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At-line gas chromatographic–mass spectrometric analysis of fatty acid profiles of green microalgae using a direct thermal desorption interface

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Abstract

Thermally assisted hydrolysis and methylation–gas chromatography (THM–GC) is an important tool to analyse fatty acid in complex matrices. Since THM–GC has major drawbacks such as isomerisation when applied to fatty acids in natural matrices, a direct thermal desorption (DTD) interface and an incubation time of 30 min were used to circumvent these problems. Using vegetable oils such as sunflower oil and triarachidonin, the conversion of triglycerides into their fatty acid methyl esters (FAMEs) was investigated. The yields using a DTD (and trimethylsulfonium hydroxide as a reagent) were found to be similar or even higher than when applying a conventional off-line method, while the FAME profiles were identical. When the procedure was applied to analyse the FAME profiles of microbial cells in a methanolic or aqueous suspension, it was found that accurate profiles are obtained for such samples. Thus, the present approach opens the route to analyse fatty acids in microbial cells in a fully automated fashion, which will allow high sample throughput.

Keywords: Thermal desorption; Methylation; Hydrolysis; Vegetable oils; *Selanstrum chaetoceras*; *Tetraedron minimum*; Fatty acids; Fatty acid methyl esters; Triglycerides

1. Introduction

Fatty acids are the principal components of most natural lipids and portray an immense diversity in terms of chain-length, degree of unsaturation, geometry and substitution pattern. The specificity of the fatty acid composition, often referred to as fatty acid profile or fingerprint, is a particularly typical characteristic of cells, e.g., algae and bacteria. The overwhelming amount of reports that have been published on this subject [1] illustrates that, in life sciences, there is a distinct need for profiling fatty acids from various natural sources.

Over the years, several well-defined procedures have been developed for the analysis of fatty acid profiles [2]. Most studies still use extraction procedures developed in the late 1950s [3,4], which involve extraction procedures with ternary, often hazardous, solvent mixtures, followed by other steps

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like hydrolysis and derivatisation prior to analysis by gas chromatography (GC). Despite the wide application of such procedures, the laborious sample handling and preparation increases the risk of contamination, sample loss and degradation by enzymes and/or oxygen. The latter aspect has been reviewed in detail by Christie [5].

1.1. Thermally assisted hydrolysis and methylation

Thermally assisted hydrolysis and methylation (THM) is increasingly becoming a tool used to determine the chemical composition of, for example, condensation polymers, esters, natural waxes, vegetable oils, animal fats and other classes of lipids in their natural matrices (e.g., Refs. [6,7]). The use of special reagents makes it possible to carry out a one-step hydrolysis/methylation, a so-called transesterification. Such a procedure partially avoids tedious hydrolysis and work-up procedures, and thus increases speed of analysis and repeatability. Still, most of the procedures involve off-line transesterification, followed by liquid–liquid extraction prior to analysis. However, some reagents are suitable for a method that involves application of the sample to a pyrolysis system, which will directly introduce the THM products on the capillary column upon heating. One of the methods often applied is pyrolysis, for instance, for the analysis of samples that do not dissolve in any organic solvent such as polymeric substances [8–10]. In line with this method, some reports describe the introduction of a transesterification reagent together with the sample into a hot injector. Performing the THM reaction in a pyrolysis unit or injector provides a significant advantage over the other procedures described in terms of faster sample handling and less risk of contamination [11,12]. THM has been reviewed in detail by Challinor [13].

When applied to fatty acids in natural matrices, off-line and on-line methods have their own advantages and disadvantages. The off-line method is widely used and is well-known from literature. However, the work-up of small samples is difficult, and contamination and sample loss, consequently, occur rather easily. The application of a pyrolysis unit will eliminate these problems but will also suffer from contamination and quantification problems due to the manual application of the, often small, samples.

