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In situ derivatisation using pressurized liquid extraction to determine phenols, sterols and carboxylic acids in environmental samples and microbial biomasses

Jürgen Pörschmann[a,*], Jörg Plugge[a], Roland Toth[b]

[a]*Centre for Environmental Research Leipzig-Halle (UFZ), Department of Remediation Research, Permoserstrasse 15, 04318 Leipzig, Germany*

[b]*Institute of Chemical Technology, University of Veszprem, Egyetem u. 10, P.O. Box 158, H-8201 Verszprem, Hungary*

Abstract

Pressurized liquid extraction was combined with in-situ derivatisation to extract polar analytes such as phenols (including chlorophenols) sterols and carboxylic acids from environmental and microbial samples. This one-step protocol uses acetic anhydride as an acetylation agent, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) as an silylation agent, and boron trifluoride–methanol, phenyltrimethyl ammoniumhydroxide and trimethyl sulfoniumhydroxide as methylation agents. It results in faster extraction rates and better or comparable extraction efficiencies when compared to classical approaches. The addition of a silylation agent also facilitates the extraction kinetics of analytes not accessible to silylation (e.g. polycyclic aromatic hydrocarbons or alkylbenzenes). This may be attributed to a dissociative action of the agent to weaken analyte–matrix interactions. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Pressurized liquid extraction; Extraction methods; Derivatisation, GC; Peat; Soil; Environmental analysis; Phenols; Sterols; Carboxylic acids; Fatty acids

1. Introduction

The relatively new pressurized liquid extraction (PLE; trademark of Dionex: ASE, accelerated solvent extraction) has been acknowledged by the US Environmental Protection Agency (EPA; e.g. approval of the Method 3545, which includes polychlorinated biphenyls, polycyclic aromatic hydrocarbons (PAHs), organo-chlorine and -phosphorous pesticides) and by the analytical community. It meets the requirements expected of an ideal sample preparation method: precise, reproducible, rapid, simple, without any degradation of the target analytes, no or little production of laboratory waste and the accessibility of the extract for chromatographic analysis (see [1] and references cited therein). To extract polar analytes from environmental matrices containing humic organic matter (HOM), the application of polar solvents is recommended to improve extraction recoveries. To extract lipids of a wide range of polarity (from triacylglycerides to phospholipids) from biological samples including bacterial biomasses, solvent mixtures consisting of a non-polar and a polar solvent (e.g. chloroform–methanol according to Folch or Bligh and Dyer, cited in [32]) have been used. As with polar modifiers in supercritical fluid extraction (SFE) [2], polar pressurized liquid extraction solvents increase the bulk solubility for the polar targets, interact with the polar analyte–matrix

*Corresponding author. Fax: +49-341-235-2492.

E-mail address: poerschm@san.ufz.de (J. Pörschmann).

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complex, cover active sites of the matrix and prevent readsorption or reabsorption[1]. Polar solvents with Hildebrand solubility parameters that are close to those of HOM matrices are capable of 'dissolving' the polymeric HOM [3]. This results in dirty extracts, requiring additional clean-up steps. (Moreover, connecting tubes might be blocked.) In this respect, preference should be given to non-polar solvents including benzene and toluene.

In the GC analysis of polar target analytes such as alcohols (sterols, phenols including chlorophenols) and fatty acids, preference should also be given to hydrophobic derivatives which can be handled much more easily. Dicarboxylic acids require inevitably a derivatisation step for GC detection. Overwhelmingly, extracts obtained by Soxhlet, SFE, pressurized liquid extraction, etc. are subsequently derivatised to give more hydrophobic analytes. The addition of small amounts of a derivatisation agent (about 0.5%) to the non-polar solvent is expected to weaken strong analyte–matrix interactions. Thus, the agent acts in a figurative sense as a polar solvent, without contributing to both overall solubility and 'matrix dissolution'. Therefore, to improve extraction efficiencies by overcoming strong matrix–analyte interactions, and to obtain 'clean' extracts readily analysed by GC, a combined derivatisation/extraction procedure under pressurized liquid extraction conditions seems to be useful. This approach was already taken in a former contribution [4], which described this less time consuming one-step protocol for the detection of phenols/benzenediols as well as fatty acids in contaminated sediments as acetates or methyl esters, respectively. This contribution is aimed at

1. extending this approach to further matrices (fatty acid pattern in bacterial biomasses) and target analytes (chlorophenols in sediments),
2. simultaneously detecting fatty acids, dicarboxylic acids and long-chain aliphatic alcohols in matrices rich in HOM such as peat,
3. quantifying target analytes in comparison with the conventional approach, and
4. determining the impact of derivatisation agents on analyte recovery, the analytes being no accessible to a derivatisation (PAHs, alkylbenzenes)

[1]Adsorption refers to the physical or chemical binding at surfaces or interfaces; absorption relates to the partitioning of a sorbate into the HOM sorbent matrix.

2. Experimental

2.1. Samples

The soil sample was taken from the floodplain of the stream Spittelgraben, which flows into the river Mulde. The soil is heavily contaminated with pesticides, organotin compounds, phenols and PAHs.

Dry sphagnum-peat moss (88%, w/w, dry organic matter; 6% ash content, pH 6.2) [5], was broken up using a mill and sieved into a 100–350 μm fraction.

Gram-negative bacterial biomass W 14 was grown on methane as carbon source. (The field of application of the cultivated bacteria is the remediation of sites contaminated with chlorinated hydrocarbons.)

2.2. Chemicals

Toluene, acetone, methanol, chloroform were purchased from Merck (Darmstadt, Germany), and degassed prior to use. The derivatisation agents acetic anhydride, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), boron trifluoride (14% in methanol), phenyltrimethyl ammoniumhydroxide (0.5 *M* in methanol) and trimethyl sulfoniumhydroxide (0.2 *M* in methanol) were from Supelco (Munich, Germany) or Promochem (Wesel, Germany). Chlorophenols including isotopically labelled surrogates were obtained from Promochem. Derivatisation agents were used as received. Attention should be paid to the storage of the reagents (at ≈4°C), otherwise a drop in activity results. Humic acid was purchased by Roth (Karlsruhe, Germany).

