *et al*., 2014), supercritical fluid extraction (Tilay *et al*., 2014) and efficient liquid chromatography (Yamamura and Shimomura, 1997). Among these methods, urea complexation is the simplest and the most efficient technique with low cost (Wanasundara and Shahidi, 1999). Liu *et al*. (2006) and Ratnayake *et al*. (1988) have successfully gained enriched eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (85.02%) from tuna oil and ω-3 PUFAs (69-85%) from fish oil, respectively via urea complexation.

In this study, urea complexation was employed to concentrate ω-3 PUFAs from Rana egg oil. Firstly, it was investigated saponification temperature, saponification time and the NaOH to Rana egg oil ratio to obtain the optimal parameters for free fatty acids extraction. Secondly, it was focused on urea complexation temperature, urea complexation time and the urea to free fatty acids ratio to optimize the reaction conditions for maximizing ω-3 PUFAs relative contents. Then it was optimized the parameters with response surface methodology. At the same time, Gas Chromatography Mass Spectrometry (GC-MS) was used to detect the relative contents of ω-3 PUFAs. At last, it was investigated that the effect of enriched ω-3 PUFAs on cell viability. This is the first time to concentrate ω-3 PUFAs from Rana egg oil. Rana egg is a waste of Rana oviductus production in China. Thus, this study was benefit the utilization of Rana waste and environmental protection.

MATERIALS AND METHODS

The study was conducted at Jilin University throughout 21 months from April, 2013 to January, 2015.

Materials: Rana egg oil was purchased from Long Pu Technology Co., Ltd in Shenyang Province (61.9%). The MTT (CAS 57360-69-7) was purchased from Sigma-Aldrich Chemical Co. The DMEM medium was purchased from Thermo Fisher Biological Chemicals Co. (Beijing). Fetal bovine serum and glutamine were provided by Gibco biological technology Co. and Difo Co. respectively.

Preparation of free fatty acid methyl esters: The optimal parameters of the preparation of free fatty acid methyl esters were studied by a one-factor-at-a-time approach. Three factors including saponification temperature, saponification time and NaOH to Rana egg oil ratio were mainly investigated. Rana egg oil was placed in a round-bottom flask and stirred with NaOH-CH_3OH (0.5 mol L^{-1}) and zeolites. In order to prevent the oxidation of fatty acid in the process, a small amount of EDTA was added into the flask (Shi *et al*., 2014). After 30 min reaction, the BF_3-CH_3OH solution (wt/wt, 15%) and the hexane solution (15 mL) were added. Then the saturated sodium chloride solution (20 mL) was added and stirred for about 1 h. The upper layer dehydrated by Na_2SO_4 was the free fatty acid methyl esters and was placed in -20°C for the next process.

Enrichment of ω-3 PUFAs by urea complexation: The enrichment of ω-3 PUFAs was studied with the free fatty acids as raw materials. The raw materials were obtained by saponification reaction under the optimal saponification conditions based on 2.2.

Based on the urea to fatty acid ratio, the urea was dissolved into appropriate amount of ethanol solution (13.1 g urea/100 mL, 95% ethanol) and was stirred constantly until the urea was completely dissolved at 60°C. Then the urea ethanol solution was poured into the beaker that was loaded with free fatty acids for inclusion. The beaker was placed in predetermined environment for urea complexation. The reaction solution was filtrated and evaporated with rotary evaporator at 80°C. The solid particles left were dissolved with distilled water and then were acidified with HCl to a pH of 1.5. The acidified solution was layered with petroleum ether. The upper layer was washed and evaporated to gain ω-3 PUFAs.

Response surface methodology design: The response surface methodology design was based on the results of one-factor-at-a-time approach for urea complexation. There were 20 groups of tests to be carried out using Design Expert software automatically. The relative contents of EPA (Y_1), DHA (Y_2) and docosapentenoic acid (DPA) (Y_3) were studied by the surface response methodology.

