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Gas chromatographic determination of isoprenoid alkylglycerol diethers in archaebacterial cultures and environmental samples

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ABSTRACT

A method has been developed for the determination of dialkylglycerol ethers in sedimentary samples using gas chromatography (GC) and GC–mass spectrometry (GS–MS). The method includes a fractionation procedure based on solvent Soxhlet extraction, acidic hydrolysis and column chromatography clean-up in which the dialkylglycerol ethers are separated in an alcohol fraction. GC or GC–MS analysis of these fractions after trimethylsilyl ether derivatization has shown no coelutions of the dialkylglycerols and other polar products present in the extracts, allowing their determination even in samples where they are only minor constituents of the total lipid mixtures. Examples of application to the determination of halophiles, methanogens and sedimentary environments containing residues of these archaebacteria are given.

INTRODUCTION

Archaebacteria represent a well defined third line of evolutionary descent different from other prokaryotes and eukaryotes. These phenotypically diverse prokaryotes show characteristics such as cell walls containing ether-linked isoprenoid lipids [1] instead of the ester-linked phospholipid fatty acids found in eubacteria. Some archaebacteria, *e.g.* the methanogens, are ubiquitous in most anoxic environments and are responsible for the generation of methane, the end-product of about 50% of anaerobically degraded organic carbon [2,3]. Other archaebacteria, *e.g.* the halophiles and the thermoacidophiles, occur in restricted environments such as extreme hypersaline habitats [4] or acyclic hot springs [5].

The isolation of these organisms in pure cultures has given a considerable understanding of their morphological and metabolic characteristics but knowledge of their biogeochemical roles depends on the feasibility of biomass measurements in recent and ancient sedimentary environments. The specific membrane composition of archaebacteria points to the use of isoprenoid dialkylglycerol ethers, namely di-O-phytanylglycerol, as biomass estimators.

Diverse methods based on high-performance liquid chromatography (HPLC) have been proposed for the determination of these dialkylglycerol ethers [6–8]. The low molar ultraviolet absorptivity of these compounds is avoided by using Fourier transform infrared spectroscopy [6,7] or by derivatization to *p*-nitrobenzoates [8]. These methods have been tested with lipid extracts from pure cultures [6,7] but only one has effectively been used for the analysis of environmental samples [8].

Unfortunately, the application of these HPLC methods to environmental samples is limited by several detection-related problems. First, neither ultraviolet nor infrared detectors are specific for dialkyl-

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glycerol ethers. Second, the procedures for the formation of ultraviolet chromophore ester derivatives are general for most hydroxy-substituted molecules which may give rise to serious interference problems. The lack of simple methods for the structural determination of the HPLC-eluted peaks is a major disadvantage in these analyses.

These difficulties have led to the use of gas chromatography (GC) for the determination of these compounds, and especially GC coupled to mass spectrometry (GC–MS). GC methods have usually been applied after cleavage of the ether linkages using boron trichloride [9] or hydroiodic acid [10,11]. Subsequent analysis of the resulting moieties, either as halogenated hydrocarbons or after the formation of other derivatives, allows the identification of the original structures from the molar proportion of the cleaved products. These procedures, developed as a consequence of the low volatility of many polyalkylglycerol ethers, have been successfully applied to the analysis of thin-layer chromatography fractionated mixtures obtained from pure cultures. Obviously, these cleavage methods are not useful for the direct analysis of complex mixtures such as the extracts resulting from environmental samples.

This paper reports the development of a GC method for the determination of entire dialkylglycerol ethers, namely di-O-phytanylglycerol and O-sesterpanyl-O-phytanylglycerol, in sedimentary environments. This method uses a clean-up procedure, essentially hydrolysis and separation by column chromatography, for the isolation of the neutral hydroxy-substituted lipid fraction. These alcohols are subsequently analysed using high-temperature capillary columns. The method is applicable to the identification of methanogenic and halophilic archaebacterial inputs in sediments. Examples illustrating the suitability of the method for the determination of these dialkylglycerol ethers in representative environments containing these two types of microorganisms are given.

