# Purification and Characterization of Deep Sea Shark *Centrophorus squamosus* Liver Oil 1-*O*-Alkylglycerol Ether Lipids

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**ABSTRACT:** The 1-*O*-alkylglycerol composition of the liver oil of the deep sea shark *Centrophorus squamosus*, a species which provides edible flesh, has been determined. After various fractionations of the oil, the unsaponifiable fraction was characterized by means of gas chromatography/mass spectrometry, electron impact, and positive-ion chemical ionization. The oil is composed of 60% unsaponifiable matter, containing 45% squalene, 4.5% cholesterol, and 10% of linear saturated and monounsaturated glycerol ethers with 14–18 carbon atoms. After a first separation by chromatography on silicic acid, monounsaturated glycerol ethers have been separated from the saturated homologues, in particular from 1-*O*-octadecylglycerol (batyl alcohol) and 1-*O*-hexadecylglycerol (chimyl alcohol), *via* urea complexation. This newer application of the urea method, already used in the past to extract saturated from polyunsaturated fatty acids, allowed the purification of the main components of the complex unsaturated glycerol ether fraction, namely, 1-*O*-octadecen-9'ylglycerol (selachyl alcohol) and 1-*O*-hexadecen-9'ylglycerol.
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The deep sea shark *Centrophorus squamosus* (1) lives at depths below 1000 m; it can provide large quantities of squalene-rich liver oil, because this fish, which can reach 1 m in length, has a liver comprising 50% of body weight (2). We recently reported (3) the characterization of fatty acid methyl esters from this liver oil by gas chromatography/mass spectrometry (GC/MS), and the purification of two esters each of docosahexaenoic acid (DHA or 22:6) and of docosamonoenoic acid (DMA or 22:1), by countercurrent chromatography. We then directed our investigations to the complementary unsaponifiable fraction, more particularly to the 1-*O*-alkylglycerol fraction, because of its potential applications in industry.

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Abbreviations: APRL, antihypertensive polar renomedullary lipid; DHA, docosahexaenoic acid; DMA, docosamonoenoic acid; EI, electron impact; EPA, eicosapentaenoic acid; FID, flame-ionization detector; GC/MS, gas chromatography/mass spectrometry; GE, glycerol ethers; PAF, platelet-activating factor; PCI, positive-ion chemical ionization; TLC, thin-layer chromatography; TMS, trimethylsilyl radical. The abbreviation "en-n'yl" for 1-*O*-alkylglycerols indicate a normal *cis* ethylenic bond on the *nth* carbon from the ether oxygen.

The unsaponifiable matter of this liver oil contains squalene and glycerol ethers. Squalene is used as a food additive and as a precursor of squalane; the latter compound is utilized largely as an excipient in cosmetics and pharmaceuticals (4). Glycerol ethers, including those of chimyl or batyl alcohol (or their monounsaturated derivatives), have enormous economic potential because they exhibit bacteriostatic and fungistatic properties (5,6), anti-inflammatory activities (7), and hemopoietic effects (8,9). They are also believed to protect against radiation damage and to possess antitumor properties (10). With the recent interest in platelet-activating factor (PAF) (11,12), and the antihypertensive polar renomedullary lipid (APRL) (13), both of which contain a glycerol ether in their structure, ether lipids can also be considered as biochemical mediators (14,15).

## MATERIALS AND METHODS

*Shark oil. Centrophorus squamosus* liver oil was obtained from the IS-FRANCE Company (Lorient, France).

*Standards.* Racemic batyl alcohol came from Fluka (Buchs, Switzerland) and (+) batyl alcohol from Extrasynthese (Genay, France). Squalene originated from Robeco Chemicals, Inc. (New York, NY) and cholesterol from Janssen Chimica (Geel, Belgium).

*Chemicals.* AR Normapur solvents and salts used came from Prolabo (Paris, France). The $BF_3$ was purchased from Fluka, chlorotrimethylsilane from Janssen Chimica, and *N,O*-Bis(trimethylsilyl)acetamide from Aldrich Chemie (Steinheim, Germany).

*Shark liver oil fractionation.* The saponification was performed according to the procedure recommended by the *Pharmacopée Française* (16). Crude oil (20 g) was diluted with 20 mL of heptane and 40 mL of methanol with 4.5 g of KOH (dissolved with heating); the reaction was carried out under magnetic stirring at 70°C for 30 min.

The potassium salts of fatty acids produced after the saponification were converted into the corresponding methyl esters by adding 150 mL of $BF_3$ in methanol (10%, by wt) (17); the reaction was achieved with magnetic stirring at 70°C for 15 min. After addition of 170 mL of heptane, the solution was washed with an equal volume of water saturated with

NaCl and dried over $Na_2SO_4$. An oily mixture (20 g), of a pale yellow color, including both neutral and polar nonfatty acid materials, was obtained after evaporation of the solvent.

