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Note

Rapid determination of unsubstituted alkylglyceryl ethers by gas chromatography–mass spectrometry

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Shark liver oil is the most abundant source of alkylglyceryl ethers. They occur mainly as diacyl derivatives and remain in the unsaponifiable fraction after hydrolysis [1]. Most of these lipids contain a saturated or unsaturated alkyl chain linked by an ether bond to the C-1 of the glycerol molecule [2,3]. The chain can also be substituted, usually with methoxy groups [1,2]. The naturally occurring glyceryl ethers possess the D-configuration [4].

Trace amounts of ether lipids are also found in the human body, *e.g.* in bone marrow, cell membranes, milk, and plasma [5,6]. These compounds represent ca. 0.1% and 0.3% of human milk and plasma total lipids, respectively. In plasma, ether lipids are found as alkylacyl and alkenylacyl glycerophospholipids [7], the latter compounds occurring especially in human erythrocytes [8]. Alkylglyceryl ethers have been reported to have antibacterial and tumour growth-inhibiting effects and to protect against cell damage following radiation therapy [6,9].

After unsaponifiable matter has been isolated, various separation techniques, including column, thin-layer [1,2,5] or high-performance liquid chromatography (HPLC) [7,8] are usually required before gas chromatographic (GC) analysis. Free alkylglyceryl ethers are commonly analysed by GC as bistrimethylsilyl [2,7,8] or dimethoxy derivatives [1,3,5]. Detailed GC studies on the composition of ether lipids in human plasma and erythrocyte phospholipids have recently been presented [7,8].

This paper reports a quantitative GC–mass spectrometric (MS) method for the analysis of alkylglyceryl ethers in human milk and plasma. For quantitative GC analysis, a simple purification procedure for the glyceryl ethers of shark liver oil is also presented.

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EXPERIMENTAL

Materials

Shark liver oil samples were made from a commercial preparation (Ecomer®) obtained from Hälsoprodukter (Forserum, Sweden). The human milk samples were supplied by the Human Milk Bank, The Children's Hospital, University of Helsinki, the plasma samples by the Kruunuhaka Medical Centre, Helsinki and erythrocyte samples from the Vagus Laboratory, Turku, Finland.

Shark liver oil samples (with fractionation)

The derivatization was carried out according to the steps described in Fig. 1. An oil sample (50 mg) was mixed with 1 ml of petroleum spirit (b.p. 40–60°C, May & Baker, Dagenham, U.K.) containing 1 mg of internal standard (I.S.) (heptadecanoic acid, Sigma, St. Louis, MO, U.S.A.). By modifying an earlier method [10] trans-esterification was performed with 0.5 ml of 0.5 *M* $NaOCH_3$ (Fluka, Buchs, Switzerland) in dry methanol (Merck, Darmstadt, F.R.G.) at 40°C for 5 min, and the sample was neutralized with an excess of 15% $NaHSO_4$ (1 ml) (Merck). Lipids were finally extracted from the acidic sample with petroleum spirit (1 ml).

After esterification the sample was fractionated on an Sep-Pak silica column (Waters Assoc., Milford, MA, U.S.A.) by subsequent elution with (1) petroleum spirit (2 ml) to remove hydrocarbons (*e.g.* squalene), (2) chloroform (2 ml) (Merck) to remove fatty acid methyl esters (FAMEs), and (3) methanol (2 ml) to obtain free glyceryl ethers and free fatty acids (FFAs) (including I.S.).

The methanol fraction was evaporated and silylated with 50 μl of bis(trimethylsilyl)trifluoroacetamide (BSTFA) [+1% trimethylchlorosilane (TMCS)] (Pierce, Rockford, IL, U.S.A.) at 60°C for 20 min in order to obtain a trimethylsilyl (TMS) derivative of I.S. and bis-TMS derivatives of glyceryl ethers (Fig. 1).