2.3. Pressurized liquid extraction

A commercial PLE 200 device (Dionex, Idstein, Germany) with 11 ml stainless steel extraction cells was used. A 20–100-mg amount of sample, depending on the sample to be extracted (e.g. 20 mg for freeze-dried bacterial cells, 100 mg for peat samples) was placed in the cartridge and the cartridge was then filled to capacity with purified and heated sea-sand. The sea-sand was free of any extractables and

contains no fines to clog the system. After filling and sealing, the cell was fully pressurized to 14 MPa and allowed to equilibrate for 6 min at the set temperature (mostly 120°C). The extraction time for 1 cycle was 20 min if not otherwise stated, irrespective of a conventional pressurized liquid extraction (without using a derivatisation reagent) or an in-situ pressurized liquid extraction. The derivatisation reagent was added to the solvent; the internal standards $[^2H_6]$phenol, $[^2H_8]$*o*-cresol, $[^2H_8]$ $[^2H_3]$2,4-dimethyl phenol, $[^2H_2]$(2,2)-palmitic acid, $[^{13}C_6]$4-chlorophenol, $[^{13}C_6]$2,4-dichlorophenol and $[^{13}C_6]$2,4,6 trichlorophenol were added to the cartridge. The extracts were gently evaporated to a final volume of 500 μl using a Turbo-Vap II (Zymark, Germany). In the case of boron trifluoride, the extracts were washed with diluted sodium hydrogen carbonate solution to remove the excessive agent. The quantification of extracts obtained by (i) conventional pressurized liquid extraction, (ii) ex-situ pressurized liquid extraction (postderivatisation of the 'conventional' extract) and (iii) in-situ pressurized liquid extraction was obtained using the labelled standards $[^{13}C_{24}]$*n*-tetracosane, palmitic acid $[CH_3(CH_2)^{13}C_2H_2COOH]$, *n*-octadecanol $[^{13}CH_3(^{13}CH_2)_{17}OH]$ and $[2,2,3,4,4,6\text{-}^2H_6]$cholesterol (CIL, MA, USA). The quantification was conducted by means of significant extracted ions; e.g. m/z 74 and m/z 76 to determine fatty acids as methyl esters.

2.4. GC–MS

An HP 5973 B GC–MS system with data acquisition and temperature program described elsewhere [4], equipped with a 30 m×0.32 mm, 0.25 μm HP-5 (Agilent, Waldbronn, Germany), or RTX-50 (Restek) column was used.

3. Results and discussion

3.1. Acetylation to detect phenols and chlorophenols in soils rich in HOM

The acetylation of phenols including chlorophenols in buffered aqueous matrices (pH ~8.5) has been a common method to monitor drinking water [6]. The application of water as solvent in the combined derivatisation–pressurized liquid extraction, which is vital to use the solventless solid-phase microextraction (SPME) [8], was not pursued further by us because of poor recoveries of phenols having medium to high hydrophobicities (e.g. log K_{OW}= 5.02 for pentachlorophenol, log K_{OW}=3.6 (average) for C_4-alkylated phenols [7]. The octanol–water partition coefficient K_{OW} is a measure of hydrophobicity.) In our experience, when using spiked samples this drawback might be overcome by multiple extractions (at least three successive pressurized liquid extraction cycles) followed by combining the extracts and application of SPME. However, when turning to real-world (aged) samples rich in HOM, our results indicate the utilization of water as a solvent to extract alkylated phenols ($C>3$) or chlorophenols ($Cl>3$) to be inappropriate. Only the mobile pollutant fraction will be recovered by this hot-water extraction, as can be anticipated from sorption/desorption studies on soils [8,9]. Combining pressurized liquid extraction using water as solvent with SPME using polar polyacrylate fiber coatings turned out to be more useful for detecting hydrophilic nitroaromatics (log K_{OW} around 2 [7]) in contaminated soils [10].

Toluene or benzene were chosen as potential solvents for in-situ acetylation/extraction (the same holds also true for silylation and methylation, see below) for two reasons:

1. The polarities of the solvents and of the target analytes (alkylphenols, chlorophenols, sterols, fatty acids) are similar (obviously, the similarity is higher than the similarity between these analyte polarities with the water polarity)
2. The Hildebrand solubility parameter of the solvent ($\delta_{Toluene}$=18.2 $J^{0.5}$ $cm^{-1.5}$, $\delta_{Benzene}$=18.8 $J^{0.5}$ $cm^{-1.5}$) as a measure of the cohesive energy is not very far away from that of the HOM matrix ($\delta_{HOM} \cong 24$ $J^{0.5}$ $cm^{-1.5}$) [11], thus ensuring relatively good swelling of the polymeric, amorphous matrix and access to interstitial pores. Clearly, diffusion is very fast in swollen HOM matrices.

In the framework of preliminary experiments (120°C, 1 cycle of 20 min, 14 MPa) using toluene–acetic anhydride–pyridine (100:1:0.5, v/v) as the solvent and sediments spiked with phenols, we found

that no non-derivatised phenols were detected. Although this protocol does not necessarily mean that phenols, which had been sequestered in the soil for several years, can be extracted quantitatively, the usefulness of the proposed in-situ derivatisation/extraction approach proved right.

Therefore, the in-situ mode was applied to determine phenols including chlorophenols. Fig. 1 shows the detection of phenol, cresols and C_2-phenols in a real-world soil sample taken from the floodplain Spittelgraben (see Experimental). Generally, acetylated derivatives can be easily recognised by extracting the ion m/z 43 $(CH_3–CO)^+$; however, this trace is obscured (mostly) by hydrocarbons. A more convenient way is to trace simultaneously the significant fragment ion, which equals the molecular ion of the native phenol, and the (less intense) molecular ion of the acetate, the latter for reasons of confirmation. Emphasis should be focused on the separation of *m*- and *p*-cresol (coelution of the native cresols on HP-5) and the complete absence of non-derivatised phenols. Subsequent extractions of the (already in-situ extracted) soil sample using both the conventional approach as well as the in situ derivatisation approach yielded phenol recoveries below 6%, thus indicating an almost complete extraction of the target analytes using the proposed in situ derivatisation approach.

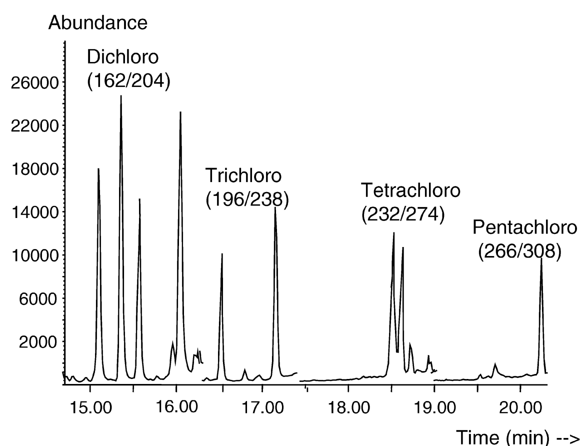

Fig. 2. Detection of chlorophenols as acetates. Pressurized liquid extraction: see legend Fig. 1; recording: sum of extracted ions m/z 162 (dichlorophenols), m/z 196 (trichlorophenols), m/z 232 (tetrachlorophenols) and m/z 266 (pentachlorophenol), the figures in parenthesis indicate the molecular ions of the virgin phenol and the acetates.