Analysis of ω-3 PUFAs by GC-MS: The relative contents of ω-3 PUFAs from the free fatty acids and the enriched PUFAs were detected by GC-MS with a capillary column (30.0 m× 250 um×0.25 um film thickness). The fatty acids to be detected were methylated completely, which would ensure the accuracy of the GC-MS detection. The preparation of fatty acid methyl esters was already mentioned in 2.2.

HepG2 cell cultures and cell viability assay: Cell viability of HepG2 cells was undertaken based on the method of Nikoloff *et al.* (2014). The ω-3 PUFAs aided by fatty free BSA (at a mole ratio of 5:1) were dissolved in DMEM. The final concentration of ω-3 PUFAs were 0.1, 0.5, 1, 5, 10, 50, 100, 250, 500, 1000 and 2000 μg mL^{-1}. The MTT solution (5 mg mL^{-1}) was then added and the absorbance was measured at 570 nm.

Statistical analysis: All experiments were carried out at least in triplicates. The optimization data were analyzed by ANOVA using Design Expert 6.0 software.

RESULTS AND DISCUSSION

Analysis of factors affecting the saponification: The preparation of free fatty acid methyl esters was studied by saponification with a one-factor-at-a-time approach. The effects of saponification temperature, saponification time and NaOH-Rana egg oil ratio on the relative contents of DHA, EPA, DPA and total ω-3 PUFAs (sum of DHA, EPA and DPA) were respectively shown in Fig. 1. Figure 1a showed that the relative contents of DHA, EPA, DPA and total ω-3 PUFAs increased

