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Measurement of phospholipid fatty acids at picomolar concentrations in biofilms and deep subsurface sediments using gas chromatography and chemical ionization mass spectrometry

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Summary

Examination of ester-linked phospholipid fatty acids (PLFA) have provided a means to characterize the community structure of microbial assemblies. Attempts to analyze such acids at low picomolar levels in environmental samples by gas chromatography and chemical ionization mass spectrometry (CIMS) using positive or negative ion detection, showed that the limit of detection (LOD) was mainly dependent on the background levels of fatty acids, introduced as contaminants during the preparation of the samples. The lowest backgrounds were obtained using a procedure preparing methyl esters of fatty acids by a mild alkaline transesterification, followed by analysis with positive ion CIMS. The blank samples of this procedure were three times as low as and less variable than the background values obtained using a procedure involving preparation of pentafluorobenzyl (PFB)-esters for negative ion CIMS. The LOD (background values + three times the standard deviation) of the positive ion CIMS method was approximately 30 pmole. The coefficient of variation ($n = 5$) for determining the proportions and amounts of PLFA in microgram amounts of *Escherichia coli* cells using the developed CIMS method were between 2–30%, depending of the amount of extracted cells and the fatty acids analyzed. The recovery of the PLFA was between 63% and 100%. The positive ion CIMS method was used to determine PLFA in deep subsurface samples with a LOD of approximately 2–3 pmoles/g dry wt corresponding to a fatty acid content of 10^5–10^6 bacterial cells/g dry wt. Measurement of PLFA in *Pseudomonas atlantica* grown in a Fowler cell adhesion module demonstrated differences in membrane composition between free and attached cells in biofilms.

Key words: Biofilm; Deep subsurface sediment; Detection limit; Fatty acid biomarker; Quantification

Introduction

Phospholipids are the major lipid components in the membrane of bacteria [1]. The

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composition of these lipids varies widely among different taxa [2] and isolation of bacteria from natural environments has shown that subsets of microbial communities contain specific fatty acids that can be utilized as biomarkers for the subset, e.g. [3–5]. Quantitative recovery and analysis of biomarker fatty acids have provided a reproducible and sensitive means to define the community structure of microbial communities in soils and sediments [6]. Furthermore, as major components of the cell membrane, the composition of fatty acids in phospholipids can influence several important physiological processes, e.g., energy generation and transport systems [7].

The most common methods to analyze phospholipid fatty acids (PLFA) involve lipid extractions with organic solvents, isolation of phospholipids by silicic acid chromatography, esterification of fatty acids by methanolysis, and separation of methyl esters by capillary gas chromatography (GC) using a flame ionization detector (FID) [6]. The detection limit of this method is limited by the sensitivity of the FID detector, which is about 2×10^{-12} mole for palmitic acid [8].

However, for the analysis of samples with very low biomass (e.g., subsurface soils) or for the analysis of organisms in specific microhabitats (e.g., biofilms), more sensitive methods are needed. Such methods have been developed using mass spectrometric (MS) detection with chemical ionization (CI) and selected ion monitoring (SIM) detection of preselected ions [9]. The sensitivity of these techniques for the detection of fatty acids, using pentafluorobenzyl (PFB) esters and negative ion CI, is approximately 2×10^{-15} mole [10]. This methodology has been succesfully applied to the detection of specific bacterial fatty acids, such as tuberculostearic acid and beta-hydroxy myristic acid, in complex biological material [10, 11]. However, problems were encountered when the procedure was applied to analyses of PLFA profiles in environmental samples, containing picomolar levels of PLFA, due to a high background level of fatty acid contaminants introduced during the preparation of the samples.

In the studies reported in this paper, these problems were minimized by developing a procedure to analyze PLFA in environmental samples at picomolar levels based on the analysis of fatty acid methyl esters using SIM detection with positive ion CIMS.

Materials and Methods

Reagents

Potassium hydroxide (analytical grade) was from Malinckrodt, Paris, KY, and NaOH was from Malinckrodt (analytical grade) or from Aldrich Chemie (99.99%) Steinheim, Germany. The 2, 3, 4, 5, 6-pentafluorobenzyl-bromide (PFB-Br) (99%) was from Sigma, St. Louis, MO, or from Aldrich Chemie (99%). Standards of fatty acid methyl esters (FAME) were obtained from NuChek Prep, Inc., Elysian, MN, and from Supelco Inc., Bellefonte, PA.