Another example indicative of the usefulness of the in-situ acetylation/extraction mode is the detection of chlorophenols in a spiked sediment (municipal river sediment, organic carbon content 7%, sediment aged for 4 weeks after spiking; see Fig. 2). The recovery of the acetates, as quantificated by the addition of ^{13}C-labelled chlorophenols into

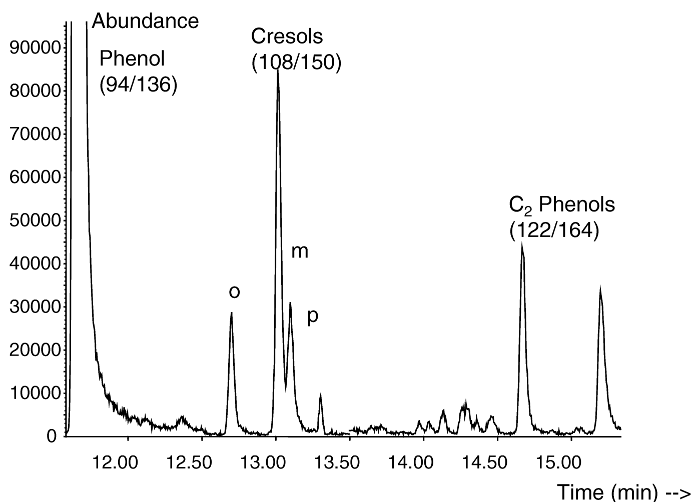

Fig. 1. Detection of phenols as acetates. Pressurized liquid extraction: solvent toluene–acetic anhydride–pyridine (100:1:0.5); temperature 120°C, one cycle 20 min, pressure 14 MPa; data acquisition in scan mode; recording by means of extracted ions m/z 94 for phenol, m/z 108 for cresols, m/z 122 for C_2 alkylated phenols. Confirmation of acetates by molecular ions m/z 136, 150 and 164, respectively.

the ASE cartridge, was between 89 and 103%, with no significant dependence of recovery on chlorine molarity. A postextraction of the already extracted samples turned out to give negligible recoveries. (The formation of bound residues between chlorophenols and the HOM matrix should be detailed elsewhere.)

These results give strong evidence that the combined in situ derivatisation/extraction is superior to conventional pressurized liquid extraction for extracting phenols due to three reasons

1. Based on a host of data, it becomes evident that the analyte recoveries obtained after one cycle of the in-situ protocol correspond roughly to recoveries obtained after three cycles of conventional pressurized liquid extraction (same conditions: 120°C, cycle 20 min, toluene). This points to a dissociative action of the derivatising agent to disrupt strong matrix–analyte interactions (see also below). Obviously, the extraction efficiency of the conventional pressurized liquid extraction using non-polar solvents can be improved by increasing the macromolecular mobility of the HOM matrix (either by sorbent swelling using polar solvents or by elevated extraction temperatures). However, both approaches result in dirty extracts.
2. The one-step protocol is less time consuming
3. On nonpolar HP-5 coatings (the same applies to polydimethylsiloxanes such as HP-1), the hydrophobic acetates show better GC behaviour than the native phenols. Even if the GC system is perfectly deactivated, the native phenols produce broader peaks due their higher non-compatibility with the non-polar phase.

3.2. Silylation to detect sterols and carboxylic acids in peat

Target functional groups of silylation include OH group (phenols, fatty and dicarboxylic acids, carbohydrates) as well as the amino group) [12]. A significant drawback of silylation agents is their susceptibility towards hydrolysis; therefore wet samples (sludge) should at least be air-dried prior to the in-situ silylation/pressurized liquid extraction. Our target analytes included sterols as well as mono- and dicarboxylic acids and long-chain alcohols in peat. Peat has been an intense matter of our interest due to (i) its sorption capabilities (remediation issue, [5]) and (ii) its role model function in recognising pathways in humification processes (diagenetic issue, [13]).

To detect sterols in environmental samples, which is important in environmental monitoring (e.g. to trace fecal contaminations via coprostanol [14]), a time consuming Soxhlet or ultrasonic extraction (see EPA method 3550A) followed by clean-up and derivatisation is recommended. The substitution of the combined in-situ derivatisation/extraction for the traditional multistep protocol should be regarded as progress in sample preparation due to the reasons outlined above. In this respect, pressurized liquid extraction is likely to be more useful than SFE because the selection of the solvent can be tailored to the specific analyte and matrix within a broader range. Solvent selection is not subjected to the restrictions occurring in SFE where the polarity and solvent strength of the almost exclusively used solvent carbon dioxide (the solubility parameter of which is close to those of *n*-alkanes [11]) can only be changed within a narrow range by means of polar modifiers. The suggested in situ silylation/pressurized liquid extraction protocol may also be of use for other purposes, e.g. for recognising metabolic disorder diseases via extraction of steroids and acids from bodies and bodily fluids [15].

Fig. 3 gives the total ion current (TIC) subsection of a peat extract, the matrix being subjected to an in-situ silylation/pressurized liquid extraction without any clean-up. A further increase in extraction temperature beyond 120°C gives no significant improvement in recoveries but darker (more humic) extracts. On comparing this TIC with the TIC obtained by conventional pressurized liquid extraction (without silylation agent, no subsequent ex-situ silylation), sterols can be unambigously identified due to a changed position in the chromatogram and a common significant fragment ion of the sterol trimethylsilyl (TMS) ethers at m/z 75 $[(CH_3)_2{=}Si^+{-}OH]$. As a rule of thumb, sterol TMS ethers (capital letters A–P in Table 1 and Fig. 3; the formulae of the selected analyses are given in Fig. 4) elute 100 Kovats index units later than the virgin sterol (Nos. 1–26 in Table 1 and Fig. 3). The identification of the sterols and the corresponding TMS ethers was done

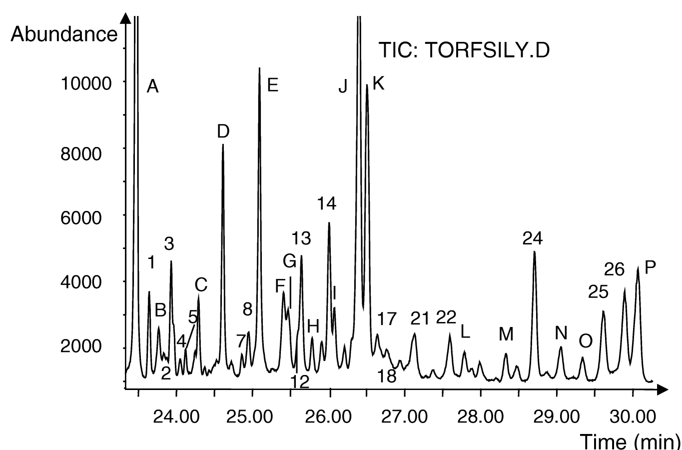

Fig. 3. Peat extract obtained by combined in situ silylation/pressurized liquid extraction [solvent toluene–BSTFA (99:1); 120°C, 1 cycle of 20 min, 14 MPa]. Capital letters: silyl derivatives; numbers: non-derivatised analytes; see Table 1.

according to guidelines given in [14] and refs. cited therein. Most of the analytes which were not subjected to silylation include hydrocarbons of the hopa(e)ne and stera(e)ne family as well as cyclic ketones (Table 1). Some sterols were also detected in Finnish peat samples subjected to solvent extraction [16]. It should be emphasised that the PLE extract (Fig. 3) comprises compounds which are physisorbed onto the HOM matrix. HOM building blocks which make up the polymeric network and which are covalently bound, cannot be observed under the conditions applied. (The application of the in situ derivatisation under more stringent conditions to reveal building blocks in the HOM backbone, as already outlined in [17], will be detailed in a forthcoming paper).