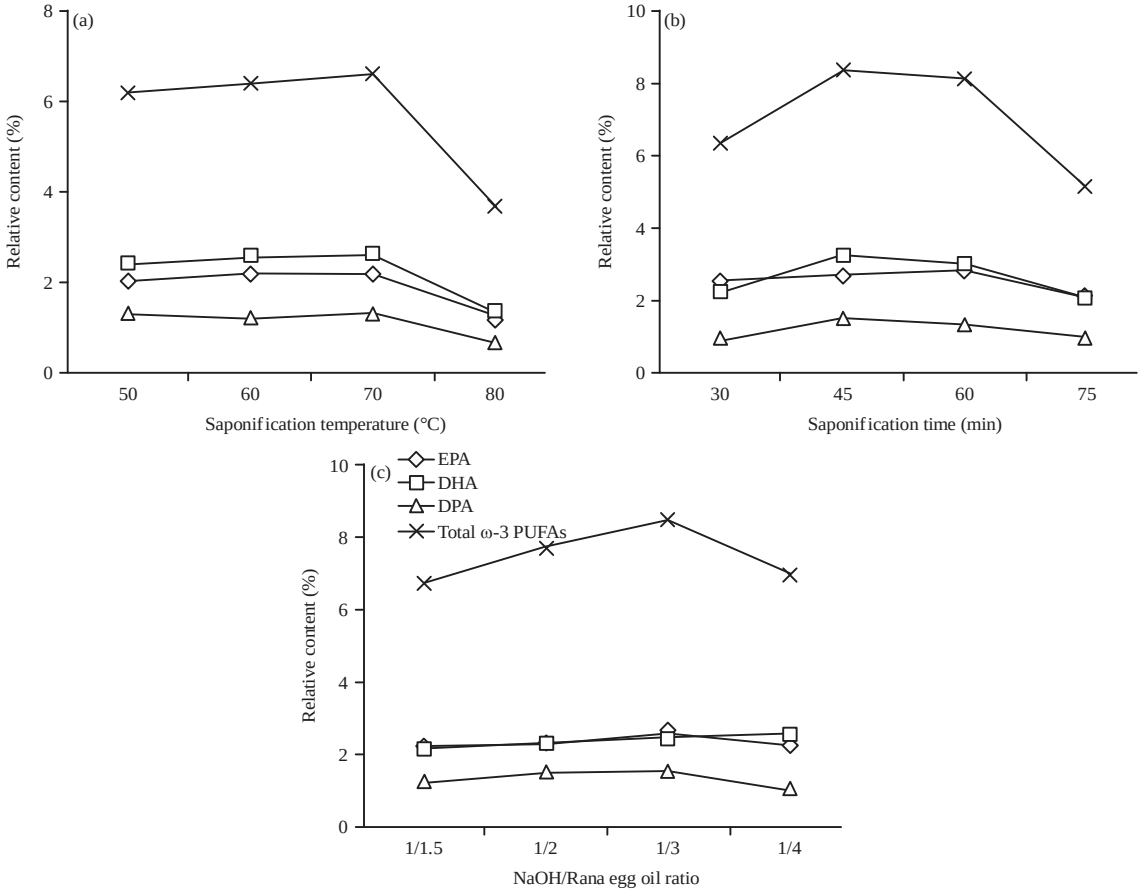

Fig. 1(a-c): Effects of (a) Saponification temperature, (b) Saponification time and (c) NaOH/Rana egg oil ratio on the relative contents of ω-3 PUFAs

gradually with the increase of saponification temperature. The result might due to the improved solubility of fatty acid salts in the system when the temperature increased (Lopez-Martinez *et al*., 2005). However, both of them declined when the saponification temperature was higher than 70°C. The most possible reason was that the free fatty acids or PUFAs started to be oxidized due to the high temperature, thus, the relative content of PUFAs was reduced. So the optimal saponification temperature was 70°C. Figure 1b showed that the relative contents of DHA, DPA and total ω-3 PUFAs increased firstly and then declined with the extension of the saponification time. The trends of DHA and DPA changed at 45 min, while EPA changed at 60 min. The longer the saponification time was, the higher the degree of oxidation of unsaturated fatty acid. The content of total ω-3 PUFAs was maximized at the specific time of 45 min, thus, it chose the optimal saponification time of 45 min. Among sodium, lithium, magnesium and potassium hydroxides and sodium hydroxide could give reasonable results in the process of saponification (Guil-Guerrero *et al*., 2007), thus, we chose NaOH as hydroxide. The effect of NaOH to Rana egg oil ratio on the relative content of ω-3 PUFAs was shown in Fig. 1c. When NaOH to Rana egg oil ratio was ranged from 1:1.5-1:3 (wt/wt), the relative contents of DPA, EPA and the total ω-3 PUFAs slowly increased. Generally, the larger the NaOH to Rana egg oil ratio was the larger the quantity of NaOH, which could make saponification reaction more completely. However, the viscosity will increase when the content of NaOH was too high, which will inhibit the saponification (Kamiouji *et al*., 2009). Thus, the optimal saponification ratio was 1:3 (wt/wt).

Analysis of factors affecting the enrichment process: In this process, response surface methodology was used to study the optimum parameters of ω-3 PUFAs concentration using Design Expert software. The results of three factors affecting the urea complexation were briefly shown in Fig. 2 by one-factor-at-a-time approach. The urea complexation belongs to an exothermic reaction, so the formation of crystals could be promoted when the temperature was