Glassware and solvents

All glass was washed in a 10% (v/v) Micro cleaner solution (American Scientific Products, McGaw Park, IL), rinsed 10 times with hot tap water and 10 times with nanopure water from a Barnsted water cleaning system. The glass was then heated overnight in a muffle furnace at 450 °C. Teflon-lined caps for test tubes were washed as above, dried at 105 °C, and washed with acetone. Organic solvents were of GC 2^{TM} grade

from Burdick and Jackson (American Scientific Products, McGaw Park, IL) and were redistilled before use. Nanopure water used in the analytical procedures was filtered through a 0.22-μm nylon filter (Gelman Sciences Inc., Ann Arbor, MI) and extracted twice with chloroform (ratio chloroform : water, 1 : 2) to remove traces of impurities. Nitrogen gas used for evaporating organic solvents was dried and purified with a GC gas purifier (Alltech Associates, Applied Science Lab, Deerfield, IL).

Biofilm experiment

Pseudomonas atlantica (ATCC 19262) were cultured in a radial flow adhesion measurement growth chamber (Fowler Cell) [12]. Briefly, the cells and culture medium were pumped through the Fowler Cell using a peristaltic pump with a 500-ml Erlenmeyer flask used as reservoir. *P. atlantica* cultures were intially grown on Difco marine broth (28 g/l, w/v) to stationary phase and then the growth medium was changed by pouring off the spent medium and replacing it with a defined minimal marine medium (30 g/l, Instant Ocean (Aquarium Systems, Mentor, OH); Tris (100 mM); NH_4Cl (19 mM); $MgCl_2$ (2 mM); K_2HPO_4 (0.5 mM); $FeCl_3$ (4 μM); riboflavin (0.66 mM); thiamine (0.74 mM); and galactose (0.1% w/v)). The cultures were grown to late log phase again before harvesting the bulk phase (nonattached cells) and removal of the stainless steel surface (series 316, 100 mm diameter, 3 mm thick) from the Fowler cell. Bulk phase cultures were harvested by centrifugation (10000$\times g$, 30 min, 4 °C) and lyophilized. The steel plates were lyophilized and samples of attached cells were scraped off the plates from sectors at a radius between 2–5 cm.

Deep subsurface sediments

Samples of subsurface sediment at a depth of 203 m were recovered from a core taken in the deep subsurface science program of the Department of Energy and Savannah River Laboratories. Great care was taken to prevent contamination with surface microorganisms and drilling muds [13]. The samples were extruded and handled aseptically under a stream of nitrogen in anaerobic chambers, the outside layer of each core was pared and the center section utilized, the samples were frozen in sterile bottles and sent to the laboratory. In the laboratory the samples were lyophilized and then extracted as described below.

Precision and recovery experiment with E. coli

To check the precision and recovery of the method to determine the composition and amount of PLFA in bacteria, cells of *E. coli* strain B (ATCC 11303) (Sigma, St. Louis, MO) were analyzed at various concentrations. Ten mg of lyophilized cells were dispersed in 10.0 ml of H_2O and repeatedly diluted to give solutions of 1.00, 0.25, 0.125, 0.063, 0.031, and 0.015 mg *E. coli* cells/ml. Aliquots of 1 ml were transferred to test tubes; chloroform, water, and methanol were added, and the samples were extracted as described below.

Calibration curves

Calibration standards were made with known ratios of bacterial fatty acids relative to the internal standard methyl nonadecanoate (19:0). The fatty acids were: a13:0, 13:0, i14:0, a15:0, 15:0, i16:0, 16:1ω7*c*, 16:1ω7*t*, 16:0, 17:0, 18:1ω9*c*, 18:1ω7*c*, 18:1ω7*t*, and 18:0.

The standards contained fatty acids at six different concentrations ranging from 0.2 to 50 pmole/μl with an internal standard (IS) amount of 5 pmole/μl.

Lipid extractions

Lyophilized samples of bacteria were extracted in 15 ml test-tubes (teflon-lined caps) with 5 ml of a single phase mixture consisting of chloroform : methanol : water (1 : 2 : 0.8, v/v/v) according to Bligh and Dyer (BD) [14]. After sonication for 1 min, the samples were extracted for 2 hours. The samples were then centrifuged in a bench-top centrifuge (approximately $4000 \times g$) and the extracts were transferred to a new set of test tubes. The residue was washed with 2 volumes of 1 ml of the one-phase BD mixture and the combined extracts were separated into two phases by adding 1.8 ml of chloroform and 1.8 ml of water. After vortexing for two minutes and centrifugation ($4000 \times g$), the aqueous layer was discarded and the organic phase evaporated under N_2 ($< 40\,^{\circ}C$).