*Separation of the neutral and polar materials.* Esters and other materials (10 g) were submitted to chromatography on a 30 cm × 3 cm column filled with 100 g of silicic acid (ASTM, 230–400 mesh; Merck, Darmstadt, Germany). The eluent was a mixture of heptane and diethyl ether of increasing polarity (18). Five fractions (F1, F2, F3, F4, and F5) were obtained; the final fraction (F5), corresponding to very polar compound(s), was eluted with methanol; the methanol of the last fraction was evaporated and diethyl ether added to the residue, the solution was then filtered on a Büchner funnel to eliminate silica residues.

Collected fractions were analyzed by thin-layer chromatography (TLC) on silica gel 60 $F_{254}$ Special from Riedel-de-Haën (Seelze, Germany), with two different eluents. The less polar fractions were eluted with a mixture of heptane/diethyl ether/formic acid (1:0.25:0.025, by vol), the more polar with the same mixture in the proportions (0.25:1:0.025, by vol) (19). Compounds were visualized by spraying with $H_2SO_4$ and heating; results are shown in Tables 1 and 2.

*Silylation of glycerol ether and cholesterol fractions.* Nonvolatile compounds such as 1-*O*-alkylglycerols (F4) and cholesterol (F3) had to be converted into more volatile silyl ethers prior to GC, which is a technique well suited for the analysis of molecules of this kind (20). Silylation was performed by adding 200 µL of *N,O*-Bis(trimethylsilyl)acetamide and 100 µL of chlorotrimethylsilane to 10 mg of such compound(s); the reaction was carried out at 70°C for 20 min with magnetic stirring. After addition of 200 µL of diethyl ether 1 µL of each silylated fraction was injected into the GC.

*Analysis of F1, F3, and F4 by GC.* Unsaponifiable fractions were first examined by GC to optimize the conditions for further analysis by GC/MS. The analytical system was a Shimadzu Model GC-14A, connected to a flame-ionization detector (FID), controlled with a chromatopac C-R5A integrator (Shimadzu Corporation, Kyoto, Japan), and equipped with a nonpolar dimethylpolysiloxane CP-Sil 5 CB (Chrompack, Delft, The Netherlands) fused-silica capillary column (25 m × 0.25 mm × 0.12 µm). The final optimized analytical conditions were: $T_{inj}$ and $T_{det}$ 285°C, $T_{col}$ isothermal at 190°C for 5 min followed by a programmed temperature gradient from 190 to 285°C at 10°C/min$^{-1}$, $P_{He}$ $10^5$ Pa, split of 80 mL/min, injected quantity 1 µL of silylated compound(s) dissolved in diethyl ether or (20 g/L) for the fraction(s) which were not silylated. See Figure 1 for the results for F4.

*Analysis of F1, F3, and F4 by GC/MS.* The structures of compounds were characterized by GC/MS experiments with a combination of a chromatograph gas Varian Model 3000 (Sunnyvale, CA), using the same column and conditions as previously described, and a Nermag R10-10C mass spectrometer controlled by a Digital PDP11-23 Plus System (Delsi-Nermag, Argenteuil, France). Electron impact (EI) spectra were recorded at 70 eV, and positive-ion chemical ionization (PCI) studies were performed with $NH_3$ (Air Liquide, Le Plessis-Robinson, France) as reagent gas at a pressure of 0.1 Torr (1 Torr = 133.3 Pa) in the ion source.

*Purification of monounsaturated glycerol ethers (GE) of F4 by urea complexation of saturated GE.* Five grams of GE fraction (F4) was dissolved at 70°C in 50 mL of ethanol and 10 mL of methanol, with 5, 15, or 30 g of urea, corresponding, respectively, to the weight ratios $R = 1$, $R = 3$, and $R = 6$ (with $R$ = U/F4, by wt). Urea and urea complexes were allowed to crystallize overnight at 4°C. The crystallized material was separated by filtration on a Büchner funnel.

To recover the noncomplexing materials held in solution, 60 mL of water was added to each filtrate. Then, 6 N HCl was added, with magnetic stirring, until the mixture reached a pH of 4–5. Each solution was then extracted with 2 × 100 mL of diethyl ether. The extracts were separately dried over $Na_2SO_4$ and filtered. After evaporation of the solvent, a white wax was obtained for each extract of the three filtrates.