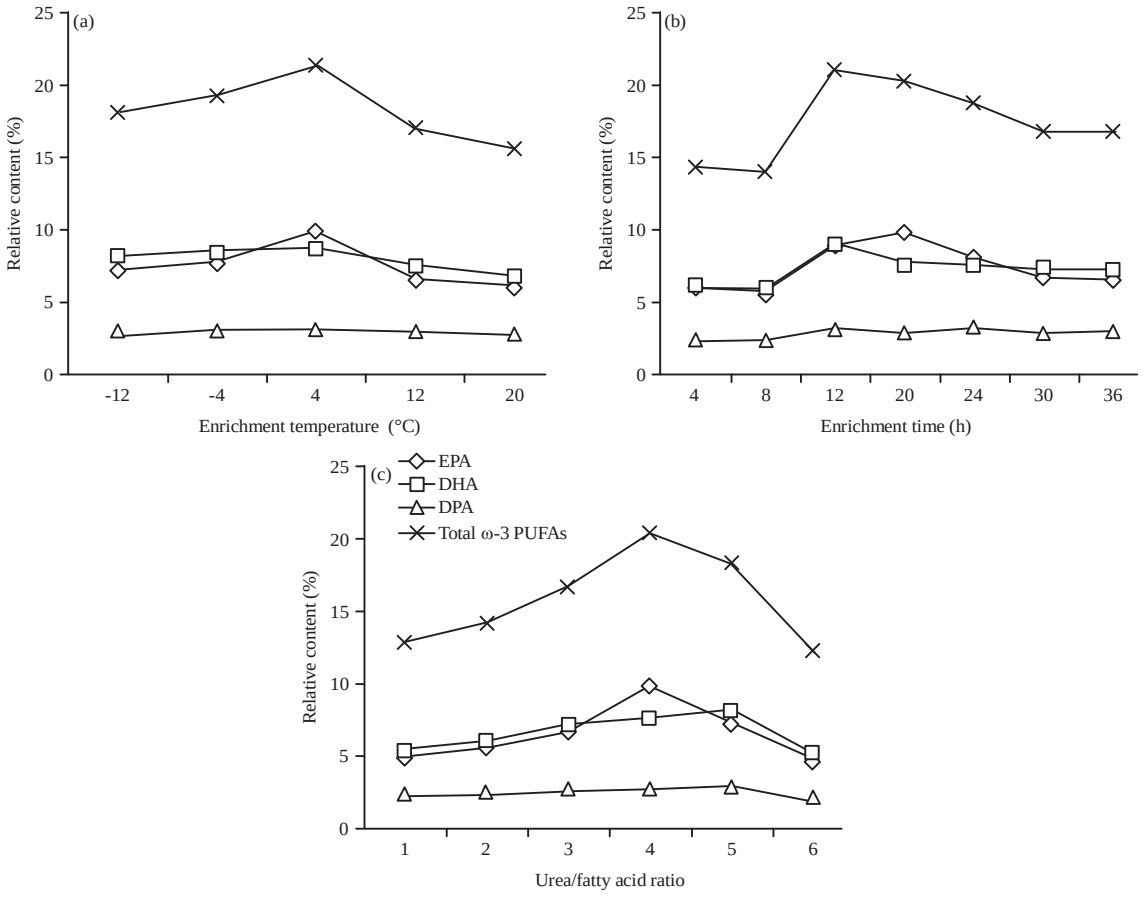

Fig. 2(a-c): Effects of (a) Enrichment temperature, (b) Enrichment time and (c) Urea to fatty acid ratio on the relative contents of ω-3 PUFAs

decreased. When the enrichment temperature was too low, the urea complexation would be prevented due to the solidification of ethanol and crystals. Thus, the content of ω-3PUFAs in un-complexed fatty acids declined relatively (Fig. 2a). This phenomenon was also observed by Brown and Kolb (1955). In Fig. 2b, the relative content of ω-3 PUFAs firstly increased and then declined with the extension of enrichment time. Only the relative content of EPA was maximized when the reaction time was 20 h. Based on the trend of total ω-3 PUFAs, the best choice was 12 h. Figure 2c showed that the relative contents of ω-3 PUFAs was maximized when the urea to fatty acid ratio was 1:4 (wt/wt).

Analysis of response surface methodology for urea complexation: The surface response methodology was employed to study the effects of enrichment temperature and urea to fatty acid ratio on DHA, EPA and DPA in Fig. 3. It could be initially concluded that enrichment temperature and urea to fatty acid ratio had bigger effects on content of ω-3 PUFAs than enrichment time. This results were similar to Liu's conclusion (Liu *et al*., 2006).