Samples from the deep subsurface sediments were extracted using a modified procedure requiring larger volumes of solvents. 20–25 g of lyophilized sediments were extracted in 250-ml centrifuge bottles by adding 100 ml of the one-phase BD mixture. After mixing and sonication for 1 min, the samples were extracted at room temperature over night. The liquid extractants were transferred to 250 ml separatory funnels and the phases were separated by adding one volume of water and chloroform. The organic phase was dried under vacuum using a rotary evaporator.

Silicic acid chromatography

Plugs of glass wool, preextracted with chloroform and methanol and heated to 450 °C overnight, were placed in a small (4 ml) 'champagne' column (Supelco, Bellefonte, PA) and 100 mg of silicic acid (Unisil, 100–200 mesh, Clarkson, Williamsport, PA) was added in a slurry of chloroform. The silicic acid had been precleaned by washing in chloroform, methanol and chloroform, and conditioned by heating to 105 °C for 1 hour. The samples were applied onto the columns with 3×50 μl of chloroform using disposable glass micro-pipettes (100 μl, Corning Pyrex, American Scientific Products), and the extracts were fractionated into neutral, glyco- and phospholipids by eluting with 1 ml of chloroform, 4 ml of acetone, and 1 ml of methanol. The methanol fractions containing phospholipids were recovered in test tubes and dried under N_2 ($< 40\,^{\circ}C$).

Preparation of FAME

Samples were transferred with 3×50 μl chloroform to 0.3-ml microvials capped with Tuf-Bond Teflon-lined septa (Pierce, Rockford, IL). Methyl nonadecanoate (typically 50 pmole) was added as internal standard and the solvents were evaporated under N_2. Transesterification was performed by a mild alkaline methanolysis with a procedure modified from [15] by adding 25 μl of methanol : toluene (1 : 1, v/v) and 25 μl 0.2M solution of KOH in methanol. The potassium hydroxide had been purified by extracting the KOH pellets with hexane (typically 1 g of pellets were extracted overnight with 10 ml of hexane). The microvials were heated at 37 °C for 15 min, and after cooling, the pH was adjusted to 7 by adding 10 μl of 1 M acetic acid. The methyl esters were extracted with 2×50 μl of hexane : chloroform (4 : 1, v/v) and the organic solvents were

evaporated under N_2. All microliter volumes of solvents and reagents were measured with glass micro-pipettes (cleaned in an oven at 450 °C, overnight).

Preparation of PFB esters

The phospholipid fractions were dried under N_2 and subjected to alkaline hydrolysis in 1 ml of 15% NaOH in a 1:1 (v/v) mixture of methanol:water at 80 °C for 30 min [10]. After cooling, unsaponified lipids were extracted by adding 0.5 ml of H_2O and 1.5 ml of hexane. Free fatty acids were extracted with 2×2 ml of methylene chloride after acidification with 0.5 ml of 25% HCl in water. The free fatty acids were dissolved in 30 μl of acetonitrile and derivatized with 10 μl of 35% PFB-Br in acetonitrile and 10 μl triethylamine as previously described [10]. In some cases the formed PFB esters were purified by extraction with a 1 M phosphate buffer (pH 7) and chromatography on a short silicic acid column [16].

The alkaline hydrolysis was also performed with smaller volumes of solvents and reagents than described above by transferring the polar lipid samples from the test tubes to microvials using 3 volumes of chloroform. Hydrolysis was accomplished with 25 μl of 15% NaOH, and unsaponified material was extracted with 25 μl of H_2O and 50 μl of hexane. Free fatty acids were extracted with 50 μl of 2 M HCl and 50 μl methylene chloride and the free fatty acids were derivatized with PFB-Br as described above.

Gas chromatography

Analysis using flame ionization detection (FID) was performed using a Hewlett Packard 5880 GC. The column was a 50 m×0.2 mm i.d. fused silica column coated with a nonpolar cross-linked methyl silicone phase (HP-1, Hewlett Packard, Palo Alto, CA). The initial oven temperature was 80 °C for 1 min, then increased at 10 °C per min to 150 °C, then at 3 °C per min to 240 °C, and finally at 5 °C per min to 300 °C. Hydrogen at a linear gas flow rate of 80 cm per second was used as the carrier gas. Injections were performed in the splitless mode. The GC data were acquired and manipulated by a Nelson data system (Nelson Analytical Inc., Paramus, NJ).

Gas chromatography/mass spectrometry (GC/MS)

GC/MS analyses were performed on a VG TRIO-3 GC/MS/MS instrument equipped with a HP5890 GC. The GC conditions were those described above but helium was used as the carrier gas at a linear flow rate of 40 cm per second. Isobutane (purity 99.99%) was used as the reagent gas in CI. The source temperature was 150 °C.

The MS was set up for SIM using six consecutive sets comprising different ions, characteristic of bacterial fatty acids. The time events and ions in positive and negative ion CIMS are presented in Table 1.