Each urea complex precipitate was also dissolved in 150 mL of water and neutralized by 6 N HCl to pH 4–5. Solutions were individually extracted with 2 × 100 mL of diethyl ether and dried over $Na_2SO_4$. After filtration and solvent evapora-

**TABLE 1**
**Thin-Layer Chromatography of the Crude, Saponified, and Esterified Deep Sea Shark Liver Oil with the Less Polar Eluent: *n*-Heptane/Diethyl Ether/Formic Acid (1:0.25:0.025, by vol)**

| $R_f$ | Crude oil | Saponified oil | Esterified oil |
|---|---|---|---|
| 0.95 | Squalene | Squalene | Squalene |
| 0.90 | Cholesterol esters | | |
| 0.65 | | | Fatty acid methyl esters |
| 0.57 | Diacyl-, monoalkylglycerols | | |
| 0.41 | Triacylglycerols | | |
| 0.27 | Fatty acids | Fatty acids | |
| 0.14 | Cholesterol | Cholesterol | Cholesterol |
| 0.06 | monoacyl-, monoalkylglycerols | | |
| 0.01 | Diacylglycerols | | |
| 0.00 | X[a] (Polar compounds) | Monoalkylglycerols, X | Monoalkylglycerols, X |

[a]X for undetermined compounds.

**TABLE 2**
**Composition and Percentages of the Different Fractions Obtained After Chromatography on Silica Gel of the Esterified Deep Sea Shark Liver Oil**

| Fraction | Compound | Percentage (%) |
|---|---|---|
| F1 | Squalene | 45.0 |
| F2 | Fatty acid methyl esters | 40.0 |
| F3 | Cholesterol | 4.5 |
| F4 | 1-*O*-alkylglycerols | 10.0 |
| F5 | X[a] | 0.5 |
| Esterified deep sea shark liver oil | | 100 |

[a]X for polar compounds eluted with methanol.

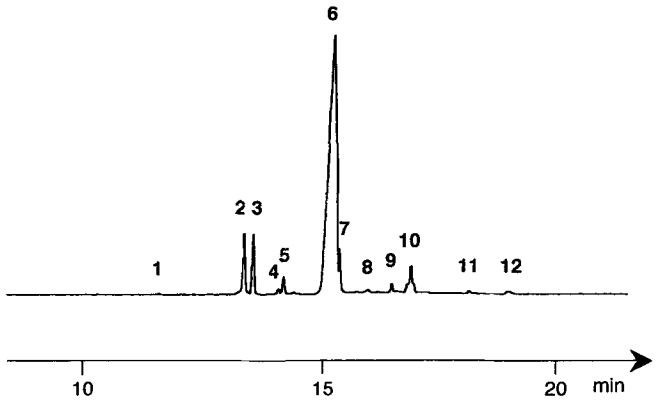


**FIG. 1.** Gas chromatogram of glycerol ethers of the silylated fraction F4. Column: CP-Sil 5 CB (25 m × 0.25 mm × 0.12 μm); pressure: $10^5$ Pa; column temperature: 190°C for 5 min then 190 to 285°C at 10°C/min.

tion, pale yellow waxes were obtained. All diethyl ether extracts were silylated prior to GC, following the experimental procedure previously described.

## RESULTS

*Chromatography of* C. squamosus *liver oil products on silica gel.* The original crude, deep sea shark liver oil, the saponified oil, and the esterified materials were first analyzed by TLC. Spots were compared with standards and with TLC profiles of other oils found in the literature (21). Table 1 shows that crude oil is composed of three kinds of molecules, i.e., squalene, free cholesterol, and fatty acids as various esters of glycerol or 1-*O*-alkylglycerols. Saponification, followed by esterification, leads to a mixture of squalene, fatty acid methyl esters, cholesterol, and the monoalkylglycerols. The monoalkylglycerols were easily recovered by column chromatography, as shown in Table 2. It appears that this species of deep sea shark has a liver oil which contains an important amount of unsaponifiable matter (60%), of which a very large proportion is squalene (see Table 2). This marine oil also contains significant amounts of ether lipids (10%) and a small quantity of cholesterol (4.5%).

*F1, F3, and F4 GC/MS analysis.* To confirm the composition of the unsaponifiable matter of the deep sea shark liver oil, fractions F1, F3, and F4 (as defined in Table 2), were first analyzed by GC, then by GC/MS experiments. GC analysis of fraction F1 showed only one chromatographic peak with a retention time of 17.69 min identical with the retention time of a squalene standard. This was confirmed by comparing the EI spectrum of F1 with that of squalene, given by the library (22). Both spectra showed the following characteristic fragments: $[M]^{+\bullet}$ of *m/z* 410, $[M - CH_3]^+$ of *m/z* 395, $[M - C_3H_7]^+$ of *m/z* 367 and $[M - C_5H_9]^+$ of *m/z* 341. The molecular ion and isoprenic fragments of the spectrum were clear enough to identify the compound from F1 as squalene.

