Technological approach of 1-*O*-alkyl-*sn*-glycerols separation from *Berryteuthis magister* squid liver oil

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Abstract Biological active compounds, 1-*O*-alkyl-*sn*-glycerols (AG), were isolated from liver oil of the squid *Berryteuthis magister*. The main components of the initial lipids were 1-*O*-alkyl-2,3-diacyl-*sn*-glycerols (38.50 %) and triacylglycerols (24.26 %). The first step of separation was the alkaline hydrolysis of oil to form a lipid mixture consisting of AG, free fatty acids and cholesterol. AG were separated by double recrystallization from acetone at −20 °C and 1 °C. A simple procedure is proposed for obtaining AG with a purity of 99.22 %, the main component of which is chimyl alcohol (94.39 %). Purity and structure of the obtained products were confirmed by GC and GC-MS technique. Isolated AG may be used in nutrition and cosmetics.

Keywords 1-*O*-alkyl-*sn*-glycerols · Biologically active compounds · Low temperature crystallization · Marine lipids

Introduction

Marine lipids are the source of many biologically active compounds. Together with the n-3 polyunsaturated fatty acids

Research highlights 1 Highly purified 1-*O*-alkyl-*sn*-glycerols was isolated from squid processing waste
2 The proposed method does not require any sophisticated equipment
3 The obtained product may be used in nutrition and cosmetics

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(PUFA), the positive effects of which have been extensively studied (Schmitz and Ecker 2008), AG, ethers of glycerol and fatty alcohols, arouse interest of many researchers. AG exhibit a variety of biological activities (Iannitti and Palmieri 2010). These compounds are precursors in the biosynthesis of plasmalogens, phospholipids with an alkenyl bond at *sn*-1 position of glycerol. The first steps of the plasmalogens biosynthesis, namely, the formation of the alkyl bond in the dihydroxyacetone phosphate molecule takes place in the peroxisomes (Nagan and Zoller 2001). Therefore peroxisomal dysfunction is one of the main reasons of neurodegenerative diseases and reduction plasmalogens in cells, for instance the level decline of plasmenyl phosphatidylethanolamine with docosahexaenoic acid (DHA, C22:6 n-3) in *sn*-2 position of glycerol in brain cells in Alzheimer's disease (Braverman and Moser 2012). The addition to the diet of plasmalogens precursors - AG and DHA – can promote the increase of their level in brain cells. In recent years, various synthetic plasmalogens precursors (Wood et al. 2011) or semisynthetic plasmalogens (Chen and Liu 2013) have been used in investigations of neurodegenerative disorders. At the same time, the inclusion of DHA in the *sn*-2 position of the glycerol was the most important condition for such preparations. However, this approach is associated with complicated chemical or biochemical synthesis and this may prevent using them in practice. Dietary lipids are known to be exposed to the lipolytic enzymes with elimination of DHA, and it significantly reduces therapeutic efficiency of preparations. Human acyl-CoA transferase in the endoplasmic reticulum of the hepatocytes has been shown to include DHA in the *sn*-2 position of AG (Watschinger and Werner 2013); it generates formation of plasmalogens; which are then transported into the brain by the blood lipoproteins (Braverman and Moser 2012).

Marine lipids are the best known and abundant in natural sources of AG and n-3 PUFA. Liver of cartilaginous fish and

some mollusks contain a high amounts of ether lipids, 1-*O*-alkyl-2,3-diacyl-*sn*-glycerols (DAGE), the content of which in the total lipids reaches 50.0 % (Bakes and Nichols 1995; Hayashi and Kishimura 2002). Diversity of molecular species of neutral ether lipids is determined by the length and double bond number of the alkyl fragments in *sn*-1 position, and also by differences in the fatty acid composition. Alkyl fragments C18:1 (30.0–61.4 %) are the most common in the DAGE composition in the shark liver lipids (Magnusson and Haraldsson 2011), while chimyl alcohol C16:0 (70.2 %) is the main component of liver oil of the gonatid squid *B. magister* (Hayashi and Kishimura 2002). Moreover, the lipids of these marine organisms contain up to 25.0 % of n-3 PUFA, DHA and eicosapentaenoic acid (EPA, C20:5 n-3) being the main fatty acids (Bakes and Nichols 1995; Hayashi and Kishimura 2002).