Although fatty acids, dicarboxylic acids and alcohols can be recognised visually as TMS ethers with significant fragment ions at m/z 75 and at $(M-15)^+$, (e.g. palmitic acid TMS ether shows a significant fragment at m/z 313), the assignment of the individual analyte class is ambiguous using only these two significant ions. As an example, palmitic acid TMS ether and heptadecanol TMS ether cannot be distinguished from each other in this way because two methylene groups correspond to the C=O group (28 each) in a low resolution benchtop MS. However, the fragment ion m/z 117 is very distinct for carboxylic acid–TMS ethers $((TMS–O–C=O)^+$ as well as methyl group elimination of the McLafferty ion $[(CH_2=C(OH)–OTMS]^{+\bullet}$ can be assumed, the proportion of these two ions might be determined by MS–MS; but this is outside the scope of this paper). Fig. 5 shows the differentiation of long-chain alcohol TMS ethers and fatty acid TMS ethers; the upper trace shows the sum of all relevant $(M-15)^+$ fragment ions (e.g. m/z 411 for tetracosanol and C_{23}-fatty acid, m/z 425 for pentacosanol and lignoceric acid; the alcohol peaks are filled), the lower trace of m/z 117 selectively extracts the fatty acid TMS ethers. As expected, the pronounced even-over-odd discrimination for both alcohols and fatty acids points to a natural origin of the sample. (The same holds also true for the lower C_{12}–C_{20} range; chromatogram-subsection not depicted here.) Dicarboxylic acid di-TMS ethers (shorthand designation in Fig. 5: 21di and 23di) can be assigned using both the $(M-15)^+$ and the $[M-CH_2=C(OH)–OTMS]^+$ fragment ion (e.g. m/z 243 for 1,12-dodecanoic acid di-TMS, m/z 271 for 1,14-tetradecanoic acid di-TMS, etc). This example shows that by using a combined in-situ silylation–pressurized liquid extraction one-step protocol the analyst can succeed in the simultaneous detection of fatty alcohols, fatty acids and dicarboxylic acids. As known, dicarboxylic acids cannot be subjected to GC in the native form at all, the GC of native fatty acids is highly biased. The unambiguous identification of native long-chain aliphatic alcohols by means of MS is also very cumbersome due to their strong spectral similarities

Table 1
Identification of the peat extract obtained by in situ silylation–pressurized liquid extraction[a]

Peak (Fig. 3)	Identification	M_r (D)[b]
1	D-Friedooelane-14-ene	410
2	Oleane-12-ene	410
3	Alkane C_{31}	436
4	D:A-Friedooleane-7-ene	410
5	Nonacosanone	414
7	C_3(en)-Hopane	410
8	C_3(en)-Hopane	410
12	C_4-Hopane	426
13	Alkane C_{32}	450
14	Friedooleane-14-ene-3-one	424
17	Friedooleane-8(?)-ene-3-one	424
18	Stigmast-7(?)-one	414
21	Stigmast-3,5-diene-7-one	410
22	Stigmast-4-ene-3-one	412
24	D:a-Friedoursane-3-one	426
25	Cholest-4-ene-3,6-dione	424
26	Ketone	470
A	Fatty acid n-C_{26}-TMS	468 (396)
B	Ergostanol-TMS	468 (396)
C	Octacosanol–TMS	482 (410)
D	Dicarboxylic acid n C_{23}-diTMS	528 (384)
E	Fatty acid n C_{28}-TMS	496 (424)
F	Ergostanol-TMS	474 (402)
G	Campesterol-TMS	472 (400)
H	Stigmasterol-TMS	484 (412)
I	Triacontanol-TMS	510 (438)
J	β-Sitosterol-TMS	486 (414)
K	Stigmastanol-TMS	488 (416)
21	Fatty acid n-C_{30}-TMS (coelution with stigmast-3,5-diene-7-one)	524 (452)
L	Lupeol-TMS	498 (426)
M	Sterol-TMS + Nor-gammaceranol	554 (482)+ 500 (428)
N	Stigmasta-triene-3-ol	482 (410)
O	Fatty acid n-C_{32}-TMS	552 (480)
P ???		

[a] Conventional pressurized liquid extraction: toluene; in situ approach: toluene–BSTFA (99:1, v/v); each 120°C, 1 cycle 20 min; formulae of selected analytes are given in Fig. 4.
[b] Molecular mass in parenthesis: parent molecule.

to alkanes and the lack of structure-indicative fragments.

Another question is quantification of the target analytes. The proposed in-situ derivatisation approach is only sensible in the case of comparable or better recoveries of target analytes when compared with the conventional approach or the multistep ex-situ approach. In other words, the derivatisation agent must not destroy the target analytes during the in-situ derivatisation–extraction procedure and should yield a single derivative. Table 2 gives concentrations of target analytes (see also Fig. 3). When considering hydrocarbons (alkanes, hopanes) and ketones (aliphatic 2-ketones, ketosteroids), the recovery is in the same order of magnitude, although there is a tendency towards higher recoveries with the in situ mode, especially with analytes of higher retention indices (see Table 2 and paragraphs below). Fatty acids as well as sterols show comparable recoveries considering the ex situ and in situ ap-