In GC experiments, silylated fraction F3 appeared as a pure compound with a single chromatographic peak with a retention time of 20.88 min, coinciding with that of the silylated cholesterol standard. The EI-spectrum of F3 was also comparable to that of silylated cholesterol given by the literature (23), confirming the previous data: *m/z* 458 represents the molecular ion of silylated cholesterol, whereas the following fragments, $[M - CH_3]^+$ of *m/z* 443, $[M - TMSOH]^{+\bullet}$ of *m/z* 368, $[M - (CH_3 + TMSOH)]^+$ of *m/z* 353, $[M - (C_3H_4 + TMSO)]^+$ of *m/z* 329, and $[M - (TMSOH + C_9H_{13})]^+$ of *m/z* 247, (TMS for trimethylsilyl radical) were also present.

GC/MS analysis of silylated fraction F4 demonstrated that it contained several silylated glycerol ethers. Comparison with silylated batyl alcohol standard, and with results found in the literature for 2-*O*-alkyl-1,3-di-*O*-TMS-glycerols, and di- and tri-*O*-alkyl-TMS-glycerols (24), proved that the silylated fraction F4 was mainly a mixture of GE in the 1-*O*-alkyl-2,3-di-*O*-TMS-glycerol form. The gas chromatogram and GE composition of the silylated fraction F4 are shown in Figure 1 and Table 3, respectively. The EI-spectrum of silylated batyl alcohol (Fig. 2) and those of peaks numbers 1–7 of the silylated fraction F4 gas chromatogram present the following similarities: a base peak of *m/z* 205; peak relative to the TMS radical appears in all cases; ions derived from the glycerol-2,3-di-*O*-TMS moiety, namely, *m/z* 147, 133, 130, 117, and 103 are present with 5–25% relative intensity; (M –

**TABLE 3**
**Composition of Glycerol Ethers of the Silylated Fraction F4 Determined from the Gas Chromatogram (GC) of Figure 1**

| GC peaks | Retention time (min) | % | Compounds[a] |
|---|---|---|---|
| 0 | 2.59 | — | Solvents |
| 1 | 11.54 | 1.0 | 14:0 |
| 2 | 13.29 | 6.0 | 16:1 |
| 3 | 13.47 | 5.0 | 16:0 |
| 4 | 13.97 | 0.5 | 17:1 |
| 5 | 14.08 | 1.5 | 17:0 |
| 6 | 15.11 | 76.0 | 18:1 |
| 7 | 15.12 | 3.0 | 18:0 |
| 8 | 15.76 | 1.0 | |
| 9 | 16.25 | 1.0 | |
| 10 | 16.64 | 4.0 | X |
| 11 | 17.83 | 0.5 | |
| 12 | 18.59 | 0.5 | |
| Silylated fraction F4 | | 100 | |

[a]Nomenclature: 16:0 for 1-*O*-hexadecylglycerol and 16:1 for 1-*O*-hexadecenylglycerol, X for undetermined compounds.