Human milk and plasma samples (no fractionation)

Lipids from human milk and plasma (1 ml) or lyophilized erythrocytes (350 mg) were extracted after addition of I.S. (500 μg) with chloroform–methanol (2:1)

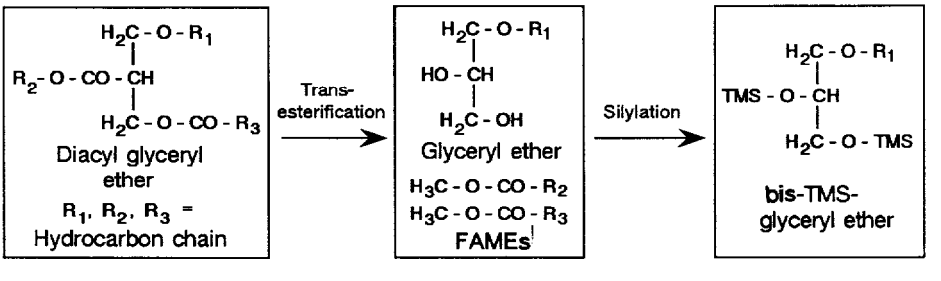

Fig. 1. Derivatization of glyceryl ethers.

(4 ml) according to Folch *et al.* [11]. After centrifugation (3500 *g*, 10 min) the chloroform layer was separated and evaporated under a flow of nitrogen. The residue was dissolved in petroleum spirit (1 ml) and transesterified. The petroleum spirit layer was separated and evaporated to a small volume and silylated as above.

GC–MS conditions

The GC–MS analyses were carried out on a Hewlett-Packard (HP) 5970 A quadrupole mass spectrometer coupled to an HP 5890 gas chromatograph. The spectrometer settings were: scan rate, 1100 a.m.u./s; electron ionization (EI) mode at 70 eV; electron multiplier voltage, 1800 V; vacuum, $1.3 \cdot 10^{-5}$ Torr; ion source temperature, 260°C. The GC conditions were: column, SE-54 fused-silica (15 m × 0.20 mm I.D.) (Nordion, Helsinki, Finland); carrier gas (He), flow-rate, 1.0 ml/min; column oven, programmed from 200 to 260°C at 7°C/min. The selected ion monitoring (SIM) analyses were carried out under the same conditions.

Gas chromatography

GC analyses with split mode were performed using a programmed temperature vapourizer (PTV) technique on a Dani 3860 GC system under the following conditions: column, SE-54 fused-silica (20 m × 0.32 mm I.D.) (Nordion); PTV-injector 70 to 250°C; flame ionization detector temperature, 250°C; column oven, programmed from 100 to 240°C at 15°C/min; carrier gas (H_2) flowrate, 3 ml/min.

Identification

The purified and silylated samples of transesterified shark liver oil were submitted to GC–MS analyses, and the spectra of bis-TMS derivatives of alkylglyceryl ethers were stored in the MS library file. The types of fragmentation of bis-TMS [12] and dimethoxy derivatives [1] presented earlier were also compared. The SIM technique was applied for the quantitative analyses of human milk and plasma alkylglyceryl ethers. In addition to the most abundant fragment *m/z* 205 (100%), the ions 147 and 103 were also selected as the typical fragments for bis-TMS derivatives of diols [13]. The ion *m/z* 117 (100%) was selected for the TMS derivative of heptadecanoic acid.

RESULTS AND DISCUSSION

Transesterified and silylated samples contain amounts of FAMEs, which coelute with silylated FFAs and glyceryl ethers (Fig. 2a). After transesterification and before silylation, it was necessary to remove the non-polar constituents (such as FAMEs and squalene) in order to improve the GC resolution (Fig. 2b). The constituents shown in Table I represented more than 86% of the total glyceryl ethers in the shark liver oil preparation.

Since unsubstituted 1-alkylglyceryl ethers are different only with respect to the