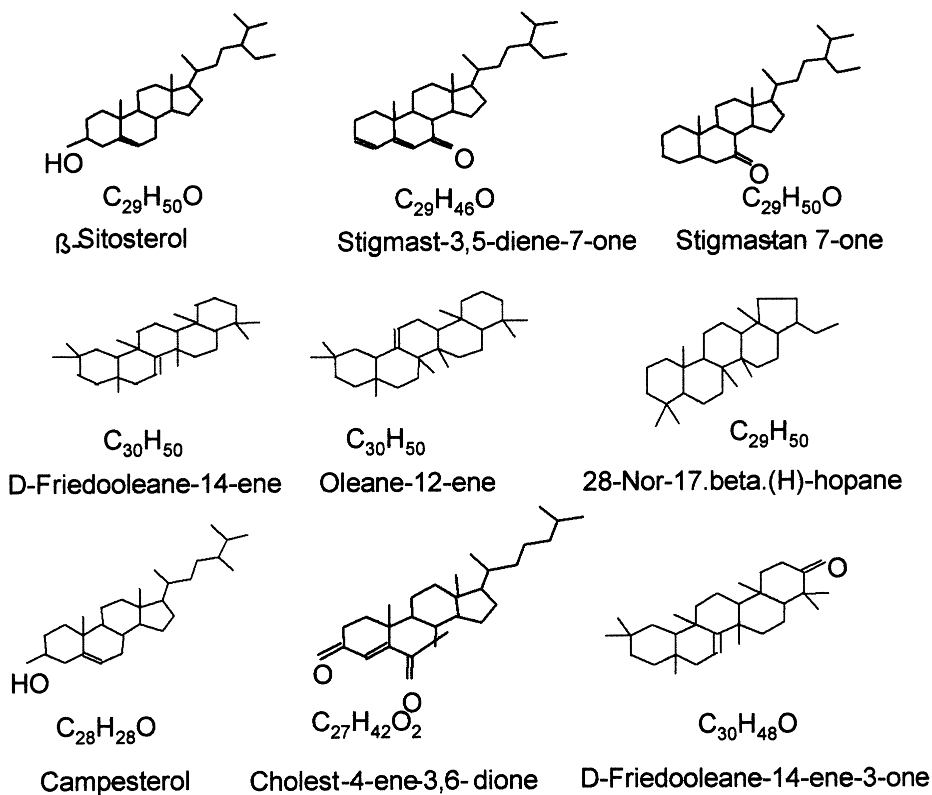

Fig. 4. Formulae of selected analytes.

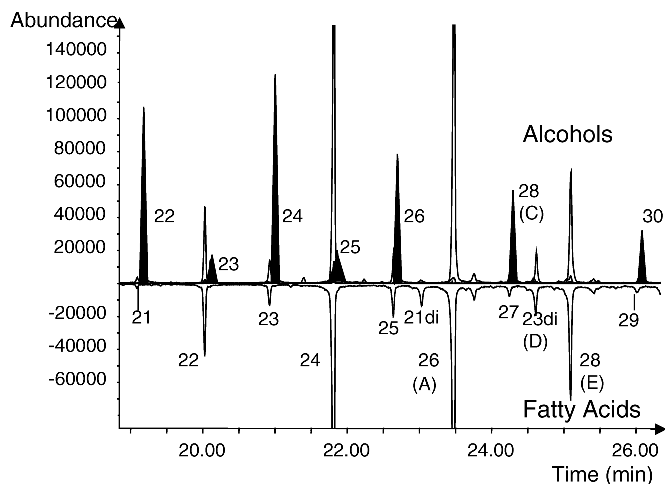

Fig. 5. Selective detection of long-chain aliphatic alcohols, mono- and dicarboxylic acids as TMS ethers (see text). Capital letters in parenthesis: see Fig. 3 and Tables 1 and 2.

Table 2
Quantification of peat extracts obtained by conventional pressurized liquid extraction, ex situ silylation and in situ silylation/pressurized liquid extraction (data in ppm, referred to mass of peat, data replicate of three, RSD between 5 and 18%)

Peak (Fig. 3)	Identification	PLE		
		Conventional	Ex-situ	In-situ
1	D-Friedooelane-14-ene	20	20	22
2	Oleane-12-ene	7.0	7.1	7.5
3	Alkane C_{31}	17	16	19
5	Nonacosanone	8.1	8.2	8.0
8	C_3(en)-Hopane	7.2	7.4	8.1
12	C_4-Hopane	7.3	7.1	7.3
13	Alkane C_{32}	12	10	14
14	Friedooleane-14-ene-3-one	24	23	29
18	Stigmast-7(?)-one	12	15	15
21	Stigmast-3,5-diene-7-one	13	13	16
22	Stigmast-4-ene-3-one	17	18	20
24	D:a-Friedoursane-3-one	36	34	48
25	Cholest-4-ene-3,6-dione	27	28	35
A	Fatty acid n-C_{26}-TMS[a]	N.n.	180	163
B	Ergostanol-TMS	7	8	8
C	Octacosanol-TMS	N.n.	21	17
	Dicarboxylic acid n-C_{21}-diTMS	N.n.	38	52
D	Dicarboxylic acid n-C_{23}-diTMS	N.n.	62	87
E	Fatty acid n-C_{28}-TMS	N.n.	84	77
F	Ergostanol-TMS	7	8	8
I	Triacontanol-TMS		15	14
J	β-Sitosterol-TMS	79	112	108
	Palmitic acid-TMS[b]	9	65	57
	Stearic acid-TMS[b]	2	17	16

[a] TMS ethers: only relevant for ex situ and in situ row.
[b] Target ion for quantification: m/z 60.

proach. Fatty acids are strongly discriminated using the conventional approach (last two lines in Table 2). The in situ derivatisation approach demonstrates its full potential with dicarboxylic acids. Obviously, the BSTFA agent is capable of disrupting the strong analyte–matrix interactions, whereas the solvent toluene (without BSTFA) fails to do so. These results provide strong evidence that — under the applied extraction protocol — the BSTFA agent does not destroy the target analytes by undesired side reactions.

The finding that hydrocarbons tend to give higher recoveries when using the addition of BSTFA to the solvent toluene (the same holds true for benzene; data not shown here), should be detailed further using a highly contaminated coal wastewater sediment (see [18]) and BSTFA as silylation agent. If this assumption is correct, the question of whether this is a kinetically or a thermodynamically related phenomenon arises. (These investigations were carried out using BSTFA silylation agent, but similar tendencies are also expected with other derivatisation agents.) There is evidence from the literature that derivatisation agents can influence the extraction efficiency of target analytes which are not accessible to a derivatisation reaction at all: as indicated in [19], the addition of silylation agents (1–2%) to the supercritical CO_2 gives better recoveries for caffeine in coffee beans in comparison with conventional SFE. Obviously, the agent does not significantly contribute to an enhancement in solubility but is capable of disrupting strong analyte–matrix interactions thus facilitating analyte desorption.