Fatty acid nomenclature

Fatty acids are designated as the total number of carbon atoms followed by: the number of double bonds. The degree of unsaturation is followed by a number indicating the position of the double bonds closest to the aliphatic end of the molecule, and by a *c* for cis and a *t* for trans. The prefixes 'i' and 'a' refer to iso and anteiso branching respectively. Cyclopropyl fatty acids are designated by using the prefix 'cy'.

TABLE 1

SETS OF ION PARAMETERS USED IN SELECTED ION MONITORING (SIM) OF BACTERIAL FATTY ACIDS PERFORMING EITHER POSITIVE OR NEGATIVE ION CHEMICAL IONIZATION (CI) MASS SPECTROMETRY

Group	Fatty acids	Positive ion CI[a]		Negative ion CI[b]	
		Retention time (min)	Monitored ions (m/z)	Retention time (min)	Monitored ions (m/z)
1	11:0	10:00 – 17:00	201.2	20:00 – 27:50	185.2
	12:0		215.2		199.2
	13:0		229.3		213.3
	14:0		243.3		227.3
2	i15:0	17:05 – 19:00	257.3	27:55 – 29:30	241.3
	a15:0		257.3		241.3
	15:0		257.3		241.3
3	16:1	19:05 – 20:50	269.3	29:35 – 31:30	253.3
	i16:0		271.3		255.3
	16:0		271.3		255.3
4	cy17:0	20:55 – 22:40	283.3	31:35 – 33:30	267.3
	i17:0		285.3		269.3
	a17:0		285.3		269.3
	17:0		285.3		269.3
5	18:2	22:45 – 24:00	295.3	33:35 – 35:00	279.3
	18:1		297.3		281.3
	18:0		299.3		283.3
6	cy19:0	24:05 – 29:00	311.4	35:05 – 39:00	295.4
	19:0		313.4		297.4
	20:0		327.4		311.4

[a] Analysis of fatty acid methyl esters (FAME). Monitored ions were the protonated molecular ions $(M+H)^+$, which are the major peak in the positive ion CI spectra of FAME [17].
[b] Analysis of pentafluorobenzyl(PFB)-esters. Monitored ions were the carboxylate anions $(M-H_2CC_6F_5)^-$, which are the base peak in the negative ion CI spectra of these derivatives [10].

Statistics

Data from the precision experiments were analyzed by the Kruskal–Wallis one-way analysis of variance, using the SYSTAT subprogram NPAR.

Results

Sensitivity

The sensitivity of positive ion CIMS for detecting fatty acids, measured as the injected amount of 16:0 methyl ester, given a signal to noise ratio of 10:1, was 0.01 pmole (Table 2). This sensitivity was approximately 50 times lower than that obtained analyzing the PFB esters by negative ion CIMS.

Background levels

Blank samples from the developed procedure for preparing FAME's for positive ion

TABLE 2

DETECTION LIMITS OF PHOSPHOLIPID FATTY ACID ANALYSIS USING CHEMICAL IONIZATION (CI) MASS SPECTROMETRY (MS) AND SELECTED ION MONITORING (SIM) WITH EITHER POSITIVE OR NEGATIVE ION DETECTION

	Positive ion CI	Negative ion CI
Derivatives[a]	FAME	PFB-ester
Sensitivity (pmole)[b]	0.01	0.0002
Background (pmole)[c]	13 (6.6)	35 (26)
Limit of detection[d]	30	110
Limit of quantification[e]	80	290

[a] FAME, fatty acid methyl esters; PFB, pentafluorobenzyl ester.
[b] Injected amount of 16:0 given a signal-to-noise ratio of 10:1, measured at m/z 271.3 for FAME 16:0 and at m/z 255.3 for PFB 16:0.
[c] Total amount, mean (SD, $n = 10$) of fatty acids in blank samples run through the whole analytical procedure.
[d] Defined as background value + three times SD [18].
[e] Defined as background value + 10 times SD [18].

CIMS contained a total amount of 4–20 pmoles of fatty acids, which was 30–100 times lower than the background in our standard procedure for FAME analysis [cf. 8]. The major components in the background from the developed procedure were 16:0 (approximately 45 mole %), 18:1ω9*c* (24 mole %) and 18:0 (21 mole %).

