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Sphingoid esters from the molecular distillation of squid oil: A preliminary bioactivity determination

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ABSTRACT

A mixture of sphingoid esters was isolated (1.4% w/w) from the molecular distillation of crude squid visceral oil. A preliminary investigation on the bioactivity profile and toxic potential of this residue was carried out by *in vitro* experiments. No cytotoxicity and a moderate lipase inhibition activity were highlighted.

1. Introduction

Squid viscera are non-edible parts and therefore "waste", produced in large quantities by the seafood processing industry. Their reutilization is appreciated not only to prevent pollution, as under current legislation squid viscera should not be dumped in the marine environment, but also by an economical point of view.

Literature method proposed for the valorization of squid viscera are the extraction of proteins and amino acids by hydrolysis (Lian, Lee, & Park, 2005; Sanchez-Alonso, Careche, & Borderıas, 2007; Uddin, Ahn, Kishimura, & Chun, 2010) and of lecithin by supercritical carbon dioxide extraction (Uddin, Ahn, Kishimura, & Chun, 2011). The aim is the use of these extracts as aquaculture feed or cosmetic ingredients, whereas Surimi and Surimi-like products can be obtained from the sole squid muscle (Cortés-Ruiz, Pacheco-Aguilar, Lugo-Sánchez, Carvallo-Ruiz, & García-Sánchez, 2008).

On industrial scale, the crude oil rendered from squid viscera is currently used as a source of omega-3 (Hwanga & Liang, 2001; Liang & Hwang, 2000; Uddin, Ahn, Kishimura, & Chun, 2011). The derived supplements are commercialized as an environmental friendly alternative to tuna oil (Boyle & Rodhouse, 2005). The procedure is based on ethanolysis and short-path molecular distillation (SPDM). The final oil results particularly enriched in docosahexaenoic acid (DHA) and eicosapentanoic acid (EPA).

Short-path molecular distillation (SPMD), also called molecular distillation, is an established good manufacturing practice for the industrial concentration of valuable compounds, on the basis of their boiling points (Krell, 1982). This method is characterized by a short exposure of the liquid to be distilled (from 10^{-1} to 10^{1} s) to an elevated temperature and high vacuum in a thin film evaporator capable to minimize heat induced side reactions and/or thermal decomposition (Breivik & Thorstad, 2005; Oterhals & Bertnissen, 2010; Oterhals, Kvamme, & Berntssen, 2010; Xu, Jacobsen, Nielsen, Heinrich, & Zhou, 2002).

By studying the residues obtained from the molecular distillation of squid oil, we isolated an unprecedented mixture of sphingoid esters. The aim of this paper is to present its chemical composition and a preliminary evaluation of its bioactivity profile, in the view of a better exploitation of this marine resource.

2. Materials and methods

2.1. Starting materials

All reagents and solvents (ACS grade) were purchased from Sigma-Aldrich and used as received without further purification. The crude visceral oils, obtained from three different species of squid (*Dosidicus gigas*, *Ilex argentinus* and *Todarodes pacificus*) by

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wet rendering, were supplied by Feng-I Co. (Kaohsiung, Taiwan). The final products object of this work were obtained in similar amounts (from 1.2% to 1.8% w/w) regardless the starting crude oil mixture used (four different batches were tested).

2.2. Short path molecular distillation (SPMD) conditions

Distillations were performed using a VTA short path molecular distillation unit (VTA, Verfahrenstechnische Anlagen GmbH, Deggendorf, Germany). Squid oil was metered from the reception chamber (500 ml) at flow rate of 1.2 ml/min onto a heated rotor under high vacuum (0.002–0.004 Mbar) and with an evaporation surface area of 400 cm^2. The distillate was collected on a condenser flask, maintained at 40 °C to prevent solidification. The un-distilled material was collected in a residue flask maintained at room temperature.

2.3. Fractionation procedure

Fractionation of the squid oil was carried out accordingly to the standard industrial procedure for the ethyl esters enrichment (Liang & Hwang, 2000). Initially, a 650 ml (600 g) of crude squid visceral oil was treated at 80 °C with a 5% aqueous solution of citric acid. The oil was thus molecular distilled at 180 °C in order to remove free fatty acids. The residue (R1, 500 ml) was submitted to transesterification by adding a 0.5 M sodium ethoxide/ethanol solution (200 ml) and by stirring at 75 °C for 5 h. The upper oily phase was washed (two portion of 200 ml of water), and evaporated under reduced pressure at 80 °C with rotavapor (waterbath B480, Buchi Labortechnik AG; Postfach, Switzerland).

The remaining material was molecular distillated at 145 °C, in order to isolate the ethyl esters (475 ml). The final residue (25 g, R2) was submitted to chromatographic separation on silica gel (Merck and Co. Ltd, Kieselgel 60, 70–230 Mesh) by eluting with dichloromethane, dichloromethane/ether (35:1) and dichloromethane/methanol (4/1). Glycerolipids, sterols and a yellow pale residue (R3, from 1.2% to 1.8% yield depending on the starting crude oil mixture) were isolated. Identification was carried out by TLC using triolein, cholesterol and methyl oleate standards for comparison. Residue R3 was submitted to a further chemical characterization. The summary of the fractioning process is reported in Table 1.