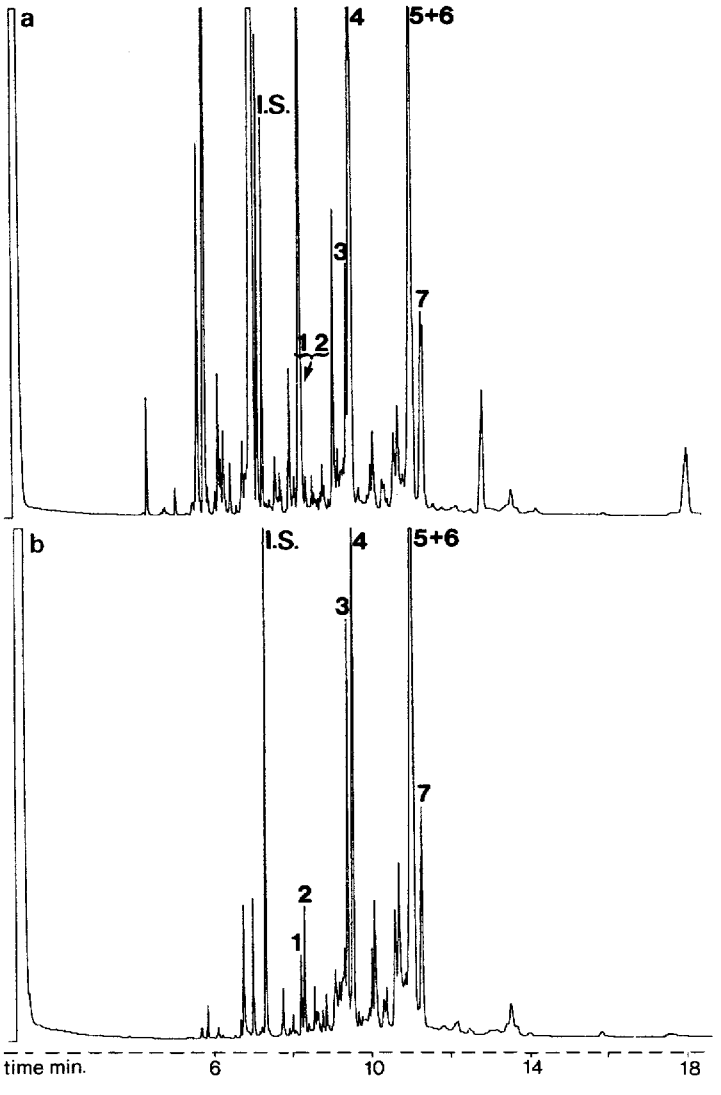

Fig. 2. Gas chromatograms of shark liver oil: (a) transesterified and silylated; (b) transesterified, purified and silylated.

alkyl chain length and the degree of unsaturation, a base peak at m/z 205 is obtained for major bis-TMS derivatives (Table I); it is caused by the loss of C-1 and the alkyl chain [12]. When the alkyl chain is saturated, the fragment M − 15 appears instead of $[M^+]$. An additional difference is found in the abundance of m/z 103, which is twice as high as that of the unsaturated chains (Table I). If the double bond in the alkyl chain is at position 7 from the terminal methyl carbon (compounds **3** and **6**), the abundance of m/z 73 is more than 90%. The ratio

TABLE I

SPECTRAL CHARACTERISTICS OF BIS-TMS DERIVATIVES OF MAJOR ALKYLGLYCERYL ETHERS

Glyceryl ether[a] (as bis-TMS derivative)	Percentage of total glyceryl ethers	Base peak	$[M^+]$	$[M-15]$	Specific fragments (relative abundance, %)	
					73	103
(**1**) Tetradecenyl	2.6	73	(430)	(415)	100	22
(**2**) Tetradecyl	5.0	205	(432)	417	56	52
(**3**) Hexadecenyl	11.5	205	458	443	93	23
(**4**) Hexadecyl[b]	20.4	205	(460)	445	57	57
(**5**) Octadecenyl[c]	35.2	205	486	471	69	21
(**6**) Octadecenyl isomer	3.5	205	486	471	95	22
(**7**) Octadecyl[d]	8.2	205	(488)	473	60	54

[a]Elution order on SE-54 column.
[b]Chimyl alcohol
[c]Selachyl alcohol
[d]Batyl alcohol.

between the fragments 73 and 103 in these isomers is also clearly the highest. Identification of compound **1** was based on such spectral similarities and retention times. The principle in the formation of the base peak (*m/z* 205) closely resembles that of the dimethoxy derivatives, where the main fragment of *m/z* 89 is obtained [1]. A total of twenty compounds with *m/z* 205 as the main fragment were detected by GC–MS from a shark liver oil sample.