The background level of the positive ion CIMS method was also lower and less variable than the background of the negative CI method (Table 2). The major fatty acids in the blank samples from the procedure for preparing the PFB-esters of bacterial PLFA were 16:0 (41 mole %), 14:0 (14 mole %), 18:0 (8 mole %), 12:0 (7 mole %), 15:0 (7 mole %), 16:1ω7*c* (7 mole %), 18:1ω9*c* (7 mole %). Experiments showed that a major part of these fatty acids was introduced as contaminants during the alkaline hydrolysis of the polar lipids. Attempts to clean-up this step by performing the hydrolysis in microvials with microliter amounts of solvents, preextracting the NaOH pellets with hexane, and by using different brands of NaOH did not bring down the background level to that of the positive ion CIMS method. Other attempts to further clean-up the PFB procedure included the use of PFB reagents from different manufacturers, and purification of the PFB-derivatives by a phosphate buffer wash and silicic acid chromatography.

Limits of detection and quantification

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated to be approximately three times lower for the positive ion CIMS method than for the negative ion CIMS method, using the definitions LOD = mean value of the background + three times the standard deviation, LOQ = mean value of the background + 10 times the standard deviation [18] (Table 2).

Calibration curves

The linearity of the calibration curves for determining fatty acids with positive ion

CIMS was satisfactory. The correlation coefficient (r) for the regression lines of the different fatty acids varied between 0.997 and 0.999. Regression data from four of the calibration curves of the bacterial fatty acid are reported in Table 3.

Precision

The precision of the developed positive ion CIMS method was examined by analysis of PLFA in microgram amounts of *E. coli* cells. The composition of PLFA in these samples showed relatively good agreement with the results obtained analyzing milligram amounts of cells (Table 4). However, there were some minor, but statistically sig-

TABLE 3

REGRESSION DATA FOR CALIBRATION CURVES OF SOME BACTERIAL FATTY ACIDS USING SELECTED ION MONITORING AND CHEMICAL IONIZATION MASS SPECTROMETRY

Fatty acids	n	slope	intercept	t	P	r
a13:0	6	1.249	0.016	34.4	<0.001	0.998
i16:0	6	0.712	0.036	25.6	<0.001	0.997
17:0	6	0.590	0.023	23.9	<0.001	0.999
18:1ω7c	6	0.402	0.014	41.8	<0.001	0.999

TABLE 4

PRECISION AND ACCURACY FOR THE ANALYSIS OF PHOSPHOLIPID FATTY ACID COMPOSITION IN VARIOUS AMOUNTS OF *E. COLI* CELLS. DATA EXPRESSED IN MOLE% ($n=5$)

Fatty acids	*E. coli* cells							
	0.025 mg[a]		0.125 mg[a]		1.00 mg[b]		50.0 mg[c]	
	mean	(SD)	mean	(SD)	mean	(SD)	mean	(SD)
14:0**[d]	2.20	(0.49)	1.95	(0.43)	3.86	(0.51)	3.99	(0.53)
15:0	1.45	(0.40)	1.43	(0.37)	1.53	(0.06)	1.46	(0.06)
16:1ω7c**	0.40	(0.05)	1.73	(0.50)	0.52	(0.09)	0.46	(0.02)
16:0*	37.50	(1.20)	41.92	(2.56)	43.29	(1.04)	42.05	(0.52)
cy17:0**	26.89	(0.49)	23.44	(0.48)	25.59	(0.60)	25.70	(0.24)
17:0**	1.07	(0.10)	1.08	(0.05)	0.77	(0.01)	0.73	(0.01)
18:1ω7c*	3.20	(0.31)	2.77	(0.04)	2.68	(0.08)	2.57	(0.07)
18:0**	2.17	(0.75)	3.52	(0.85)	0.82	(0.09)	0.75	(0.03)
cy19:0*	25.12	(1.57)	22.26	(2.72)	20.94	(0.99)	22.29	(0.52)
Amount								
(pmoles)	935	(129)	6240	(343)	62200	(3360)	-	
(pmoles/mg)	37400	(5160)	49900	(270)	62200	(3360)	61800	(1284)

[a] Analysis using mass spectrometric detection (CI^+).
[b] Analysis using flame ionization detector (FID).
[c] Values obtained using a standard analytical method [8].
[d] Indicate significant differences between means by Kruskal-Wallis one-way analysis of variance (*, $0.01 \leq P < 0.05$; **, $0.001 \leq P < 0.01$).

nificant, differences in the PLFA profiles between different amounts of extracted cells.

The coefficient of variation (CV) ($n=5$) for the determination of the amount (pmoles) of PLFA in these samples was between 2 and 30% (Fig. 1). The highest variation was found for the analysis of fatty acids in the samples containing the lowest amount of *E. coli* cells. Furthermore, the CV was higher for short chain fatty acids (14 : 0 and 15 : 0) and for fatty acids present in low proportions (18 : 1ω7*c*) than for long chain fatty acids and the more common acids.