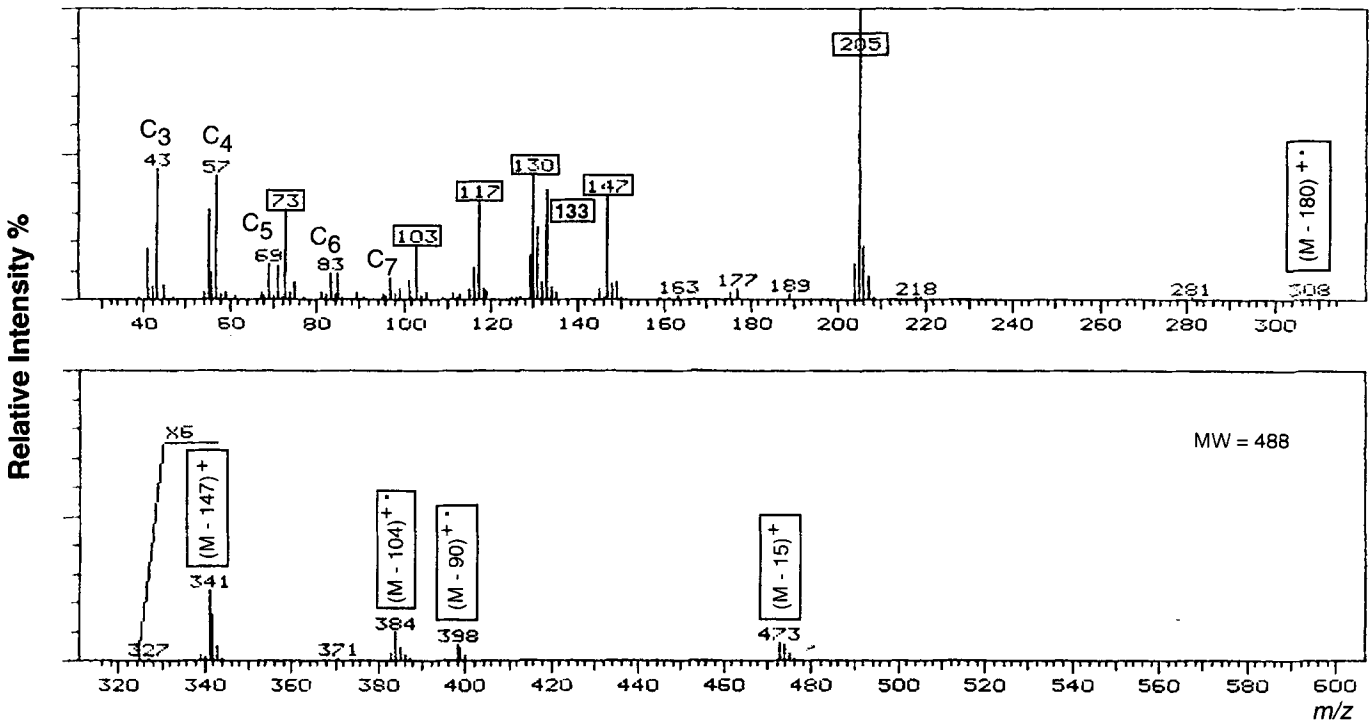


**FIG. 2.** Electron impact-spectrum of 1-O-octadecyl-2,3-di-O-trimethylsilyl-glycerol (silylated batyl alcohol) with its characteristic fragmentations. MW, molecular weight.

15)$^+$ is always present with 5–10% relative abundance, as well as (M – 90)$^{+\bullet}$, (M – 104)$^{+\bullet}$, (M – 147)$^+$, and (M – 180)$^{+\bullet}$ with lower relative intensities; in the lower mass range, the alkyl ions with *m/z* 43, 57, 71, 85, etc., for the saturated compounds and *m/z* 41, 55, 69, 83, etc., for the unsaturated ones, are of significant intensity; the molecular ion with low relative abundance could be observed only in unsaturated GE; the molecular weight of saturated GE was verified by PCI experiments. Assigments of fragment ions are indicated in Figure 3. Examples of EI and PCI-spectra of 1-*O*-hexadecyl-,2,3-di-*O*-TMS-glycerol and EI-spectrum of 1-*O*-hexadecenyl-,2,3-di-*O*-TMS-glycerol (Fig. 4) found in the silylated fraction F4 show the characteristic fragmentations of 1-*O*-alkyl-2,3-di-*O*-TMS-glycerols. Our GC/MS experiments demonstrate that the ether lipids of the esterified deep sea shark liver oil are a mixture of saturated and monounsaturated 1-*O*-alkylglycerols with linear alkyl chains composed of 14–18 carbon atoms, the major compound of which is 1-*O*-octadecenylglycerol. Although substituted GE, namely, 1-*O*-(2-methoxyalkyl) and 1-*O*-(2-hydroxyalkyl) (27) glycerols, were previously isolated by Hallgren *et al.* (25–27) from Greenland shark and other marine animals, we have not detected these compounds in *C. squamosus* liver oil.

*Purification of 1-*O*-octadecen-9′yl and 1-*O*-hexadecen-9′ylglycerol mixture* via *urea complexation.* Urea complexation of saturated fatty acids has been widely used for their removal from a mixture containing polyunsaturated fatty acids such as eicosapentaenoic acid (EPA or 20:5n-3) and DHA or 22:6n-3 (28,29). Due to their flexible chain, saturated molecules form urea complexes (host-guest molecules) more easily than their unsaturated homologues which have *cis* double bonds and are therefore partly rigid (30). We successfully applied this procedure to the purification of unsaturated glycerol ethers by selectively removing their saturated congeners with urea from fraction F4.