Transesterification by sodium methoxide converts bound fatty acids into methyl esters, which are usually analysed by GC. The same esterified plasma or human milk sample, when silylated, can be used for GC–MS-SIM analysis of alkylglyceryl ethers. Based on the retention times and comparison with specific fragments, three major ether lipids (compounds **4, 5** and **7**) were quantified from human milk and plasma (Fig. 3a and b). In human milk these compounds mainly come from neutral lipids, whereas in total plasma they come from phospholipids [9]. The relative proportions of the three major compounds in total plasma (Table I) correspond to those obtained by GC analysis of alkylglycerol moieties of alkylacyl glycerophosphocholine [7], indicating a similar alkylglyceryl ether profile for these samples.

Human plasma phospholipids contain 1-alkenylglyceryl ethers, which as bis-TMS derivatives should also give the fragment *m/z* 205. They elute on a non-polar column just ahead of the alkylglyceryl ethers of corresponding chain length and unsaturation [14]. The phosphatidylethanolamine fraction of human erythrocyte phospholipids is especially rich in these compounds [8]. A part of the GC–MS-SIM iongram for ether lipids of lyophilized erythrocytes is presented in

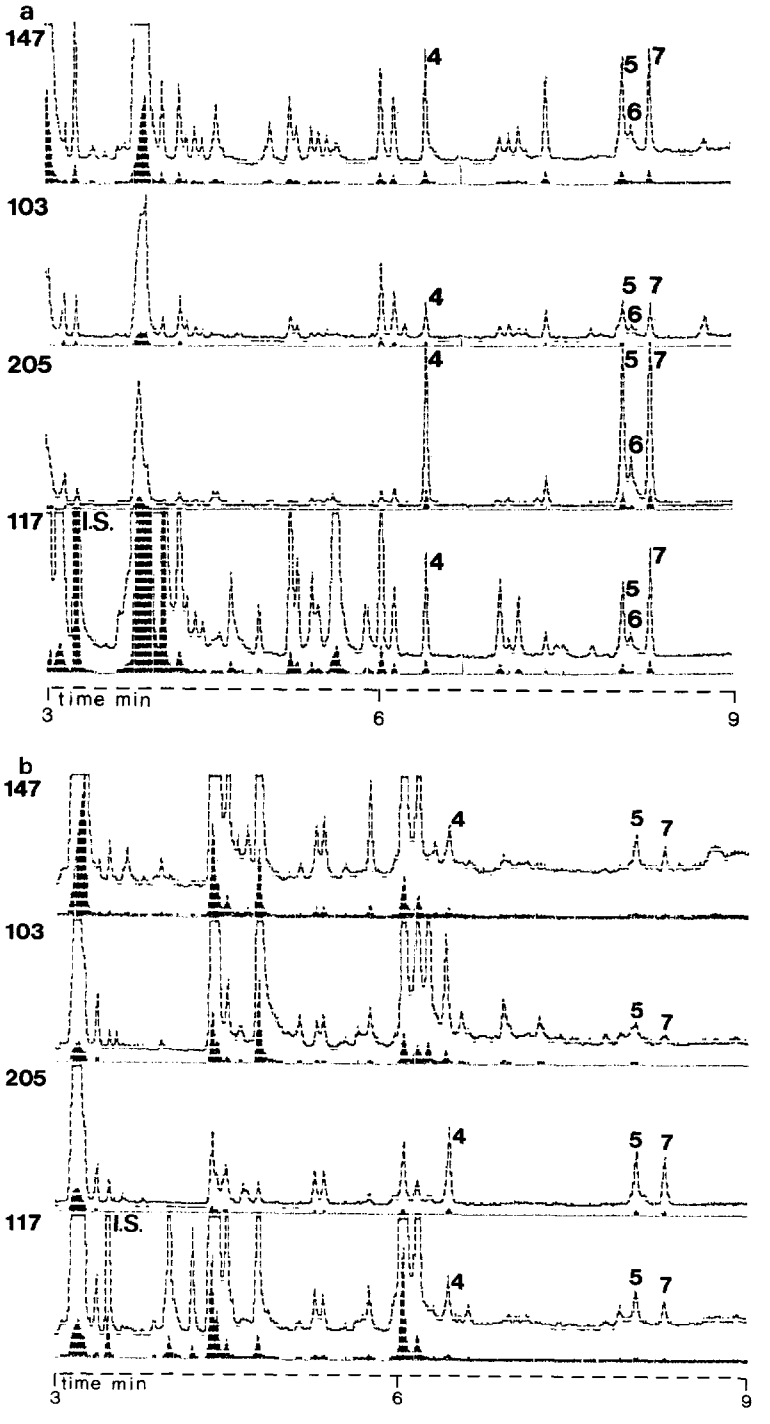

Fig. 3. GC–MS-SIM ion chromatograms of alkylglyceryl ethers of (a) human milk and (b) plasma samples. Peak numbers refer to constituents in Table I.