EXPERIMENTAL

Materials

Pestipur grade *n*-hexane and methanol were purchased from SDS (Peypin, France). Resi-analyzed grade dichloromethane and chloroform were from Baker (Phillipsburg, NJ, USA). Analytical-reagent grade acetone was from Carlo Erba (Milan, Italy). Analytical-reagent grade hydrochloric acid (25%), neutral silica gel (Kieselgel 40, 70–230 mesh) and alumina (aluminum oxide 90 active, 70–230 mesh) were from Merck (Darmstadt, Germany). Potassium hydroxide was purchased from Fluka Chemie (Buchs, Switzerland). Soxhlet thimbles were from Schleicher and Schuel (Dassel, Germany).

The potassium hydroxide was cleaned by sonication in dichloromethane. Silica gel, alumina and the Soxhlet thimbles were extracted with dichloromethane–methanol (2:1, v/v) in a Soxhlet apparatus for 24 h. After solvent evaporation, silica and alumina were heated for 12 h at 120 and 350°C, respectively. A total of 5% (w/w) of Milli-Q grade water was then added to the chromatographic adsorbents for deactivation.

The purity of the solvents was checked by concentrating 100 ml of solvent to 10 μl under vacuum for GC analysis. Blank requirements were as follows: splitless injection of 2.5 μl should result in chromatograms with no unresolved GC envelope and only very few peaks, representing up to 1 ng in terms of their flame ionization detector response. This threshold, under the above dilution factor, is equivalent to 0.08 ng/g when referred to 30 g of sediment.

Extraction of archaebacterial cultures

The cultures of halobacteria and methanogens were extracted in a Soxhlet apparatus with 150 ml of dichloromethane–methanol (2:1) for 18 h. The extract was evaporated under vacuum to 0.5 ml and hydrolysed by reflux with chloroform–methanol–concentrated hydrochloric acid (10:1:0.5); 2 ml of Milli-Q grade water were added to the mixture after 1 h of reflux. The alkylglycerols were recovered by extraction with chloroform (3 × 2 ml). The combined extracts were evaporated under vacuum almost to dryness and derivatized with N,O-bis(trimethylsilyl)trifluoroacetamide (BSFTA; 100 μl, 20 min, 200°C) before instrumental analysis.

Extraction and fractionation of sedimentary environmental samples

Before extraction the halite samples were grinded in a ring mill and the mud samples were freeze-dried. The lipids were Soxhlet-extracted with chloroform for 36 h. The extract was vacuum evaporat-

ed to about 1 ml and hydrolysed with 15 ml of 6% methanolic KOH for 12 h at room temperature. After digestion 15 ml of Milli-Q grade water were added and the solution was extracted with *n*-hexane (3 × 10 ml). The combined extracts were vacuum evaporated to about 0.5 ml and fractionated by column chromatography according to previously established methods [12]. A column filled with 0.8 g each of 5% water–deactivated alumina (top) and silica (bottom) was used. The alkylglycerols were collected in a third fraction of 6 ml of dichloromethane–methanol (4:1) after elution with 3 ml of *n*-hexane and 6 ml of *n*-hexane–dichloromethane (4:1). This alcohol fraction was evaporated under vacuum to a small volume and derivatized with BSTFA as described in the previous section.

Instrumental analysis

The samples were analysed by GC with a Hewlett-Packard Model 5890 Series II chromatograph equipped with a flame ionization detector and a splitless injector. Two fused-silica columns were used for separation: (1) 30 m × 0.32 mm I.D. SPB-5 (film thickness 0.25 μm) (Supelco, Bellefonte, PA, USA) and (2) 10 m × 0.25 I.D. OV-1 (film thickness 0.15 μm) high-temperature column (Rescom, Kortrijk, Belgium). The carrier gas was helium. The oven temperatures were (1) from 60 to 310°C at 6°C/min and (2) from 60 to 250°C at 10°C/min and from 250 to 350°C at 4°C/min. The injector and detector were maintained at 300 and 350°C, respectively. The injection was in the splitless mode (solvent, iso-octane, hot-needle technique) keeping the split valve closed for 40 s. Nitrogen was used as the make-up gas (flow-rate 30 ml/min). Detector gas flow-rates were: hydrogen 30 ml/min and air 300 ml/min.