Recovery

The recovery study showed that the CV for quantifying the total amount of PLFA in microgram amounts of *E. coli* cells varied between 3 and 10% ($n=3$). The equation for the regression line of the recovery curve was $Y=53X-564$ and the correlation coefficient 0.992 ($t=14.0$, $P<0.001$) (Fig. 2). The recovery of PLFA in the samples, compared to the concentration of PLFA determined in 50 mg of *E. coli* with our standard procedure (Table 4), was 63% for the 16 μg sample, 70% for 31 μg, 77% for 63 μg, 84% for 125 μg, 92% for 250 μg, 100% for 500 μg, and 100% for the 1000 μg sample of *E. coli*.

Applications

Samples from a deep subsurface sediment were analyzed to demonstrate the application of the positive CIMS method for the analysis of PLFA biomarkers in environmental samples containing low amount of microorganisms (Fig. 3). The total amount of PLFA in these samples were 125 pmole (mean) which were above the LOQ of the method (Table 4). Expressed by weight of the analyzed samples, the concentration of PLFA was 5.8 pmoles/g dry wt (CV=12%, $n=3$).

Analysis of PLFA in cells of *P. atlantica* grown in the Fowler Cell adhesion module

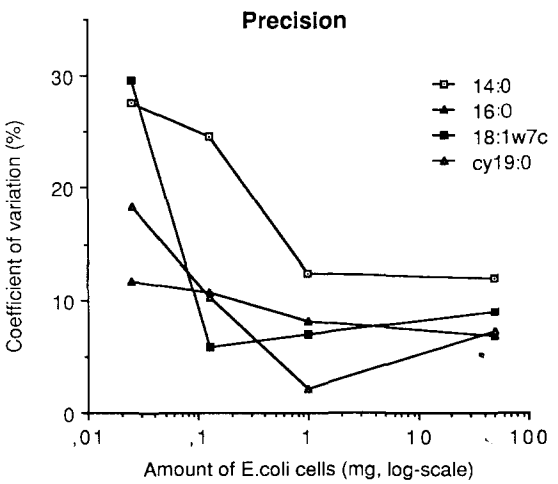

Fig. 1. Precision, expressed as coefficient of variation ($n=5$), for the determination of the concentration (pmoles/g dry wt) of some phospholipid fatty acids in *Escherichia coli* cells, as a function of the amount of extracted bacterial cells. The fatty acids were analyzed as methyl esters using gas chromatography and positive ion chemical ionization mass spectrometry.

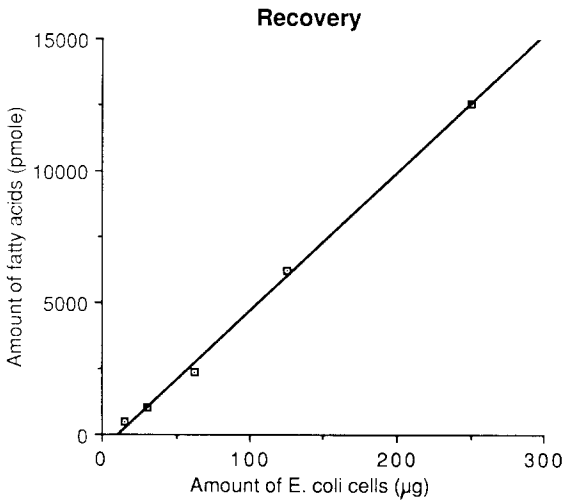

Fig. 2. Recovery curve for analysis of the total amount of phospholipid fatty acids from *E. coli* cells. Bars indicate 1 standard deviation ($n=3$). The fatty acids were analyzed as methyl esters using gas chromatography and positive ion chemical ionization mass spectrometry.

showed that there were differences in the composition of phospholipids between free and attached cells (Table 5). Attached cells had higher proportions of saturated fatty acids and *trans* monounsaturated fatty acids, but lower proportions of *cis* unsaturated fatty acids in their phospholipids than free cells. The total amount of PLFA in these samples was between 500–2000 pmoles.

Discussion

Attempts to analyze ester-linked PLFA at low picomolar levels by GC and MS revealed the major factor influencing the limit of detection was a high background of contaminating fatty acids introduced during sample preparation. Acceptable blanks were achieved by direct transesterification of the acylated fatty acids with a mild alkaline methanolysis and analyzing the methyl esters with SIM and positive ion CIMS. The background level from this procedure was lower than that obtained by preparing and analyzing PFB-esters with negative ion CIMS.