The effects of independent variables (enrichment temperature, enrichment time, urea to fatty acid ratio) on relative contents of ω-3 PUFAs were optimized. And equations reflecting the empirical relationship between response variable and the independent variables were presented as follows:

$$Y_1 = +7.06+0.30\times A-0.53\times B+0.30\times C-0.33\times A\times B +0.11\times A\times C+0.10\times B\times C+0.22\times A^2-0.12\times B^2-0.38\times C^2$$

$$Y_2 = +7.40+0.40\times A-0.57\times B+0.082\times C-0.53\times A\times B +0.23\times A\times C+0.18\times B\times C+0.21\times A^2-0.20\times B^2-0.38\times C^2$$

$$Y_3 = +2.56+0.11\times A-0.17\times B+0.096\times C-0.12\times A\times B+0.063 \times A\times C+0.029\times B\times C+0.079\times A^2-0.068\times B^2-0.13\times C^2$$

All of the above three models were highly significant ($p<0.01$).The lack of fit was not significant, which indicated that the degree of fit for the three models was all good and could be used for analysis. The relative contents of ω-3 PUFAs were calculated using the Expert Design software. The maximized relative contents of DHA, EPA and DPA were 8.81, 8.31 and 3.01%, respectively at an enrichment temperature of 0°C an enrichment time of 16 h and a urea to fatty acid ratio of 4.5:1 (wt/wt). Though it was successfully concentrated DHA and EPA, the relative contents of them were much lower than those concentrated from fish oil (60.6%) (Zhang *et al*., 2012). This might due to the initial content of these fatty acids (Kahveci and Xu, 2011).

Identification and detection of the relative contents for ω-3 PUFAs: The ω-3 PUFAs were detected by GC-MS and were shown in Fig. 4. The specific retention times and the relative contents of common ω-3 PUFAs were shown in Table 1. Five

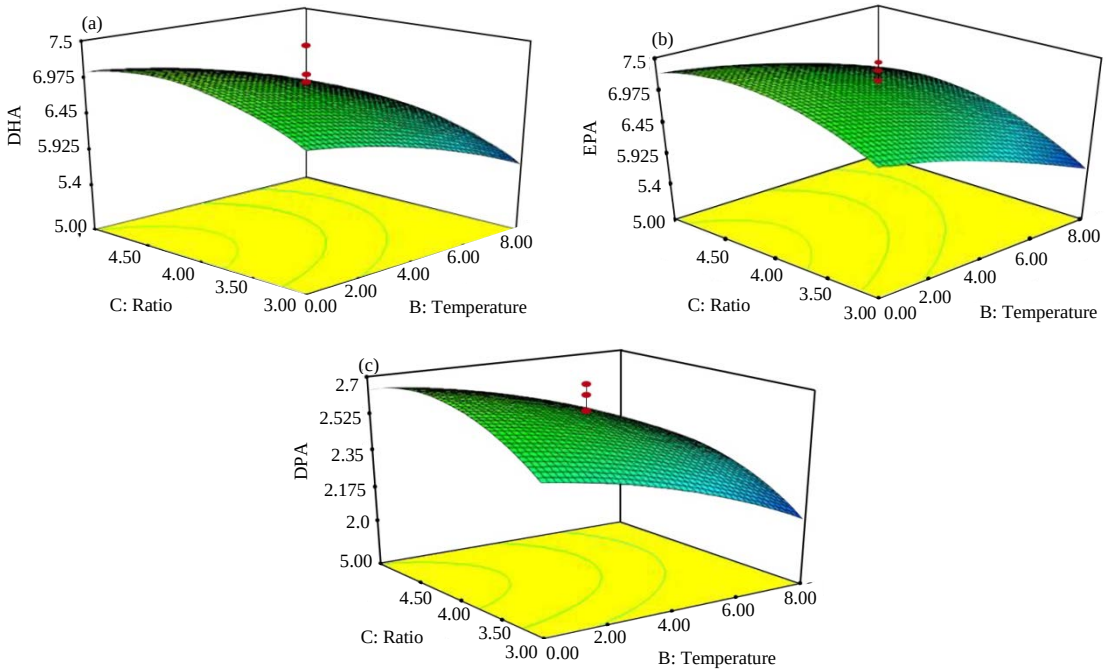

Fig. 3(a-c): Surface response analysis of enrichment temperature and urea to fatty acid ratio (a) DHA: Docosahexaenoic acid, (b) EPA: Eicosapentaenoic and (c) DPA: Docosapentenoic on the relative contents of ω-3 PUFAs