These manual steps will not only adversely affect the repeatability of the measurements but, also, hamper the analysis of large series of samples. A number of reagents are available for the transesterification of fatty acids. Common reagents like hydrochloric acid in methanol, sodium methanoate and BF_3–methanol are only used in off-line procedures, while reagents such as tetramethylammonium hydroxide (TMAH), trimethylphenylammonium hydroxide (TMPAH) and trimethylsulfonium hydroxide (TMSH) are most widely used in on-line procedures. Although TMAH is often used for the analysis of complex polymeric structures under pyrolysis conditions [8], it is not suitable for transesterification of unsaturated fatty acids, since it gives rise to isomerisation and degradation reactions [7,14]. Ishida et al. [7] elegantly demonstrated these isomerisation reactions by using triarachidonin, which is a synthetic triglyceride composed of $C_{20:4\ (5,8,11,14\ \text{all-}cis)}$ fatty acids bound to the glycerol backbone. As a result of the high number of double bonds, this lipid is particularly sensitive to isomerisation of double bonds. Under ideal conditions, triarachidonin will generate only a $C_{20:4\ (5,8,11,14\ \text{all-}cis)}$ fatty acid methyl ester (FAME) upon transesterification, but isomerisation often occurs in actual practice. TMPAH is presented as a milder reagent for the on-line analysis of unsaturated fatty acids and is mostly used for applications involving pyrolysis techniques [15,16]. TMSH is a commonly used reagent for the transesterification of triglycerides and applied for the analysis of vegetable oils [11,17,18] and also other organic acids, phenols [19] and pesticides [20]. TMSH has also been applied for the identification of *Legionella* [21] and *Mycobacterium* [22,23] species by fatty acid profiling and even to determine specific metabolic deficiencies in human blood serum [24]. Most of the reports mentioned above use TMSH for off-line transesterification followed by extraction of the reaction mixture to avoid contamination of the injector by high-boiling or charring components.

1.2. Development of procedure

The present paper studies the possibility of using a system that allows automated exchange of the liner

(with or without an insert) of a programmed-temperature vaporiser (PTV) injector. The so-called direct thermal desorption (DTD) interface has been used as the reaction chamber for the transmethylation of microbial fatty acids in their natural matrix. Its capability to automatically exchange the liner will circumvent the problems caused by residues remaining in the liner after the transesterification. Using the DTD interface in combination with an automated sample processor will allow a fully automated analysis sequence, without any manual steps.

Despite the fact that the DTD set-up does not allow hot injection, the rapid heating of the PTV after introduction of the sample and hydrolysis/methylation reagent will create THM conditions in an on-line fashion. Since the final objective of this study is the development of an automated procedure for fatty acid profiling of microbial cells in an aqueous medium, the individual steps involved in the sample preparation must be as straightforward as possible and allow automation. The present procedure involves (i) the injection of an aqueous cell suspension into a special micro-vial placed inside a DTD liner, (ii) subsequent drying by means of applying vacuum, (iii) addition of a THM reagent, followed by a second drying step and (iv) THM–GC–MS analysis. The novel procedure should, of course, provide information that is identical to that which is obtained by a conventional off-line procedure (in this study BF_3–methanol transesterification).

2. Experimental

2.1. Instrumentation

A 3800 GC system (Varian, Walnut Creek, CA, USA) equipped with an Optic 2/200 temperature and pressure programmable injection system (ATAS, Veldhoven, The Netherlands) and an ion trap detector (ITD-MS) (Saturn 2000, Varian) was used. A DTD interface (ATAS) was mounted on top of the Optic 2 injector. With this set-up, the liner of the GC injection interface can be used as a sample container and/or reaction chamber. The combination of a DTD and a FOCUS automated sample processor (ATAS) allows, next to sample and reagent injection into a vial or liner, the automated exchange of liners between a liner tray and the DTD. The injection interface can be heated up to 600 °C, which creates the possibility to perform pyrolysis/chemolysis experiments at heating rates between 1 and 16 °C/s. The GC system was operated at a constant column pressure of 70 kPa using a CP-SIL 8CB/MS (Varian) fused-silica capillary column (15 m×0.32 mm I.D., 0.25 μm film thickness), which was connected to the ion source of the ITD via a 1-m fused-silica restriction (0.5 m×75 μm I.D.). For detection and identification, the ITD settings were: trap temperature, 220 °C; interface temperature, 270 °C; manifold temperature, 50 °C; A/M amplitude, 4.0 V; emission current, 10 μA; AGC (Automatic Gain Control) target set at 10 000; scan time, 1.7 s and scan range, m/z 40–650.