The background level of the positive ion CIMS procedure was further decreased by using minimal volumes of solvents and reagents. However, in some applications, as demonstrated for the analysis of the deep subsurface samples, relatively large amounts of solvents are needed. In such cases, it was important to use freshly distilled solvents and to purify the water by filtration and extraction with chloroform to remove trace amounts of contaminants. Furthermore, lower levels of fatty acid contaminants were introduced when using disposable glass micropipettes than microliter syringes for transferring small volumes of reagents and solvents.

The amount of solvents required for the silicic acid fractionation step was decreased

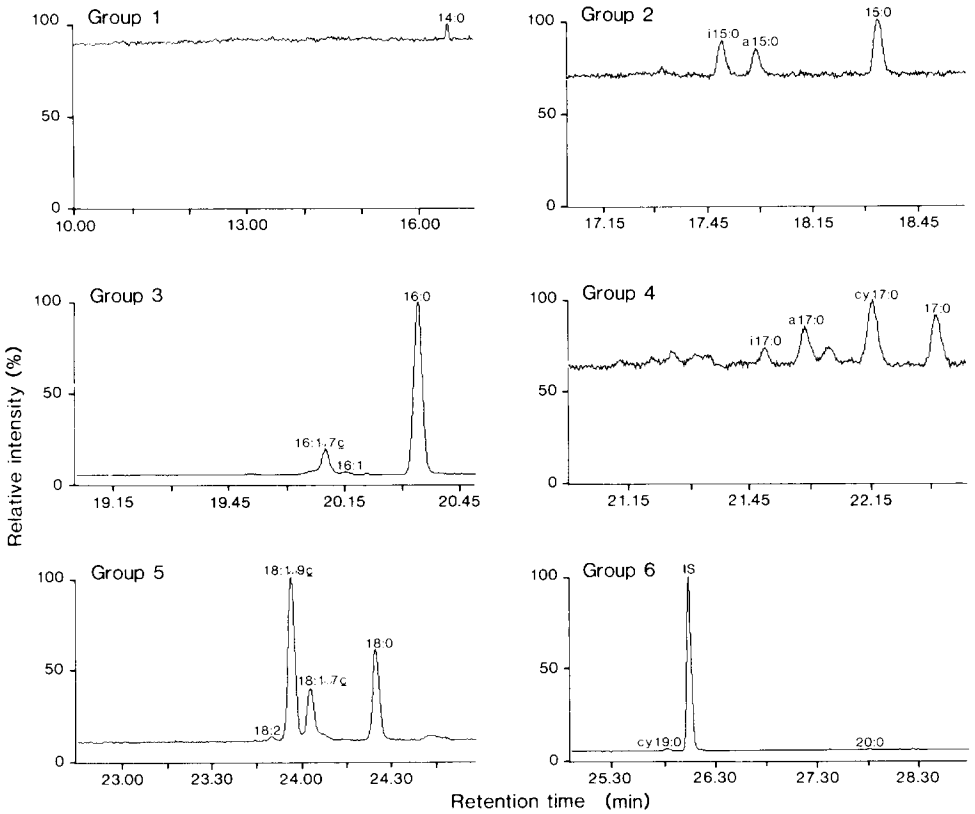

Fig. 3. Selected ion monitoring (SIM) chromatogram using positive ion CIMS of methyl esters of phospholipid fatty acids from a deep subsurface sediment collected at a depth of 203 m. The data was recorded in six consecutive groups of ion parameters (cf. Table 1). The concentrations (pmoles/g dry wt) of the identified fatty acids in this sample, after subtraction of blanks from the analytical procedure, were: 0.02 pmole i15:0, 0.02 pmole a15:0, 0.04 pmole 15:0, 0.5 pmole 16:1ω7*c*, 2.9 pmole 16:0, 0.02 pmole i17:0, 0.06 pmole a17:0, 0.09 pmole cy17:0, 0.10 pmole 17:0, 0.01 pmole 18:2, 1.3 pmole 18:1ω9*c*, 0.7 pmole 18:1ω7*c* and 18:0, 0.06 pmole cy19:0 and 0.01 pmole 20:0.

by using a smaller amount of silicic acid gel than in our standard procedure [8]. 100 mg of silica gel has the capacity of fractionating 1–2 mg of lipids [19] which is sufficient in these applications. The columns from Supelco provided an easy tall-glass system and were relatively easy to pack with the used amount of silica gel. However, it might be possible to speed up the silicic acid fractionation step by using prepacked bonded phase columns to fractionate the lipids [20].