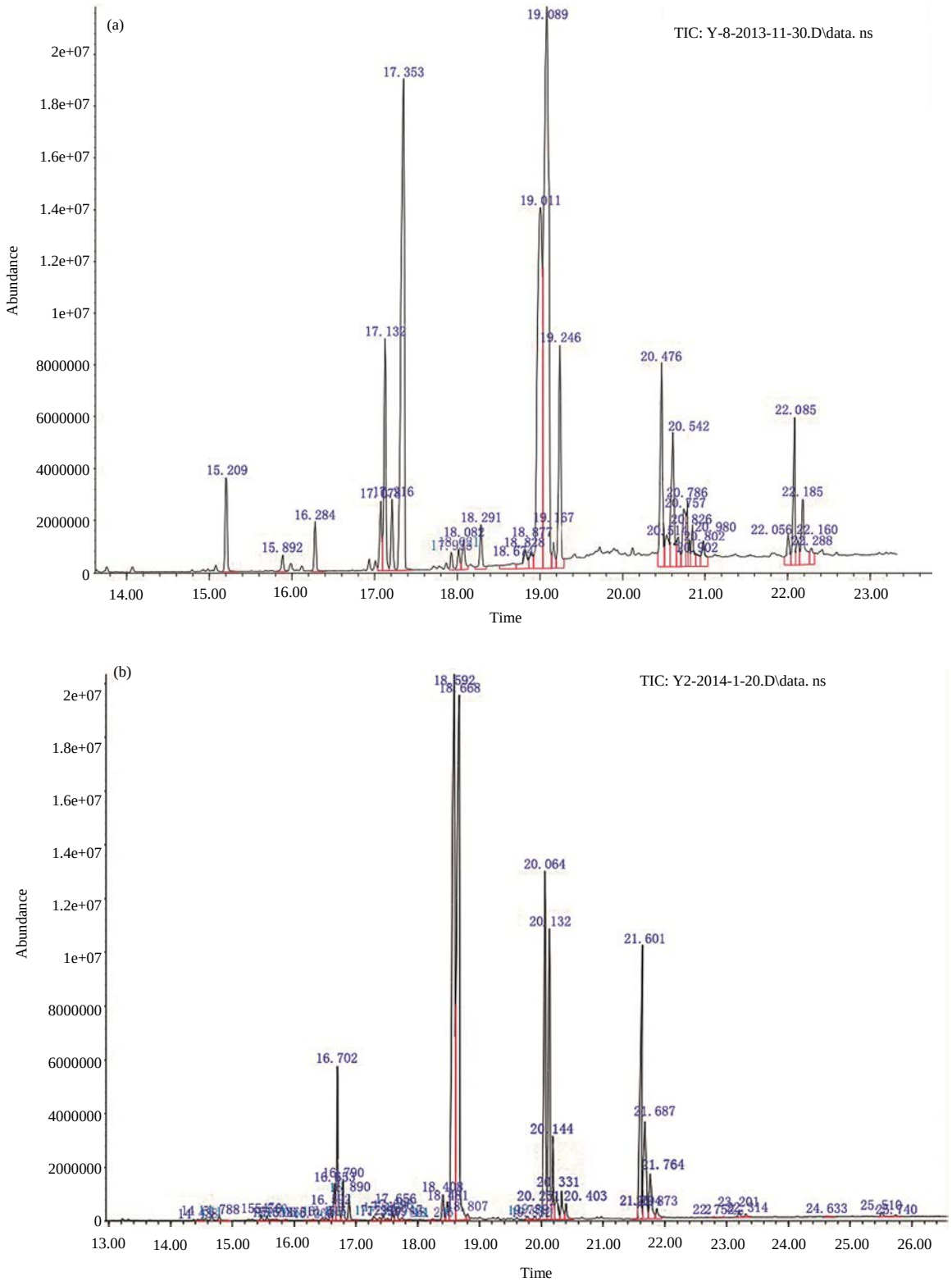

Fig. 4(a-b): Chromatograms of the ω-3 PUFAs after (a) Saponification process and (b) Enrichment process

ω-3 PUFAs were detected and the retention time of some ω-3 PUFAs had a little change after the optimization process. However, this situation occasionally happened due to the instrument SNR or the type of columns (Christie, 1989).