2.2. Materials

For THM the following reagents were tested: TMAH (0.25 *M* in MeOH, Acros, Belgium), TMPAH (0.1 *M* in MeOH, Fluka, Buchs, Switzerland) and TMSH (0.25 *M* in MeOH, Fluka). For reference purposes, transesterification with BF_3–MeOH (12:88, Acros) was used. As THM samples, the following vegetable oils were purchased from local stores: sunflower oil, hazelnut oil, walnut oil, almond oil and olive oil. To test isomerisation reactions, triarachidonin (Sigma, St. Louis, MO, USA) was subjected to THM. Test microorganisms were the freshwater green micro algae *Selanstrum chaetoceras* obtained from a continuous culture and *Tetraedron minimum* from a batch culture.

2.3. THM of triglycerides

The following dichloromethane solutions of triglycerides were used: 0.41 mg/ml sunflower oil, 0.33 mg/ml hazelnut oil, 0.49 mg/ml walnut oil, 0.41 mg/ml almond oil, 0.28 mg/ml olive oil and 0.10 mg/ml triarachidonin. To study the dependence of FAME yields upon THM of triglycerides, the FOCUS was programmed to transfer 1 μl of a triglyceride solution into a GC sample vial with insert. Subsequently, 9 μl of a 0.1 *M* TMSH solution was added (0.025 *M* in the case of triarachidonin) and the two solutions were mixed by multiple strokes

of the syringe plunger. Next, 1 μl was injected into the DTD liner at 40 °C and dried for 5 s under solvent vent conditions. After venting, the temperature was increased to 350 °C at 16 °C/s under splitless conditions. The GC was programmed from 70 °C (3 min hold time) to 120 °C at 30 °C/min and next, at 8 °C/min to 320 °C (5 min hold time).

2.4. THM of S. chaetoceras and T. minimum

After careful optimisation (see Results) the final procedure was as follows: 2 μl of a 0.90 (*S. chaetoceras*) or 0.85 (*T. minimum*) mg/ml aqueous suspension were injected into a 40-μl vial placed inside a capped liner. Subsequently, the liner was placed in an in-built vacuum system, courtesy the magnetic tip of the autosampler, and allowed to dry. After complete drying, 1 μl of a 0.05 *M* TMSH solution in methanol was injected and the reaction mixture allowed to incubate for 30 min. Next, the content of the insert was dried and transferred to the DTD interface. Finally, THM was performed at 350 °C with subsequent GC under the conditions described in Section 2.3. To increase sample throughput, preparation of $(n+1)$th sample was performed simultaneously with GC analysis of the nth sample.

2.5. Off-line transesterification

A 0.5-ml volume of a sample solution or aqueous suspension (cf. Section 2.4) was dried under N_2 and 0.5 ml of BF_3–MeOH (12:88, Fluka) was added. The reaction mixture was heated to 80 °C for 30 min. Subsequently, 0.5 ml water was added. The aqueous layer was extracted with 1 ml hexane (3×) and the combined organic extracts were concentrated to 500 μl. A 2-μl volume of the final solutions was injected in the splitless mode at an injector temperature of 40 °C. GC–MS conditions were the same as those reported above.

2.6. Deactivation of liner inserts

New liner inserts (ATAS) were soaked for 12 h in aqueous 2 *M* HCl. After washing with double-distilled water, they were heated at 500 °C in an oven for 3 h. Next, they were placed for 12 h in a 25% solution of dimethyldichlorosilane (Supelco, Bellefonte, PA, USA) and finally washed with toluene (3×), ethyl acetate (3×) and methanol, followed by 30 min drying at 100 °C.

3. Results and discussion

3.1. DTD set-up

Since DTD is, as yet, not widely used as an injection device for GC, a brief introduction of the technique is required. Rather than in conventional injection techniques, samples are applied onto a newly developed liner, which is capped with a crimp cap made of stainless steel. The liners are placed in an ordinary sample tray. In order to be analysed, each liner is transported to the injector for thermal desorption. A special pneumatic opening/closure device has been developed to facilitate this option. Both liner transport/exchange (which occurs after each analysis) and opening/closure of the injector are automated. The system is especially suitable for analyses in which sample constituents remain in the liner after transfer of the analytes to the GC column, i.e., for solid samples and samples containing high-boiling by-products. In order to study the potential of the above system for the determination of fatty acids in biological material, three main parameters had to be studied, viz: (i) hydrolysis of the fatty acids from the membranes, (ii) their chemical conversion to methylesters and (iii) the quantitative transfer to the GC column. Next, the performance of the system was demonstrated by running several applications.