Fig. 6a and b shows the profile of alkylbenzenes C_6–C_{25} and alkanes n-C_{12}–n C_{32}, respectively, at different extraction temperatures. The y axis indicates the ratio of the measured concentration of the analyte when using BSTFA–toluene (1:99) as the

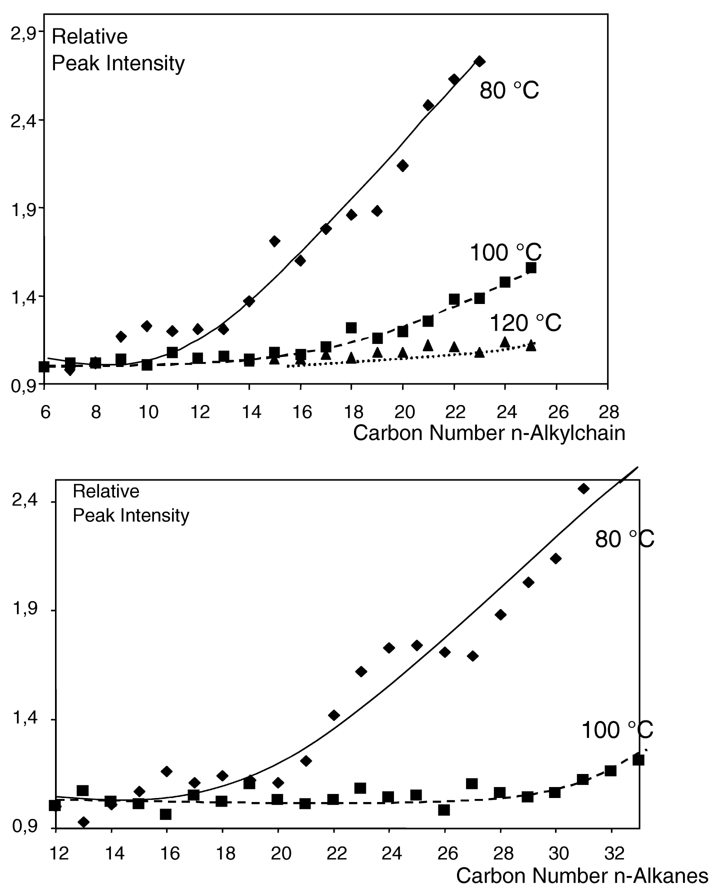

Fig. 6. Relative peak intensity (in ppm/ppm) of alkylbenzenes (a) and *n*-alkanes (b) using BSTFA as silylation agent (1% in toluene) referred to sole use of toluene (see text). Extracted ions: m/z 92 for alkylbenzenes, m/z 57 for alkanes.

solvent to the concentration measured when using solely toluene as the solvent (conditions each: 20 min extraction time, 1 cycle, 100 mg sample). It is evident that the silylation agent has no effect on the analyte recovery when considering analytes up to *n*-hexadecane and *n*-decylbenzene within the selected extraction temperature interval. This also applies to high extraction temperatures of 120°C and beyond, regardless of the chain length (data measured at 140°C, which are not shown here, are quite identical to the 120°C ratios.) On turning to lower extraction temperatures (80°C, 100°C), both higher-molecular-mass alkanes and alkylbenzenes are significantly better extracted when 1% BSTFA is added to the toluene solvent. Again, we assume that BSTFA does not significantly influence the overall solvent solubility but has a dissociative impact on the analyte–matrix bonding. The discrimination effect is more pronounced with alkylbenzenes indicating that BSTFA tends to disrupt stronger interactions[2]. Fig. 7a illustrates the alkane profile using an extraction time of 10 min at 80°C: The longer the carbon chain length, the higher the impact of the silylation agent on the analyte recovery. (The same tendency holds true with alkylbenzenes, data not shown here.) On turning to longer extraction times (60 min, see Fig. 7b), the BSTFA-impact levels off as indicated with alkylbenzenes (see Fig. 7b; alkane data are similar.).

[2]In this respect, analytes having identical carbon numbers should be compared; e.g. *n*-tetradecane (boiling point = 253°C) with *n*-octylbenzene (boiling point = 262°C).