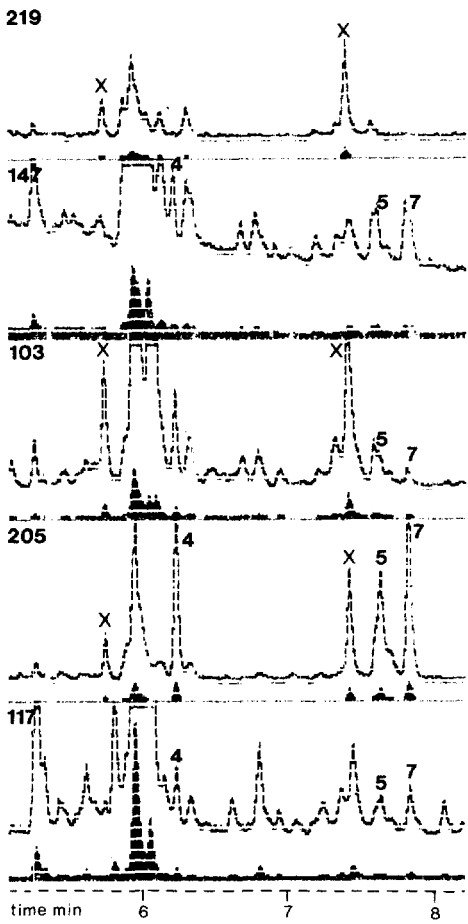

Fig. 4. GC–MS-SIM ion chromatogram of glyceryl ethers of human erythrocytes. Peak numbers refer to constituents in Table I.

Fig. 4. Based on their retention times and fragmentation properties, the unknown compounds (indicated by ×) may be 1-alkenylglyceryl ethers. This is supported by the fact that fragment *m/z* 219, which is formed by the elimination of the ethereal oxygen and the alkenyl chain linked to it [12] is also present. However, when total plasma samples of 1 ml were analysed, the concentrations of possible 1-alkenyl compounds were too low for quantification.

The reproducibility of the method, expressed as the mean relative standard deviation (R.S.D.), was 2.6% for human milk and 6.0% for plasma analyses when the total amount of alkylglyceryl ethers was quantified (Table II). The reproducibility for individual compounds was on average 3.6% and 8.4% (R.S.D.), respectively. Pooled human milk with a fat content of 3.7% contained

TABLE II

REPRODUCIBILITY OF THE METHOD IN HUMAN MILK AND PLASMA ANALYSES

Standard addition, extraction, transesterification, silylation and GC–MS-SIM included; $n = 6$.

Compound[a]	Human milk (1 ml)		Human plasma (1 ml)	
	Mean (μg/ml)	R.S.D. (%)	Mean (μg/ml)	R.S.D. (%)
4	12.7	2.9	0.75	8.1
5	12.2	2.4	0.42	9.3
6	3.0	6.0	–	–
7	14.6	3.0	0.37	7.7
	Mean	3.6	Mean	8.4
Total amount	42.5	2.6	1.54	6.0

[a]Numbering as in Table I and Figs. 2–4.

42.5 μg/ml of alkylglyceryl ethers, representing 0.11% of total lipids, a finding which corresponds to the level reported earlier [9]. The reproducibility of plasma alkylglyceryl ethers was determined at one of the lowest concentrations found in these experiments. Considerable quantitative variation (0.6–9.0 μg/ml) was found when the plasma alkylglyceryl ethers of six different persons were determined.

In conclusion, the GC–MS of bis-TMS derivatives of major alkylglyceryl ethers leads to specific fragmentation with a base peak of m/z 205. The results show that major alkylglyceryl ethers in human milk and plasma samples (1.0 ml) can be reliably quantified by the GC–MS-SIM technique without laborious isolation and purification steps.

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