2.4. Hydrolysis of R3

The product R3 (10 mg) was suspended in 15 ml of methanol and 3 ml of concentrated hydrochloric acid in a closed vial and maintained at 70 °C for 5 h under stirring. After cooling, the solution was extracted with hexane (2 × 5 ml). The extract was dried under a flow of nitrogen and submitted to GC analysis. The methanol/water phase was treated with 5 ml of water and 30% sodium hydroxide solution added dropwise until pH 10 was reached, and then it was extracted with diethyl ether (2 × 5 ml). The ether extract was dried under a stream of nitrogen to give a residue named as $R3_{hyd}$.

Table 1
Treatment and fractionation of squid oil for the preparation of Ω3 enriched fatty acid ethyl esters.

Step	Process	Material isolated	Material discarded
1	Collecting squid visceral oil	Polar and neutral lipid protein, sugars, others	–
2	Degumming and bleaching	Polar and neutral lipid	Proteins, sugars, lecithin others
3	Molecular distillation at 180 °C	Polar and neutral lipid (R1)	Fatty acids
4	Ethanol treatment	Esterified and not esterified lipid	Glycerol
5	Distillation at 145 °C	Ethyl ester, monoglycerides (D2)	Tryglycerides, sterols, ceramides, others (R2)
6	Silica gel chromatography of R2	R3	Tryglycerides, sterols, others

R3 is the final residue of the procedure object of the present study.

2.5. Analytical methods

TLC separations were performed on silica gel P254 (Merck) plates (20 cm × 10 cm). IR spectra were recorded on a FTIR/ATR Nicolet Avatar (Thermo Scientific) in attenuated total reflectance (ATR) in the range of 4000–550 cm^{-1} with 64 scans at 2 cm^{-1} of resolution.

Infusion APCI-MS were performed with a Shimadzu 201A instrument on products dissolved in isopropanol/methanol mixture (3:10 v/V). APCI-MS conditions were: nitrogen flow of 2.5 ml/min; detector voltage at 1.5 kV; interface voltage at 4.5 kV; CDL temperature at 250 °C; interface temperature at 400 °C; block heater temperature at 200 °C. Mass acquisition was obtained in full scan from 150 to 1500 amu with a scan speed of 50 amu/s. Shimadzu Infusion kit was used for infusion analysis. The infusion flow was 10 μl/min.

LC-ESI-QTOF analysis were performed using a 1200 Infinity HPLC (Agilent Technologies, USA), coupled with a Jet Stream ESI interface (Agilent) with a Quadrupole-Time of Flight tandem mass spectrometer 6530 Infinity (Agilent Technologies). ESI operating conditions were the same used previously for the identification of lipids in various samples (Saliu, Degano, & Colombini, 2014). High resolution MS spectra were acquired in positive mode in the range 100–1400 *m/z*. MS/MS spectra were acquired in the range 100–1400 *m/z*. The collision energy for the MS/MS experiments was set at 50 V. Data were collected by auto MS/MS acquisition with a MS scan rate of 1.03 spectra/s and MS/MS scan rate of 1.05 spectra/s; only one precursor was acquired per cycle. The chromatographic separation of the products was achieved by an Agilent Poroshell 120 EC-C18 column (3.0 mm × 50 mm, 2.7 μm) with a Zorbax eclipse plus C-18 guard column (4.6 mm × 12.5 mm, 5 μm). A gradient of methanol (eluent A) and isopropanol (eluent B) was used. Ammonium formate (0.1%) was added as a mobile phase modifier. The elution gradient was: 90% A for 3 min, followed by a linear gradient to 90% B in 16 min, then B held for 5 min at 90%. Re-equilibration time for each analysis was 10 min. The chromatographic runs were performed at a flow rate of 0.6 ml/min. The injection volume was 2 μl and the column temperature was 30 °C.

GC analysis of the methyl esters was carried out with a 6850 Agilent chromatograph (Agilent, Santa Clara, CA, USA) using an HP-1 fused-silica capillary column (Agilent, Santa Clara, CA, USA): 100% dimethylpolysiloxane, 30 m, 0.25-mm inner diameter, 0.25-μm film thickness. Helium was used as a gas carrier (1.4 ml/min). Injection of samples was performed by using a split mode (10:1 split ratio). The oven temperature was held at 140 °C for 3 min and then raised at 6 °C/min to 190 °C and held for 1 min, then raised at 6 °C/min to 235 °C and held for 1 min, and finally raised at 15 °C/min to 300 °C and held for 3 min. The thermal conductivity detector operated at 250 °C under a 20 ml/min helium flow and 6.0 ml/min nitrogen makeup flow. For identification, the retention times of each methyl ester were related to the retention time of the internal standard octadecane.

NMR spectra were recorded on a Varian Mercury 400 instrument at room temperature using $CDCl_3$ as solvent. The chemical shifts were referred to the solvent signal. Relaxation delay of 4 s