The composition of the filtrate, determined by GC, and the yield in monounsaturated GE, are shown in Figure 5 for three different ratios of urea to that of fraction F4, $R$ = U/F4 (by wt). For each ratio $R$, the presence of saturated GE and other impurities in the filtrate is negligible compared with that of monounsaturated GE. Increasing the amount of urea results in a decrease in both yield and purity of the monounsaturated GE, and as a consequence does not favor their purification. This is, of course, a very important parameter in large-scale preparation, because it minimizes the amount of urea to be used. The proportion of the two main compounds found in the filtrate, namely 1-*O*-octadecen-9′yl and 1-*O*-hexadecen-9′yl-glycerol, varies with the ratio $R$ = U/F4 as well, as shown in Figure 6. These results prove that the ratio $R$ also has an effect on the efficiency of the purification procedure because monounsaturated GE do not have identical behaviors toward urea complexation. Behavior seems to depend upon the carbon chain length of the monounsaturated GE; a higher $R$ does not result in discrimination based on the carbon chain length, whereas a lower $R$ allows purification of longer monounsaturated GE from shorter ones.

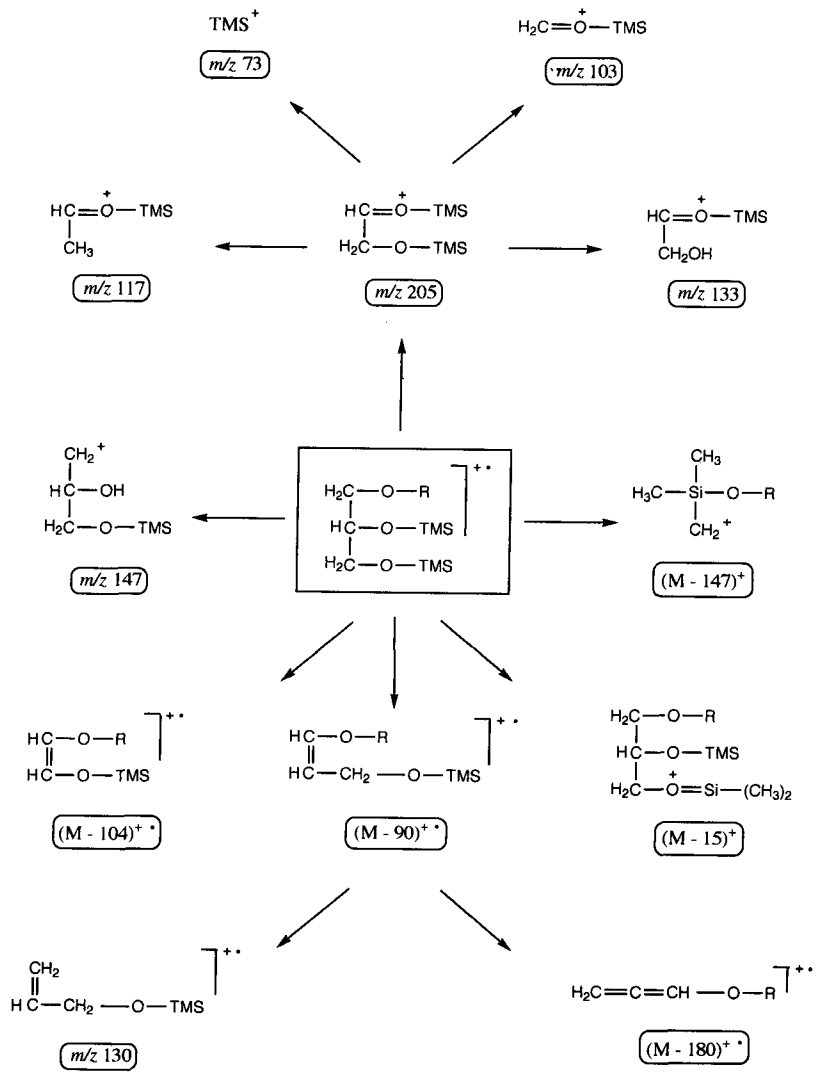


**FIG. 3.** Scheme of specific fragmentations of 1-*O*-alkyl-2,3-di-*O*-TMS-glycerols (TMS-trimethylsilyl radical).

## DISCUSSION

*Significance of the notable ether lipid and squalene contents of the liver oil of C. squamosus.* Elasmobranch fish (sharks, skates and rays) usually have large livers, containing important quantities of low-density oil, presumably to serve as a substitute for the commonly found gas-filled swim bladder of teleost species (31). The liver oil of *C. squamosus* differs from that at pelagic or sedentary deep sea sharks and contains a larger proportion of low-density lipids (squalene and ether lipids) (32), probably because it is a fast-swimming deep sea shark species (33). Various hypotheses have been put forward to explain the high percentages of these compounds in the hepatic oil of this kind of fish (34). One is the possibility that the fast-moving deep sea sharks use these molecules, and particularly squalene (35), or diacyl- and monoalkylglycerols (36), to maintain an appropriate buoyancy during vertical migrations. Another explanation must be sought for the high percentage of squalene, a molecule that is highly susceptible to oxidation (37).