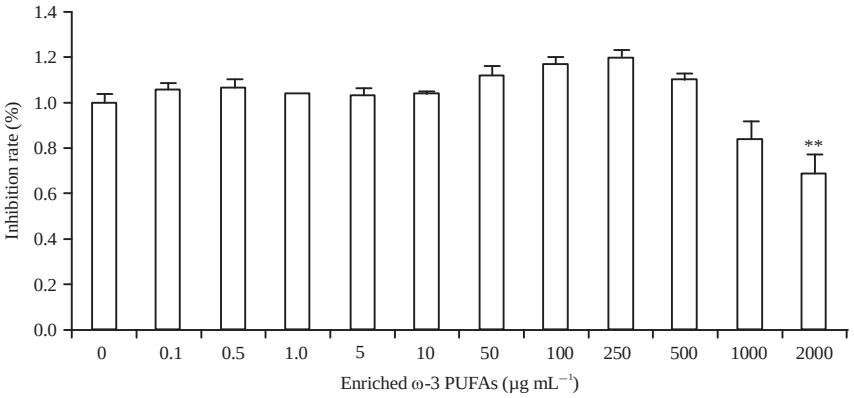

Fig. 5: Effects of enriched ω-3 PUFAs on cell viability of HepG2 cell, **p<0.01 compared with control (0 μg mL^{-1})

Table 1: Retention time and the relative content of common ω-3 PUFAs

		Average relative content (%)	
ω-3 PUFAs	Retention time (min)	Free fatty acid after saponification	Polyunsaturated fatty acid after enrichment
EPA (C20:5)	20.514	2.62	8.31
DHA (C22:6)	22.056	2.67	8.81
DPA (C22:5)	22.160	1.57	3.01
ETE (C20:3)	20.802	0.97	0.72
ETA (C20:4)	18.877	0.56	1.42

ω-3 PUFAs: Omega-3 polyunsaturated fatty acids, EPA: Eicosapentaenoic acid, DHA: Docosahexaenoic acid, DPA: Docosapentenoic acid and ETA: Eicosatrienoic acid

From Table 1, the relative contents of ω-3 PUFAs were significantly increased especially DHA, EPA and DPA. Five ω-3 PUFAs were detected by GC-MS including DHA, EPA, DPA, ETE and ETA.

Effect of ω-3 PUFA on cells viability: The cells viability of HepG2 cells treated with ω-3 PUFAs were shown in Fig. 5. It was concluded that ω-3 PUFAs could inhibit the HepG2 cell viability at dose of 2000 μg mL^{-1}. That it is to say, ω-3 PUFAs was safe to HepG2 cell under the dose of 2000 μg mL^{-1}, whereas, ω-3 PUFAs had antitumor effect at dose of 2000 μg mL^{-1}. The antitumor effect against HepG2 was consistent with the finding of (Lim *et al*., 2009).

CONCLUSION

The optimum parameters of ω-3 PUFAs concentration from Rana egg oil were gained by urea complexation and response surface methodology. As a results, we got below optimum parameters: Enrichment temperature of 0°C, enrichment time of 16 h, urea to fatty acid ratio of 4.5:1 (wt/wt), saponification temperature of 70°C, saponification time of 45 min and NaOH to Rana egg oil ratio of 1:3 (wt/wt). Under this condition, the relative contents of total ω-3 PUFAs from Rana egg oil were greatly increased. Five ω-3 PUFAs were detected by GC-MS including DHA, EPA, DPA, ETE and ETA. In addition, the ω-3 PUFAs obtained from Rana egg oil had antitumor effect against HepG2 cells at dose of 2000 μg mL^{-1}.

ACKNOWLEDGMENT

The authors are grateful to Ministry of Science and Technology of China and government of Jilin Province for financial support (2013BAD16B09 and 2011-YS9).

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