Selected samples were analysed by GC–MS using a HP 5890 Series II gas chromatograph coupled to a HP 5970 mass-selective detector equipped with an HP 300 data system. Spectra were obtained in the electron-impact mode (70 eV), scanning between 50 and 600 mass units at 1.5 scans/s. The transfer line temperature was 320°C. The chromatographic conditions were the same as described earlier.

RESULTS AND DISCUSSION

The mass spectrum of the trimethylsilyl derivative of bis-O-phytanylglycerol, the target compound for methanogen biomass estimation [8], is shown in Fig. 1. The structures corresponding to the mass ions are given in Table I. The structural assignments have been performed by comparison with the mass spectra of a synthetic standard, bis-O-hexadecyl-*rac*-glycerol (Sigma, St. Louis, MO, USA), that has also been run as trimethylsilyl ether under the same instrumental conditions.

In both compounds the molecular ion and other related mass fragments such as M − CH_3, M − $CH_2OSi(CH_3)_3$ (see Table I) are very minor, and are of limited use for structural identification. The most prominent and informative ions correspond to the loss of C_nH_{2n+1}, namely m/z = M − $C_nH_{2n+1}OH$ and m/z = M − $C_nH_{2n+1}OCH_3$, and to the combination between alcoxy and trimethylsilyl groups, namely m/z = $C_nH_{2n+1}O$ + $Si(CH_3)_3$ − H. These are the mass fragments of choice for the identification of the compounds contained in the samples. Accordingly, the selection of wide mass scan ranges for the inclusion of the molecular ions are of limited use and may involve important losses in sensitivity.

The mass spectrum of bis-O-hexadecyltrimethylsilylglycerol shown in Fig. 1 is similar to that previously reported [13], which was obtained by packed-column GC–MS, although the intensity of the mass fragments >300 is higher in the mass spectrum of this study. Another difference between the two mass spectra concerns the m/z 130/133 ratio; m/z 130 predominates in Fig. 1 but m/z 133 was the base peak in the previous study. As a result of this, the m/z 133 ion was proposed instead of m/z 130 for dialkyltrimethylsilylglycerol monitoring [13] because m/z 130 is also the base peak of the 1-alkyl-2-acyltrimethylsilylglycerols [14]. Nevertheless, the mass spectra shown in Fig. 1 indicate that m/z 130 have also to be considered as bis-O-phytanyltrimethylsilylglycerol does not show a significant m/z 133 peak.

Bis-O-phytanylglycerol is the dominant compound in the alcohol fraction resulting from the application of the described experimental procedure to solvent extracts of halobacteria (Fig. 2A and B). Some of these halobacteria also contain O-sesterpanyl-O-phytanylglycerol that elutes in the same fraction (Fig. 2B). The analysis of this latter glycerol as the trimethylsilyl derivative requires the use of short capillary columns (about 10 m, column 2 in the experimental procedure). Bis-O-phytanylglycerol is