*The use of silylated glycerol ethers to resolve the structures of these compounds of natural complex oils by GC/MS.* While the potential of GC/MS for the identification of ether lipids in natural samples is enormous, there have been few reports of the use of silylated glycerol ether derivatives, although they were described by Myher *et al.* (38). Isopropylidene derivatives are usually preferred to identify 1-O-monoalkylglycerols (39). Silylated ether lipids are generally examined by capillary GC on polar liquid phases. This work demonstrates that capillary GC on a nonpolar liquid phase, coupled with MS detection of silylated ether lipids, is also an effective method which can be used to sucessfully separate and determine ether lipid compositions from complex mixtures such as natural extracts of marine origin. For many natural samples, the retention times relative to authentic standards should serve as identification guides. However, this procedure is not suitable to identify the double bond positions of such compounds, even through further use of isopropylidene derivatives on nonpolar GC phases. The unsaturation posi-

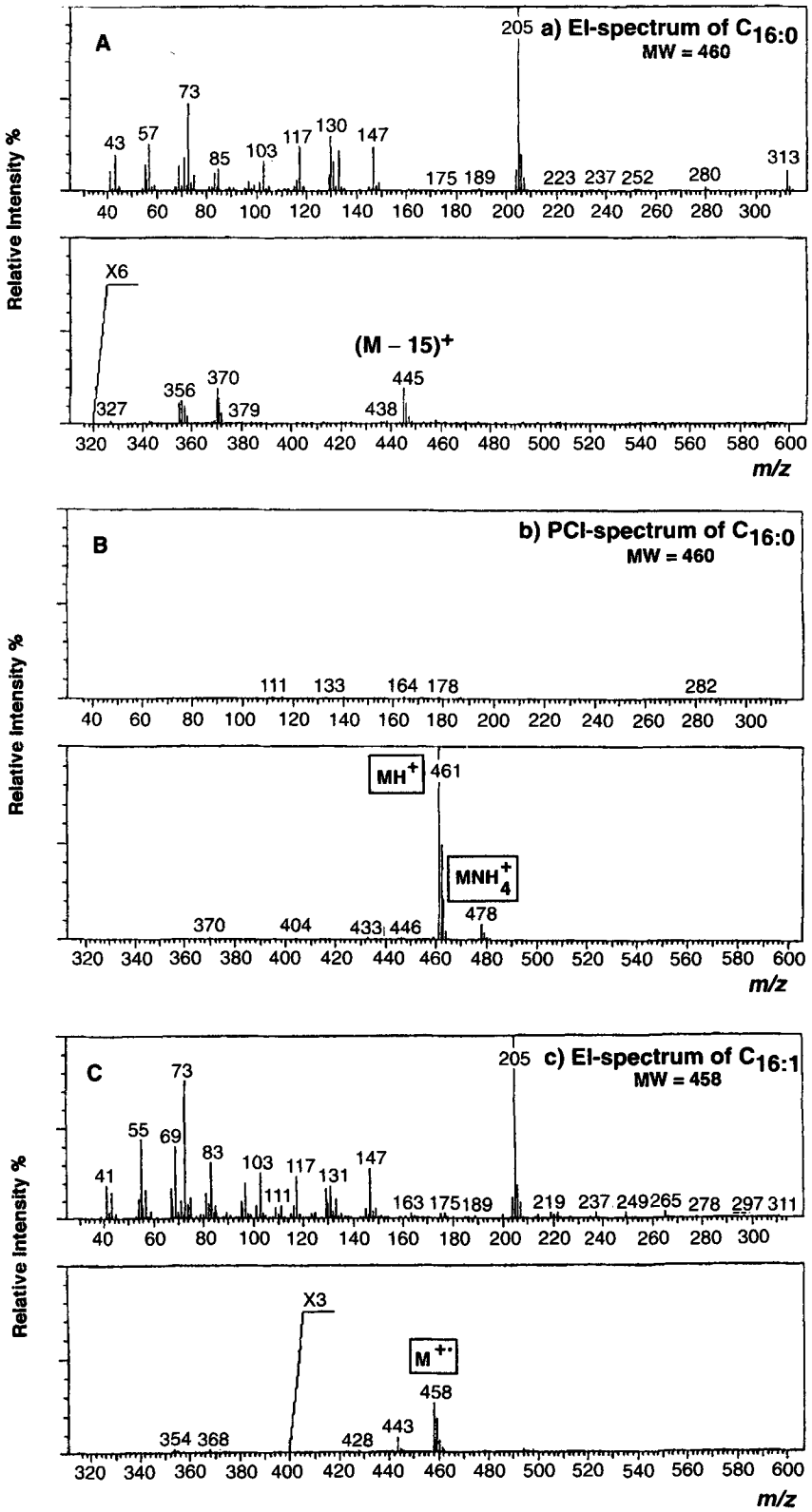


**FIG. 4.** A, Electron impact (EI)-spectrum of 1-*O*-hexadecyl-2,3-di-*O*-TMS-glycerol of the silylated fraction F4; E = 70 eV. B, Positive-ion chemical ionization (PCI)-spectrum of 1-*O*-hexadecyl-2,3-di-*O*-TMS-glycerol of the silylated fraction F4; reagent gas, $NH_3$ at 13.33 Pa. C, EI-spectrum of 1-*O*-hexadecenyl-2,3-di-*O*-TMS-glycerol of the silylated fraction F4; E = 70 eV. Abbreviations as in Figures 2 and 3.

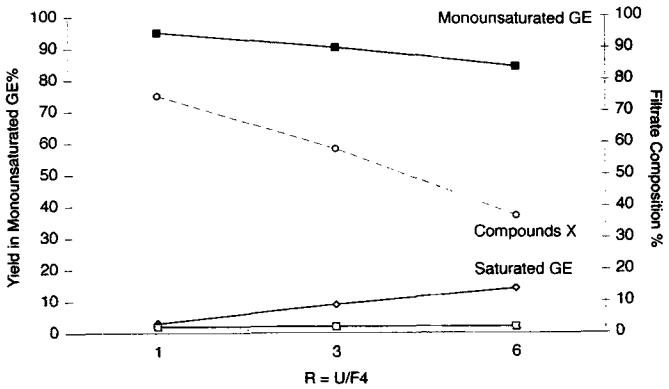


**FIG. 5.** Yield of monounsaturated glycerol ethers (GE) and composition of monounsaturated, saturated GE, and undetermined compounds X, obtained in the filtrate from the GE fraction F4 after overnight urea complexation at 4°C, with various amounts of urea (U), given by the ratio *R* = U/F4 (by wt); percentages acquired by gas chromatography.

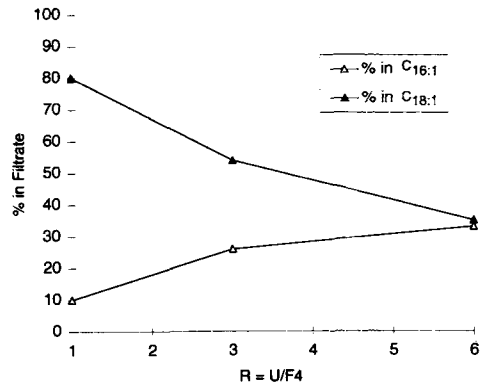