Despite the *B. magister* squid liver lipids are the source of AG, EPA and DHA, the high content of free fatty acids (FFA) and cholesterol (CHOL) imposes restrictions on the use of the oil and requires a special approach to processing it.

Separation of highly purified AG is a rather difficult problem, despite the simple structure and high concentration in the marine oils. The proximity of the physicochemical properties of triacylglycerols (TAG) and DAGE complicates a single-stage separation. Lipase-catalysed transesterification (Fernández et al. 2013), supercritical fluid extraction and vacuum distillation (Tenllado et al. 2011) was allowed to obtain products rich in DAGE and AG. However these methods do not permit to isolate highly purified AG. The column chromatography on silica gel used for isolation of AG from soft coral lipids (Martinez Diaz et al. 2015), shark liver oil (Bordier et al. 1996) is limiting stage for technological application.

The purpose of this study was to design a technological approach of separation of highly purified AG from *B. magister* squid liver oil.

Material and methods

Squid was fished in the Bering Sea in September 2013. After squid processing, the liver was separated and stored for 3 months at -12°C . All chemical reagents used in this study are of analytical grade (Sigma–Aldrich, USA). All solvents were of HPLC grade and supplied by Sigma–Aldrich (USA).

Separation and hydrolysis of squid liver oil

Extraction of total lipids was conducted according to Bligh and Dyer (1959). Lipids (120 g) were mixed with 300 ml of 2 M KOH solution in 70 % aqueous ethanol with following heating to 70°C under stirring during 1 h. The resulting solution was diluted with 400 ml water and acidified with 400 ml of 1 N H_2SO_4 aqueous

solution. Mixture of FFA, CHOL and AG was extracted from aqueous ethanol solution with hexane (3×300 ml). The hexane extract was washed with water and dried with anhydrous Na_2SO_4 ; the solvent was evaporated.

Analysis of the obtained lipid mixture was conducted by thin layer chromatography (TLC). AG was isolated by preparative TLC in the system hexane:diethyl ether:acetic acid in the ratios of 50:50:1 (v/v/v) followed by determination of the composition of AG as trimethylsilyl derivatives (TMS-AG) using gas chromatography (GC) and gas chromatography–mass spectrometry (GC-MS). The composition FA as methyl esters was determined by GC.

Isolation of AG

Precipitation of AG was carried out by double crystallization in acetone at different temperatures. The mixture of saponified lipids (30 g) was dissolved in acetone at a ratio of 1:5 (w/w). The solution was kept at -20°C for 24 h. The sediment 1 was separated by filtration at a temperature of crystallization followed by dissolving in acetone at a ratio of 1:5 (w/w). The second crystallization was carried out at 1°C for 24 h. The sediment 2 was separated by filtration at 1°C , washed with cold acetone, dried and weighed. The AG yield was 57.47 % of the AG content in saponified lipids. Lipid composition analysis of the sediments and filtrates was performed by TLC. Composition of AG as TMS-AG was determined by GC and GC-MS.

Analytical methods

Determination of lipid composition

The lipid composition was determined by TLC on the precoated Merck Kieselgel 60 silica gel G plates (10×10 cm) according to Imbs et al. (2006). The plates were developed in the system hexane:diethyl ether:acetic acid in the ratios of 80:20:1 (v/v/v) for analysis of total lipid composition and 70:30:1 (v/v/v) for analysis of saponified lipids, filtrates and sediments. After drying in a stream of air, plates were sprayed with 7 % solution of $\text{H}_2\text{SO}_4/\text{EtOH}$, with following heating. The chromatograms were scanned using a scanner Epson Perfection 2400 PHOTO (Japan) in grayscale mode. The percentage of lipid components was determined by the intensity of the spots by an image processing program Sorbfil TLC Videodensitometer DV (Russia). The units were calibrated using the known standards for each lipid class.

Determination of AG composition

TMS-AG were prepared as follows: 50 μl of *N,O*-Bis(trimethylsilyl)trifluoroacetamide were added to 5 mg

AG; the mixture was heated to 80 °C for 1 h. After addition of 200 µl of hexane 1 µl of each silylated fraction was injected into the GC. The composition of TMS-AG was determined by GC using a chromatograph Shimadzu GC-2010 plus with a flame ionization detector (Japan) and a capillary column Supelco SLB™-5 ms 30 m×0.25 mm i.d. (USA). Separation of mixture components was carried out under the following conditions: initial temperature 200 °C, heating rate of 2 °C/min to 290 °C, which was held at this temperature for 35 min. Injector temperature was 270 °C, detector temperature 250 °C. AG identification was performed by comparison with available known standards. GC-MS was used to identify the structure of TMS-AG. Electronic impact spectra were recorded using an instrument Shimadzu TQ-8040 (Japan) with the column Supelco SLB™-5 ms (USA) at 70 eV under the same temperature conditions as during GC.