The regression line for the recovery curve of *E. coli* did not pass through the origin and this was probably due to a decrease in the recovery of PLFA from the samples containing the smallest amounts of lyophilized cells. The reasons for the low recovery values as well as for the differences in the composition of PLFA in samples with low microgram amounts of cells compared to in samples containing milligram amounts cells are at present not known. However, it can be assumed that losses of material occur

TABLE 5

PHOSPHOLIPID FATTY ACID COMPOSITION IN FREE AND ATTACHED CELLS OF *PSEUDOMONAS ATLANTICA* GROWN IN A FOWLER CELL ADHESION MODULE. DATA EXPRESSED IN MOLE%, MEAN (SD, $n=3$)

Fatty acid	Free cells	Attached cells
14:0	0.9 (0.6)	1.4 (0.6)
15:1	1.6 (0.9)	1.5 (0.6)
15:0	2.8 (1.0)	3.0 (0.8)
16:1ω7*c*	28.0 (4.4)	26.7 (3.0)
16:1ω7*t*	3.6 (1.7)	12.2 (1.1)
16:0	25.7 (4.2)	31.6 (0.6)
17:1ω8	7.3 (3.1)	4.5 (0.6)
17:0	3.0 (1.2)	4.2 (1.5)
18:1ω7*c*	23.3 (2.9)	10.1 (3.3)
18:1ω7*t*/9*c*	0.6 (0.1)	0.5 (0.2)
18:0	3.3 (2.1)	4.3 (1.3)
Saturated	35.6 (2.5)	44.5 (3.2)
Unsat *cis*	58.7 (3.7)	41.2 (3.1)
Unsat *trans*	4.1 (1.7)	12.7 (1.1)

during transference of samples, evaporation of solvents, and due to adsorption of lipids to glass walls of test tubes and the silica gel. The increment in CV with the analysis of low levels of samples will effect the precision of the PLFA measurements, since the most commonly used criteria for analytical measurements, the 95% confidence interval, is approximately two standard deviations [21]. The recovery values, as well as the precision of the analysis, can probably be improved by adding an internal standard earlier in the analytical procedure. For example, a synthetic phospholipid containing 19:0 could be added during the initial lipid extraction step.

Even though the sensitivity of the developed positive CIMS method is considerably lower than the negative ion method, it has several advantages. First the positive CIMS method has a lower background level of fatty acid contaminants than the negative CIMS method. Second, the mild alkaline methanolysis will leave plasmalogen vinylethers, amide and ether linkages intact, and these can be analyzed by appropriate methods [15, 22]. Furthermore, if enough material is present for scanning spectra, electron impact (EI) MS analysis can be used for structural identification of FAME's [23]. Recently, several techniques using positive ion CIMS have also been developed to localize double bonds in unsaturated FAME [24–25]. On the other hand, the major ions in the EI spectra of PFB-esters of fatty acids are related to the PFB group, and negative ion CI spectra only give information about the molecular weight of the compound [10, 26].

Groundwater is becoming increasingly important as a resource of clean and fresh water for industrial and domestic use. At the same time, more of this vital resource is found to be contaminated with potentially toxic water. Therefore, it is important to define the structure of the microbial community and its biodegradation potential. Examination of the microflora in deep subsurface sediments has revealed the presence

of a sparse and specific microflora [13]. The concentration of PLFA in the analyzed samples was 5.8 pmoles/g dry wt. By using appropriate conversion factors [27], it can be calculated that this value corresponds to 1.3×10^6 bacterial cells/g dry wt. The number of bacterial cells estimated by MPN (most probably number) and plate counts were 2×10^6 and 3×10^4/g fresh weight, respectively, with a moisture content less than 20% [13]. The detection limit of the positive CI MS method to analyze phospholipid fatty acids for biomass determination in the deep subsurface sediments can be calculated to about 1 pmole/g dry wt using the LOD values given in Table 2 and assuming 20 g of extracted material. This level corresponds to the PLFA content of approximately $10^5 - 10^6$ cells/g dry wt.

Analysis of the samples from the Fowler Cell adhesion module demonstrates that the developed method can be utilized to examine the composition of PLFA from microorganisms in specific microenvironments. Shifts in the composition of phospholipids in bacteria can influene important physiological functions, e.g. transport mechanisms, which might effect the activity and metabolic functions of the organism [7]. The FAME analysis revealed differences in PLFA composition between free and attached cells in a biofilm of *P. atlantica*. Similar differences were found in the lipid composition between free and initially adhered cells of a pseudomonas isolated from soil [28].

To further improve the detection limit and precision of analysis of PLFA, new approaches need to be developed to further reduce the background levels of fatty acid contaminants. The amounts of solvents and reagents can be minimized by performing derivatization i a vapour phase using capillary reaction vessels [29]. Tandem mass spectrometry (MS-MS) enables further simplification of cleaning-up procedures and thereby decreases the risk of introducing contaminants during preparation of the samples [30].

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