**FIG. 6.** Influence of the quantity of urea (U) given by the ratio *R* = U/F4 (by wt), used for the urea complexation overnight at 4°C, on the proportion of 1-*O*-hexadecenylglycerol ($C_{16:1}$) and 1-*O*-octadecenylglycerol ($C_{18:1}$), the main constituents of the glycerol ether fraction F4; percentages given by gas chromatography.

tions in the alkyl chains of the shark liver oil ether lipids have been determined recently in our laboratory by GC/MS, using and comparing two different approaches to fix the position of the ethylenic bond. First, a classical procedure was used, namely, the preparation of methoxy ether derivatives at the ethylenic have position of the alkylchain (40); second, an original technique, the use of soft chemical ionization with nitric oxide as reagent gas, developed by Malosse and Einhorn (41) for straight long-chain unsaturated olefins, alcohols, acetates, and aldehydes, was also performed satisfactorily on monounsaturated 1-*O*-alkylglycerols. These results will be published elsewhere.

*The use of urea complexation to produce substantial amounts of pure 1-*O*-monoalkyl and monoalkenylglycerols.* From the data of our urea complexation experiments, it is possible to imagine an efficient purification procedure as follows. Step 1: urea complexation of the initial fraction containing both saturated and monounsaturated GE with a high ratio of urea to GE (*R*), which will lead to the recovery of a pool of pure monounsaturated GE in the filtrate, but with a poor yield (40%); the yield can of course be improved by reprocessing the precipitate. Step 2: urea complexation of the monounsaturated GE fraction with a low *R*, to separate the GE as a function of the carbon chain length.

This procedure will be reduced to step 2 if only long-chain monounsaturated GE are of interest, and this makes urea complexation a powerful means for removal of the longer-chain monounsaturated ethers from a mixture of saturated and monounsaturated glycerol ethers. This novel use of the urea complexation method can provide useful amounts of pure 1-*O*-monoalkylglycerols. These compounds could be utilized as precursors in the large-scale synthesis of a series of molecules with important potential uses in cosmetic as well as pharmaceutical areas.

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