3.2. Methylation and hydrolysis of fatty acids

Initially, TMPAH, TMAH and TMSH were tested for the methylation of free fatty acids in standard solutions, viz, saturated fatty acids from $C_{14:0}$ to $C_{24:0}$ and unsaturated ones from $C_{14:1}$ to $C_{24:1}$.The reagent and sample solutions were separately injected into the cold liner under solvent vent conditions.

Upon heating the injector, methylation and transfer of the reaction products took place. Preliminary

GC–ITD-MS analyses showed that, with our set-up, methylation can indeed be performed inside the glass liner of the interface. To test the combined hydrolysis-plus-methylation capability of the reagents when using the DTD set-up, experiments were performed with triglycerides present in common vegetable oils and triarachidonin. Preliminary results showed that TMAH is not suitable for our studies. First, much double-bond isomerisation was found to occur, as was also reported by Ishida et al. [7] and Jun-Kai et al. [14]. The high degree of isomerisation (>80% of total area) does not only reduce the amount of proper or expected unsaturated fatty acids, but also makes distinction of naturally occurring unsaturated fatty acid from a product difficult. Fig. 1a illustrates the undesired effect of isomerisation; all asterix (*) labelled peaks should not have been in the chromatogram at all. In addition, partial methylation occurred at the C2 position of the FAMEs generated by this reaction, as could be deduced from the presence of compounds containing both m/z 88 and 101 as important fragment ions corresponding to $[CH_3–CH_2=COOCH_3]^+$ and $[CH_2=CH(CH_3)–COOCH_3]^+$ ions, respectively. Therefore, further experiments were limited to the milder reagents, TMPAH and TMSH. The next step was to optimise the THM reaction in the DTD interface/PTV set-up to obtain acceptable results for triglyceride mixtures.

3.3. THM of triarachidonin with TMPAH

The optimum ratio of triarachidonin and TMPAH for THM was experimentally determined by injecting different amounts of reagent into a liner after application of a fixed amount of triarachidonin and solvent evaporation. GC–ITD-MS showed that, although this reagent performs much better than TMAH, the isomerisation reactions as described in the previous section for TMAH cannot be avoided when using the DTD interface and aiming at a high conversion of the triglycerides.

For fast hydrolysis of the triglycerides, a large amount of reagent is required. Once the free fatty acids have been produced, the remaining reagent causes isomerisation. Moreover, intermolecular reactions of the reagent molecules gives rise to the formation of various phenyl- and nitrogen-containing side-products which co-elute with the FAMEs. As a consequence, lower reagent concentrations had to be used. This resulted in less isomerisation, but also yielded unacceptably low recoveries of the FAMEs: there simply was not sufficient reagent to complete both hydrolysis and methylation (Fig. 1b).

Attempts to use different heating regimes (2–16 °C/s) and reaction temperatures (240–350 °C) did not improve the situation significantly.