Determination of FA composition

Methyl esters of FA from squid oil were prepared according to the accepted procedure (Carreau and Dubacq 1978). An analysis of FA esters was conducted by gas chromatography on a Shimadzu GC-17A chromatograph (Japan) with a flame-ionization detector, a capillary column 30 m×0.25 mm i.d. Supelcowax 10 (USA). An analysis was performed under the following conditions: column temperature 190°C, the injector and detector temperature 240 °C. Helium was used as a carrier gas. The peaks of methyl esters of FA were identified by retention times of the individual FA esters through comparison of their equivalent carbon length numbers with the authentic standards (PUFA-3 mix from menhaden oil was purchased from Supelco, USA) (Stransky et al. 1997).

Results and discussion

Lipid composition of *B. magister* squid liver oil

The total lipid content was 53 % of the wet weight of the liver. TLC analysis demonstrated that the squid liver oil consisted of CHOL and its esterified form, DAGE, TAG, FFA, mono- and diacylglycerols (MAG and DAG), as well as minor amounts of polar lipids (Table 1). The contents of the main components (DAGE, TAG and FFA) were 38.49, 24.26 and 20.24 %, respectively. CHOL, and its esterified form, and polar components were present in small amounts, which is in consistency with the previously published data for oil of the *B. magister* liver (Hayashi and Kishimura 2002). Considerable amounts of FFA, MAG and DAG were found in the lipids, which is not characteristic of normal tissues. The high levels of the above compounds may be produced by enzymatic hydrolysis of the TAG and DAGE during a prolonged storage at −12 °C and

Table 1 Composition of the original squid liver oil and the saponified lipids after alkaline hydrolysis

Class of lipids	Liver oil	Saponified lipids
Cholesterol esters	2.12±0.32 ^a	ND
1- <i>O</i> -alkyl-2,3-diacyl-glycerol	38.49±2.71	ND
Triacylglycerols	24.26±2.14	ND
Free fatty acids	20.24±4.16	74.51±0.91
Cholesterol	5.45±0.41	6.35±0.55
Mono-diacylglycerols	6.87±2.19	ND
1- <i>O</i> -alkyl- <i>sn</i> -glycerols	tr	19.14±0.51
Polar components	2.57±0.25	ND

tr in traces (less than 0.05 %), ND not detected

^a mean % of sum peak areas± standard deviation (*n* = 5)

thawing (Hayashi and Kishimura 2002). Digestive systems of marine invertebrates, as it was shown before, contain highly active lipases and phospholipases (Patton 1975).

All acyl-containing lipids were completely deacylated after alkaline hydrolysis, and the composition of the obtained lipid mixture is given in Table 1.

Composition of AG in saponified squid liver oil

oil and silylation of preliminary isolated AG determination of TMS-AG composition was performed by GC (Table 2) TMS-AG were identified by GC-MS. Identification was performed by characteristic mass peaks of molecular ion M^+ , $(M-15)^+$ and base peak of m/z 205 indicating a TMS-AG structure (Bordier et al. 1996).

Analysis of the AG composition has shown that the main component is chimyl alcohol (75.36 %) in the saponified lipids (Table 2). Unsaturated AG (15.80 %) were represented mostly by isomers of C18:1 and C20:1, which is in consistency with previously published results (Hayashi and Yamamoto 1987).