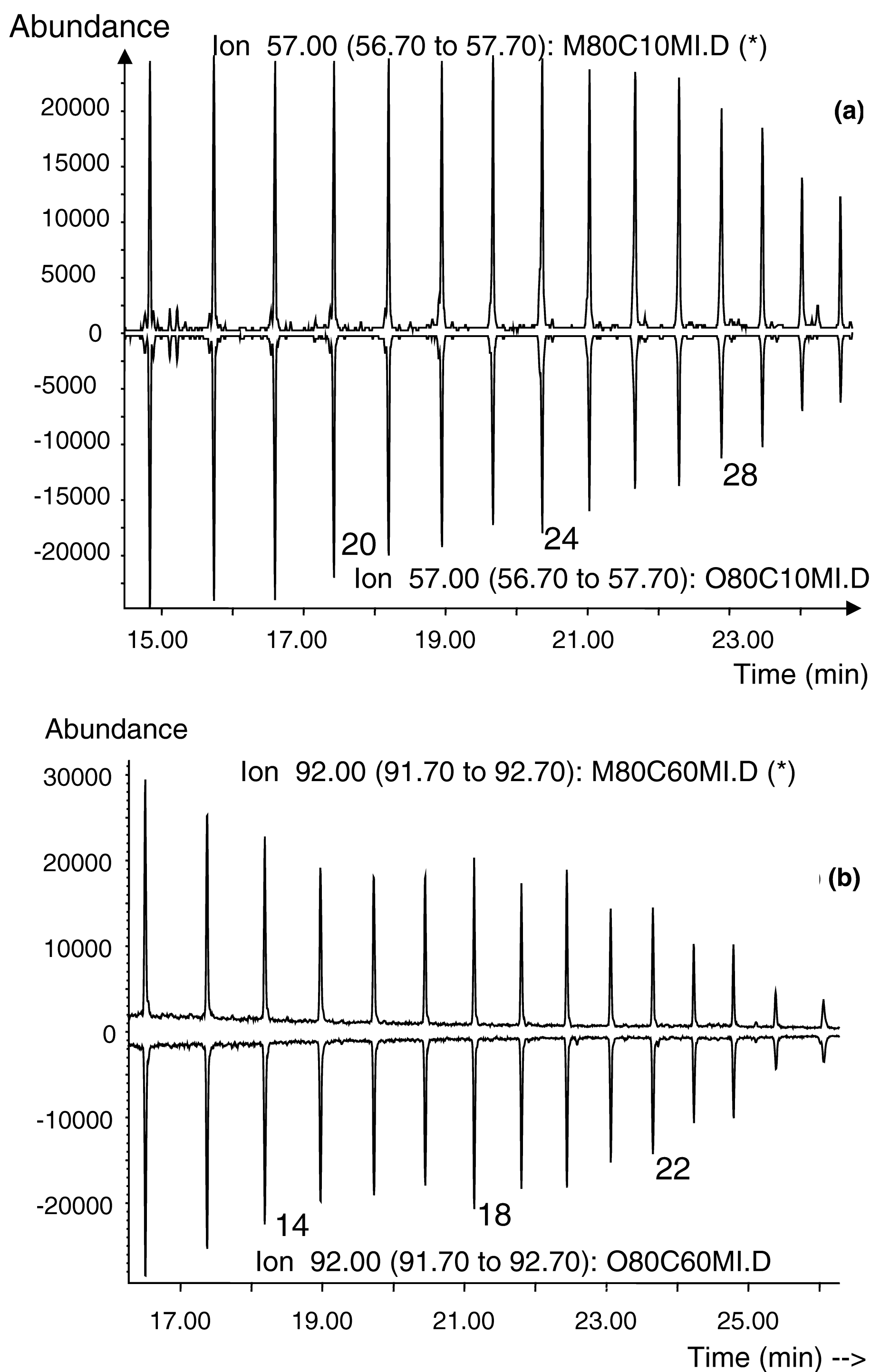

Fig. 7. Alkane profiles with BSTFA–toluene (1:99) as solvent (a, upper trace) and sole use of toluene as solvent (a, lower trace); alkylbenzene profiles (b) in dependence on BSTFA addition (upper trace: with BSTFA, lower trace: without BSTFA; pressurized liquid extraction each: 80°C, 1 cycle, 10 min extraction time in (a), 60 min in (b).

Obviously, the analyte mass transfer from the inner core of the HOM matrix to its surface as well as the transfer from the HOM surface through the layer of stagnant toluene into the bulk solvent cannot be changed significantly by small amounts of derivatisation agents; the diffusion coefficients in both toluene and BSTFA–toluene (1:99) are expected to be very similar. This levelling off provides strong evidence that the silylation agent influences the desorption kinetics. The beneficial effect of a derivatisation agent may be very useful in the extraction of thermolabile analytes which require moderate or low temperatures.

3.3. Methylation to profile fatty acids in microbial biomasses

The identification and classification of microorganisms have been based on their biochemical, morphological, serological, and toxigenic characteristics. Fatty acids can be used as biomarkers and beyond this, certain fatty acids and their ratio among each other (fingerprint) might be used to assess the physiological state of microbial communities [20]. Profiling fatty acids may also contribute to solve further problems, e.g. to differentiate strains of food-borne pathogens responsible for disease outbreaks [21]. Limitations of the established methods (e.g. cell assemblages and soil aggregation mask microorganisms resulting in wrong counting of microorganisms) have led to the analytical microbiology, including profiling fatty acids. To profile fatty acids in environmental and biological samples, volatile and hydrophobic methyl esters (FAMEs) have been used for decades. To extract lipids, the application of PLE is a good alternative to conventional methods (SFE, sonication, Soxhlet) [22,23].

There have been two mainstream approaches used to obtain FAMEs

1. (i) The (alkaline) saponification followed by removal of non-saponificable compounds (e.g. hydrocarbons including squalene), release of free fatty acids from their salts, and finally methylation using (mostly) boron trifluoride–methanol.
2. (ii) Extraction of lipids and free fatty acids by means of solvent mixtures (e.g. chloroform–methanol according to Folch), followed by trans esterification of the extracted lipids.

In both cases, fatty acids in both non-polar and polar lipids are obtained (e.g. triacylglycerides and phospholipids, respectively); whereas fatty acids including hydroxy fatty acids bound in lipopolysaccharides[3], can only be detected using the first approach, because lipopolysaccharides cannot be extracted using chloroform–methanol mixtures. In this respect, hot phenol–water or phenol–guanidinium thiocyanate reagents are necessary [25]. However, it is these hydroxy fatty acids located in the external layer of the cell envelope of Gram-negative bacteria which are of distinct taxonomic significance [26].

The proposed one-step protocol should be beneficial in comparison to the time consuming approaches (i) and (ii). The first step in evaluating the new in-situ approach consisted of utilising a normal fat containing no hydroxy fatty acids, the latter being expected to be difficult to handle (see below). Peanuts, the lipids of which consist mainly of a triacylglycerol and a phospholipid fraction [27], were considered as convenient role model. Table 3 compares the FAME pattern of peanuts obtained by the

Table 3
FAME profile in peanuts obtained by different sample preparation methods (data in ppm, replicate of three; RSD between 5 and 21%)

FAME (shorthand designation)	Alkaline saponification[a]	In situ PLE (BF_3)[b]
14:0	34	37
15:0	9	8
16:1	835	780
16:0	7 180	8 550
17:0	141	128
18:2	19 300	16 800
18:1	47 100	45 600
18:0	2 840	2 770
20:0	1 110	880
22:1	1 735	1210

[a] Alkaline saponification: Experimental, see [32].
[b] Pressurized solvent extraction: 1 cycle 100°C, 20 min, quantification using m/z 74 for saturated FAME, m/z 236, 296, 294 for palmitoleic, oleic and linoleic FAMEs, respectively, internal standard: 500 ppm deuterated [2,2-$^2H_2$]palmitic acid, target ion: m/z 76.

[3] Lipopolysaccharides are commonly subdivided in lipid A, core polysaccharide and O-antigen [24], the lipid A functionality is highly immunogenic and important in diseases of bacterial origin.

common alkaline saponification and by in-situ methylation/pressurized liquid extraction using 0.2% boron trifluoride–methanol in toluene. The good correlation indicates that — under the chosen pressurized liquid extraction conditions — the processes of saponification, methylation and extraction take place. The in situ methylation was conducted at 100°C (instead of 120°C used throughout the experiments), because there was a significant loss of unsaturated FAMEs at higher temperatures. However, the careful validation of this approach (centered around the variation of temperatures, extraction times, number of cycles, application of different solvents, which should be free of radicals, and more gentle derivatisation agents, etc.) is outside the scope of this paper. The aim of this paragraph is to convey the idea that the proposed in-situ methylation–pressurized liquid extraction protocol is an effective and time saving tool for determining fatty acid patterns and might be used for rapid screening of lipids in a wide variety of matrices.

This good correspondence between the classical approach and the in-situ approach prompted us to turn to microbial biomasses, the phospholipid fraction being the main source of fatty acids as a rule of thumb [28], and which are expected to contain hydroxy fatty acids. A saponification of bacterial lipids and derivatisation of the fatty acids using the in-situ derivatisation/SFE approach with phenyltrimethyl ammoniumhydroxide has already been described [29,30].

Fig. 8 compares the selected ion chromatograms at both m/z 74 obtained via both protocols for a Gram-negative bacterial biomass. The correspondence is striking. Similar chromatograms were obtained when using the ion-pair agents phenyltrimethyl ammoniumhydroxide and trimethylsulfonium hydroxide [3]. No distinct difference in the fatty acid profile was observed when using a splitless or on-column injection, which indicates that methylation has taken place before injection.