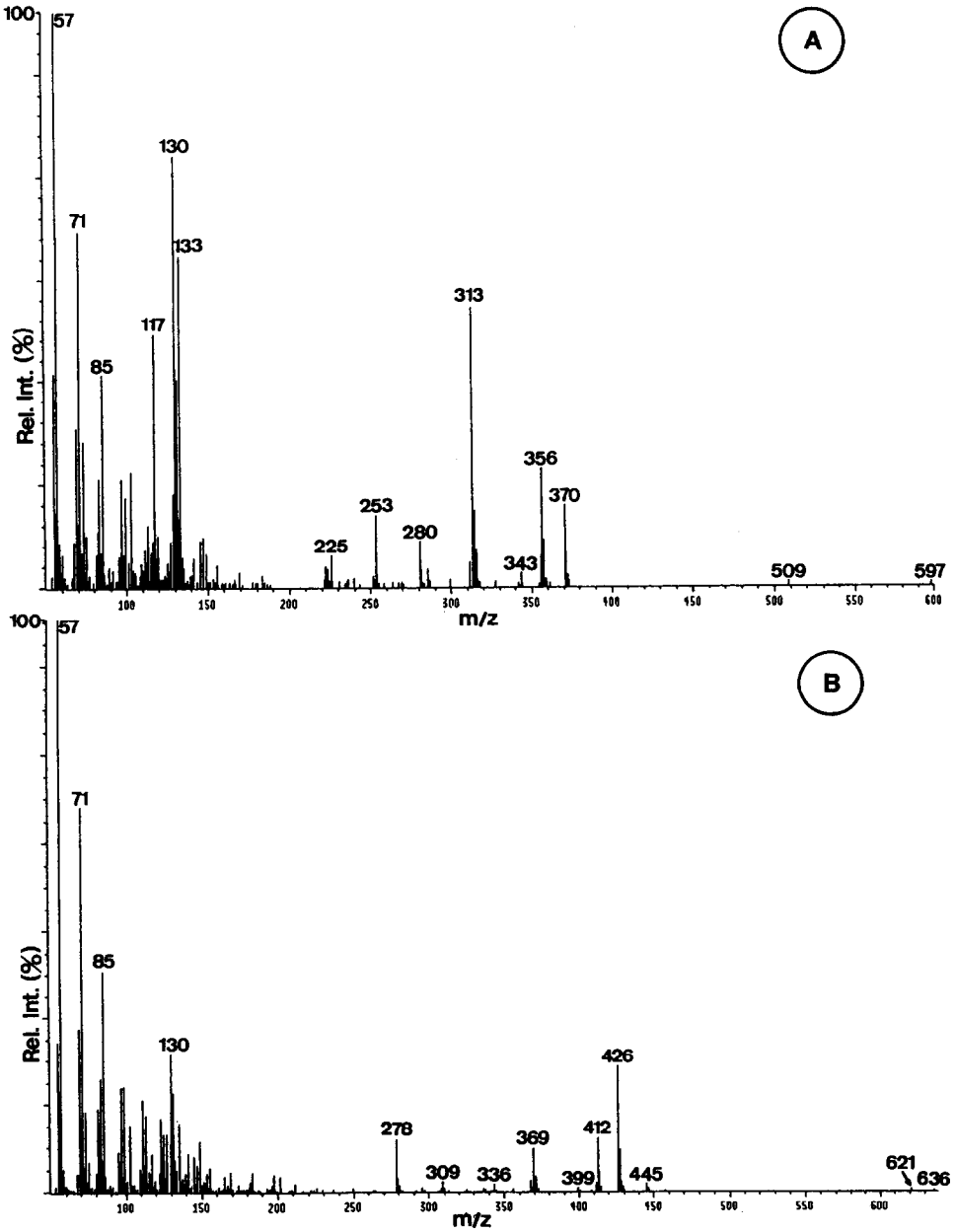

Fig. 1. Mass spectra of (A) bis-O-hexadecyltrimethylsilylglycerol and (B) bis-O-phytanyltrimethylsilylglycerol. Ion structure assignments in Table I.

also the major compound in the alcohol fraction of halite deposits (Fig. 2C) where it occurs together with other alcohols and O-sesterpanyl-O-phytanylglycerol.

Bis-O-phytanylglycerol is also the dominant compound in the equivalent alcohol fraction of methanogens such as *Methanosarcina* sp. (Fig. 3A). Conversely, this glycerol may be a minor lipid con-

TABLE I

MAJOR MASS FRAGMENTS OF THE MASS SPECTRA OF FIG. 1 CORRESPONDING TO THE TRIMETHYLSILYL (TMS) DERIVATIVES OF BIS-O-HEXADECYLGLYCEROL (16/16) AND BIS-O-PHYTANYLGLYCEROL (20/20)

Mass fragment		Ion structure
16/16 ($n=16$)	20/20 ($n=20$)	
Molecular groups		
612	724	M
597	709	M − CH_3
524[a]	636	M − $OSi(CH_3)_3$ + H
509	621	M − $CH_2OSi(CH_3)_3$
Loss of C_nH_{2n+1} groups		
389[a]	445	M − C_nH_{2n-1}
370	426	M − $C_nH_{2n+1}OH$
356	412	M − $C_nH_{2n+1}OCH_3$
C_nH_{2n+1} groups		
343	399	$H_{2n+1}C_nOCH_2O$ + $Si(CH_3)_3$ − H
313	369	$C_nH_{2n+1}O$ + $Si(CH_3)_3$ − H
280	336	$CH_2CHCH_2OC_nH_{2n+1}$ − 2H
253	309	$CH_2OC_nH_{2n+1}$ − 2H
225	281	C_nH_{2n+1}
223	279	C_nH_{2n-1}
222	278	C_nH_{2n-2}
TMS-glycerol groups		
133	133	$HOCHCH_2OSi(CH_3)_3$
130	130	$CH_2CHCH_2OSi(CH_3)_3$
117	117	$CH_2CH_2OSi(CH_3)_3$
Alkyl groups		
85	85	C_6H_{13}
71	71	C_5H_{11}
57	57	C_4H_9