AG isolation

Saponification of squid liver oil

Alkaline hydrolysis of lipids was used in the first stage to isolate the most significant components (AG and PUFA) from squid liver oil. The formation of AG and fatty acid salts was occurred in the result of DAGE cleavage. The other acyl-containing components were also deacylated. The procedure made it possible to obtain a mixture of FA salts and unsaponifiable lipids (CHOL and AG). Extraction of unsaponifiable compounds from the hydrolysis reaction mixture is an important stage in the separation of AG. Hayashi (1986) separated unsaponifiable lipids from FA salts by extraction with diethyl ether. However, the use of diethyl ether is permitted only to a limited extent because of the risk of working with large amounts of this solvent. In addition,

Table 2 AG composition of saponified lipids, AG after double crystallization

Alkyl chain	Saponified lipids	AG after double crystallization
C14:0 ^a	1.77±0.47 ^b	1.26±0.16
C15:0	0.28±0.01	ND
C16:0	76.36±0.81	94.39±0.03
C16:1	0.53±0.11	ND
C17:0	0.22±0.01	ND
C17:1	0.31±0.03	ND
C18:0	4.77±0.50	4.35±0.37
C18:1	7.01±0.04	ND
C18:1	5.45±0.55	ND
C18:1	0.65±0.02	ND
C18:2	0.12±0.08	ND
C20:1	0.55±0.05	ND
C20:1	0.99±0.05	ND
C22:1	0.19±0.01	ND
ΣBranched ^c	0.80±0.10	
Σsat	84.20±0.77	100±0.00
Σunsat	15.80±0.77	

ND not detected, *sat* saturated AG, *unsat* unsaturated AG

^a indicated by chain length and double bond of alkyl chain in 1-*O*-alkyl-*sn*-glycerols (AG)

^b mean weigh %± standard deviation (*n*=5)

^c branched - 16:0i, 17:0i, 18:0i

the FA salts and the AG form stable emulsions during the non-polar solvent extraction of unsaponifiable compounds from the mixture obtained after hydrolysis; this complicates the extraction process and does not permit to separate unsaponifiable compounds quantitatively. According to the approach of Bordier et al. (1996), the FA salts were converted into FA methyl esters after hydrolysis followed by the separation of a mixture of squalene, FA methyl esters, CHOL and AG by the column chromatography on silica gel. The use of column chromatography makes it possible to obtain preparative amounts of AG. In this work, just after hydrolysis, the FA salts were transferred in FFA by acidification. As a result, we have obtained the fractions of FFA (74.51 %), CHOL (6.35 %) and AG (19.14 %) (Table 1).

AG precipitation from acetone

Crystallization of the organic solvent at low temperatures is a simple method for isolating FA. This method is widely used, in particular for pre-enrichment of PUFA (Shahidi and Wanasundara 1998).

As it has been shown before, squid liver lipids are abundant in saturated AG (84.20 %). First of all, we had to remove the maximum FFA in the stage of AG separation. Acetone was used as a solvent for crystallization. The lipid mixture after the

hydrolysis and acidification was dissolved in acetone in a ratio of 1:5 at room temperature and incubated for 24 h at −20 °C. Saturated FA and CHOL precipitated together with AG in the sediment 1 at low temperature crystallization, while PUFA remained in the filtrate 1. Recrystallization of sediment 1 was conducted for a complete separation of AG from free saturated FA and CHOL residues at 1:5 and temperature of 1 °C for 24 h. As a result, analysis of the obtained product by TLC and GC revealed that the AG content was 99.22 %, the main component being chimyl alcohol (Table 2). The total yield of AG was 57.47 % from initial AG content in saponified lipids. The filtrate 2 contained mostly saturated FFA, CHOL and AG.

Separation of AG and PUFA

Based on previous work (Latyshev et al. 2014) and the results obtained in this study, we have designed a new technological approach of isolation AG and n-3 PUFA from lipids of squid liver (Fig. 1). It is important that full removal of CHOL from preparations of AG, EPA and DHA occurs during oil processing. The proposed methods of separation and concentration of

Fig. 1 The scheme of separation of AG and PUFA from squid liver oil. *CHOL* cholesterol, *AG* 1-*O*-alkyl-*sn*-glycerols, *FFA* free fatty acids, *PUFA* polyunsaturated fatty acids, *EPA* eicosapentaenoic acid, *DHA* docosahexaenoic acid

biologically active components allow to obtain highly purified preparations for plasmalogens-replacement nutritional therapy of neurodegenerative diseases and for the cosmetic use (Johnson 2011).

Conclusion

Separation of highly purified AG from liver lipids of the squid *B. magister* was carried out. Chimyl alcohol (94.39 %) was the main component of the obtained preparation. The proposed method does not require any sophisticated equipment and costly reagents; the method allows the use of low-grade oil obtained from wastes of the squid processing; this significantly reduces the cost of the final products.

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Conflict of interest The authors declare no conflict of interest

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