Special attention should be focused on the fact that

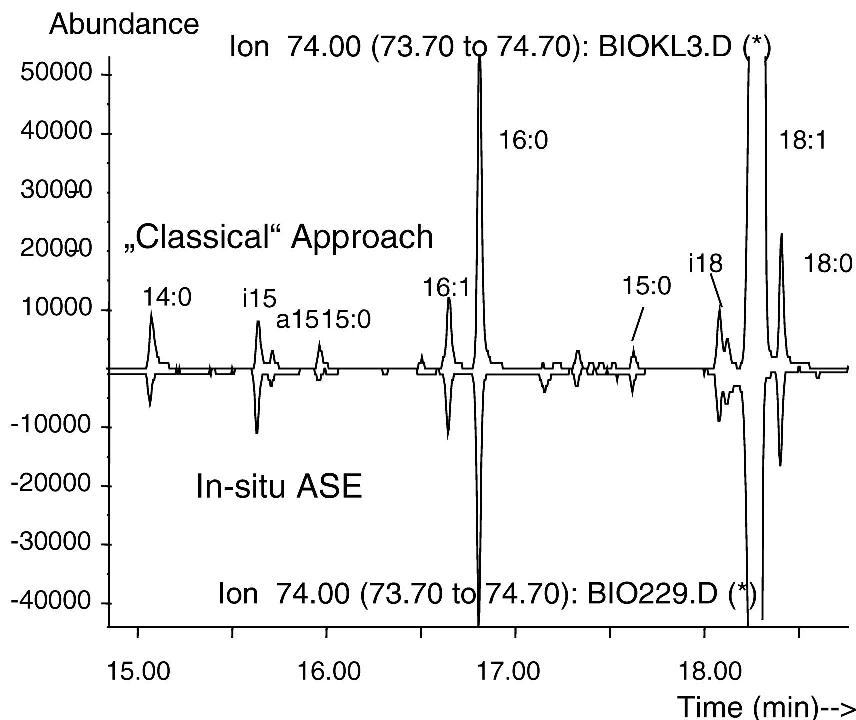

Fig. 8. FAME pattern of a Gram-negative bacterial biomass obtained by the classical saponification protocol (upper trace) and by the in-situ methylation–pressurized liquid extraction approach. Conditions: 100°C, 1 cycle 20 min, 0.2% boron trifluoride–methanol added to toluene. Target ion: m/z 74.

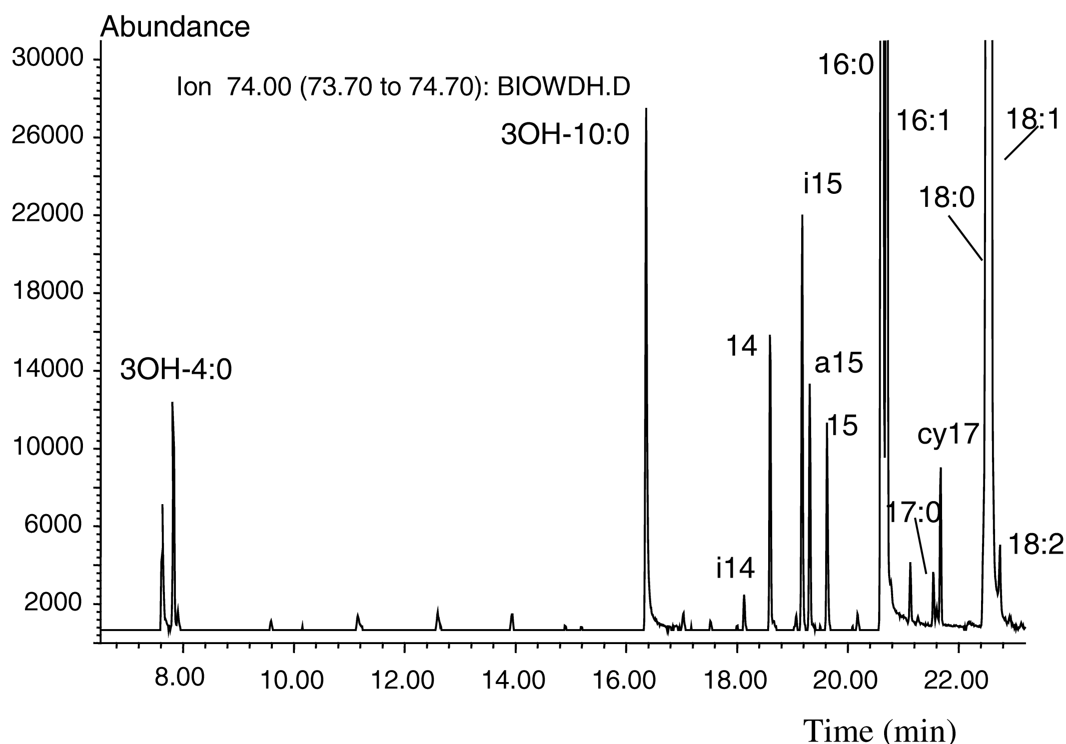

Fig. 9. FAMEs in a Gram negative bacterial biomass subjected to in situ methylation (boron trifluoride–methanol). Pressurized liquid extraction: see legend to Fig. 8.

hydroxy-FAMEs could also be detected: Fig. 9 gives the selected ion chromatogram at *m/z* 74 using a capillary coated with RTX-5[4]. Both 3-hydroxybutyric and 3-hydroxycaprinic acid methyl esters could be detected. The same finding was observed when the sample was subjected to the classical saponification procedure. The detection of hydroxy species is partly contrary to a former contribution [30]. In this pioneering paper, hydoxy FAMEs could not be detected in *E. coli*, which was subjected to an in-situ derivatisation–SFE using phenyltrimethyl ammoniumhydroxide, although *E. coli* definitely contains hydroxy fatty acids (mainly 3-OH-C_{14} according to our experience and results given in [31]). In contrast to that, our results provide strong evidence that an in-situ methylation–pressurized liquid extraction combination using boron trifluoride–methanol (the same holds also true for phenyltrimethyl ammoniumhydroxide as well as trimethylsulfonium hydroxide) also leads to hydroxy-FAMEs in the chromatogram (no methoxy-FAMEs were observed) (see [3]). These hydroxy acids could not be detected when using a solvent extraction according to Folch, i.e. they must have been covalently linked in the polymeric lipopolysaccharide network. This, in turn, points to a dissociative mechanism of the derivatisation agents including boron trifluoride–methanol, phenyltrimethyl ammoniumhydroxide and trimethyl sulfoniumhydroxide. In a forthcoming paper [3] the in-situ methylation using different agents should be detailed further, along with the application of this approach to evaluate fermentation processes and to rapid screening of microbial biomasses.

[4]This phase was used to better separate branched and unsaturated FAMEs; both groups coelute on nonpolar coatings such as HP-1 or HP-5.

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