[a] Not detected.

stituent in anoxic sediments in which methanogens are active, with the corresponding alcohol fraction being dominated by other products such as *n*-alkan-1-ols, *n*-alkandiols, sterols, hopanols, tetrahymanol and other triterpanols (Fig. 3B). Nevertheless, even in these instances no coelution between bis-O-phytanylglycerol and these other polar compounds has been observed in the GC traces, which allows an easy determination for biomass measurement.

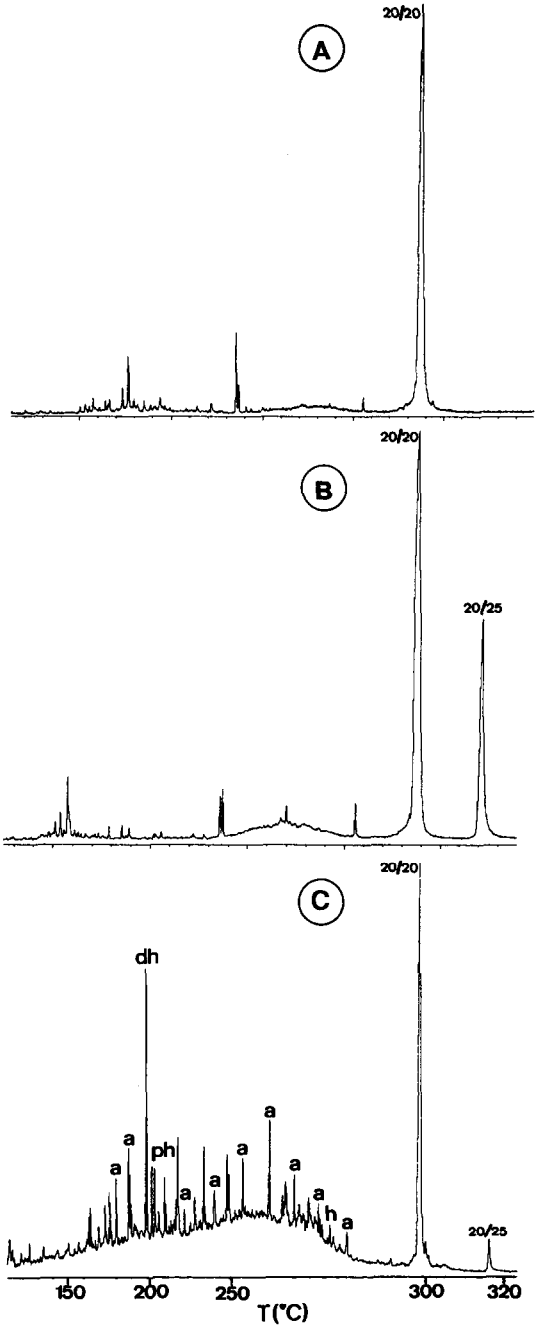

Fig. 2. Gas chromatograms of the trimethylsilyl-derivatized alcohol fraction of two halophile bacteria (A) Ma 2.38 [15] and (B) Ma 2.20 [15], and a halite deposit (C) Bonmatí, Santa Pola, Comunitat Valenciana, Spain. The instrumental conditions correspond to column 2 described under Experimental. 20/20 and 20/25 refer to bis-O-phytanylglycerol and O-sesterpanyl-O-phytanylglycerol, respectively. a = *n*-Alkan-1-ols; dh = dihydrophytol; ph = phytol; h = hopanols.

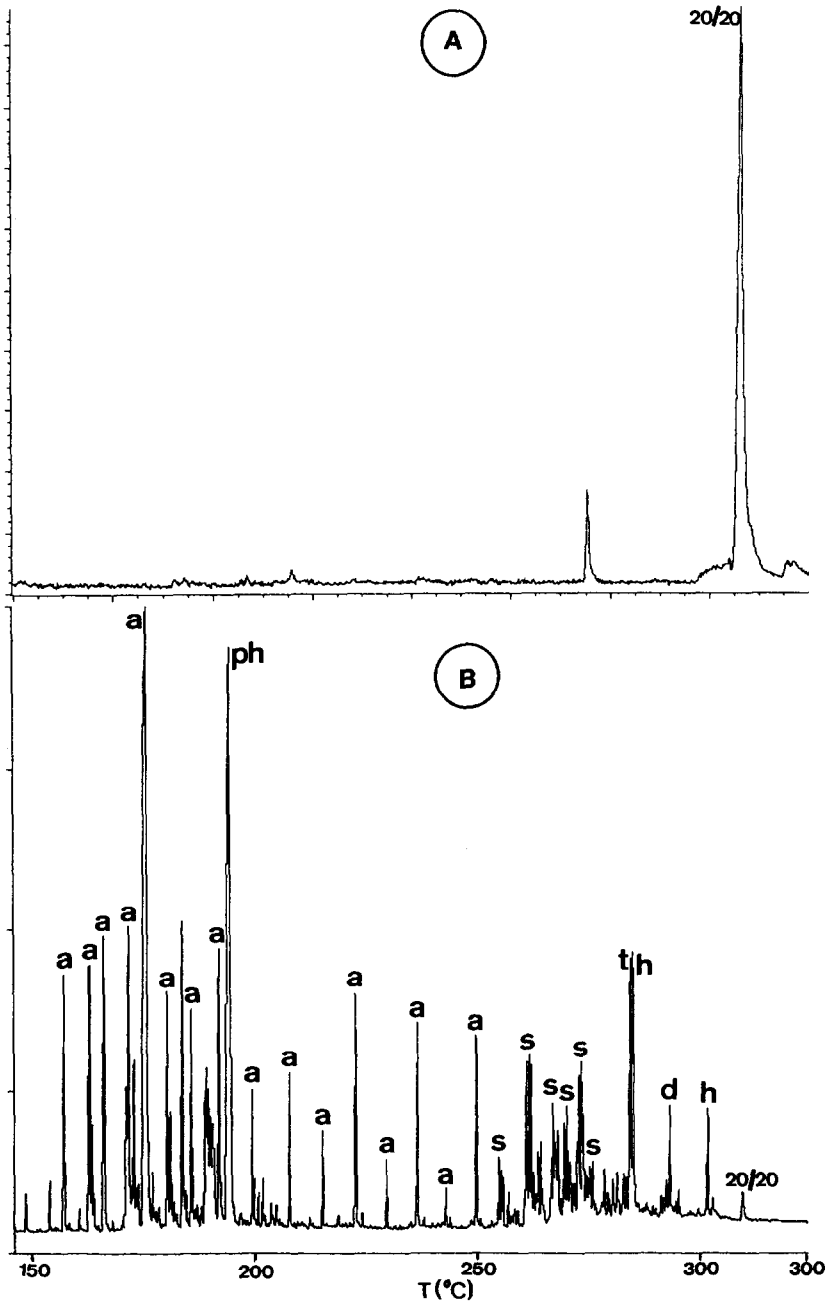

Fig. 3. Gas chromatograms of the alcohol fraction of (A) *Methanosarcina* sp. and (B) an anoxic sediment from Santa Olalla lagoon (Southwest Spain). The instrumental conditions correspond to column 1 described under Experimental. 20/20 refers to bis-O-phytanylglycerol. a = *n*-Alkan-1-ols; ph = phytol; d = *n*-alkandiols; s = sterols; h = hopanols; t = tetrahymanol.

CONCLUSIONS

Dialkylglycerols, namely di-O-phytanylglycerol, can be used for biomass estimation of halophilic and methanogenic archaebacteria. The method proposed here allows the GC or the GC–MS determination of these compounds in sedimentary samples even when they are only present as minor constituents in the total lipid mixtures. No coelutions between the sedimentary dialkylglycerols and other polar products have been detected after isolation of these compounds by the column chromatography clean-up procedure described in the method.

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