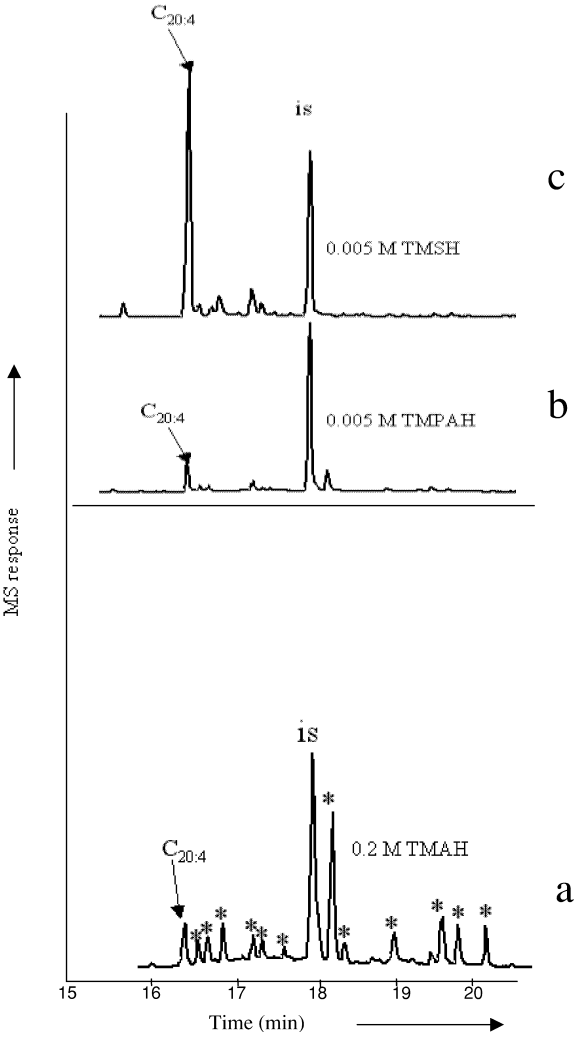

Fig. 1. Total ion current (TIC) response of TMAH, TMPAH and TMSH illustrating the difference in conversion efficiency of triarachidonin into $C_{20:4}$ FAME (all-*cis*). *: $C_{20:4}$ isomers; is= internal standard (C_{22} *n*-alkane).

3.4. THM of triarachidonin and vegetable oils with TMSH

When TMSH was used instead of TMPAH, two observations were made. When identical concentrations were used, the yield of $C_{20:4\ (5,8,11,14\ all\text{-}cis)}$ FAME was significantly higher and the degree of isomerisation much lower (Fig. 1c). Despite this significant improvement of the quality of the analysis, isomerisation at high reagent concentrations made optimisation of the reaction conditions necessary. Furthermore, care had to be taken to avoid the introduction of un-reacted TMSH into the capillary column, since it reacts with the CP-Sil 8 CB stationary phase, as was also observed by Amijee et al. [25]. After testing different injection schemes and conditions, the best results for triarachidonin were found to be obtained by mixing of sample and reagent prior to injection into the liner at 40 °C, followed by a solvent vent and heating to 350 °C. Obviously, local low/high reagent/analyte ratios in the liner, which may occur when no premixing is done, should be avoided as they can cause an overall low FAME yield and a higher degree of isomerisation. On the other hand, isomerisation was less than when a premixing step was applied. Evaluation of the reagent/analyte ratio resulted in a distinct optimum. Fig. 2 illustrates that the reagent/analyte ratio should lie between 1400 and 3800 if a 90–100% yield, compared with the optimum value, is set as the target. At low excesses of TMSH, the yields are low due to insufficient availability of the reagent for transesterification; at high concentrations, isomerisation of double bonds decreases the yield of the methylesters of the original unsaturated fatty acids. When the same approach was used to analyse vegetable oils, the FAME yields were found to be similar to those obtained by the off-line procedure except for walnut oil, as is illustrated in Fig. 3.

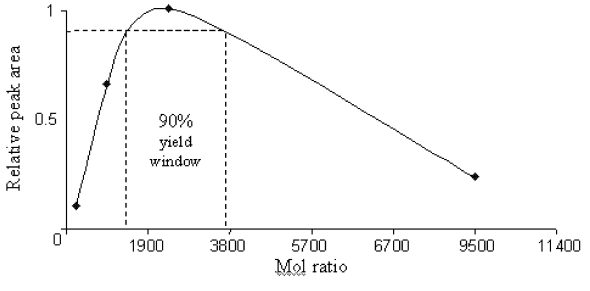

Fig. 2. Relative peak area of the $C_{20:4(5,8,11,14\ all\text{-}cis)}$ FAME released upon THM of triarachidonin with TMSH. For conditions, see text.

3.5. THM of microbial fatty acids from S. chaetoceras and T. minimum with TMSH

The strategy derived from the experiments with the triglycerides was used to optimise the FAME profile analysis of microbial cells. As our intention was to use aqueous cell suspensions for these experiments, a drying step had to be performed prior to analysis in order to prevent water from entering the GC column.

Furthermore, the introduction of water into the reaction mixture had to be avoided, since it may cause unwanted side-reactions as will be described below. Therefore, the microbial cell suspension had to be introduced prior to the reagent, with drying being performed in between. In order to obtain, in the end, a fully automated system, a vacuum set-up, which should allow at-line drying of the liner and its content prior to transfer to the DTD and analysis, was tested. In order to effect the pre-mixing recommended in Section 3.4, the sample and reagent

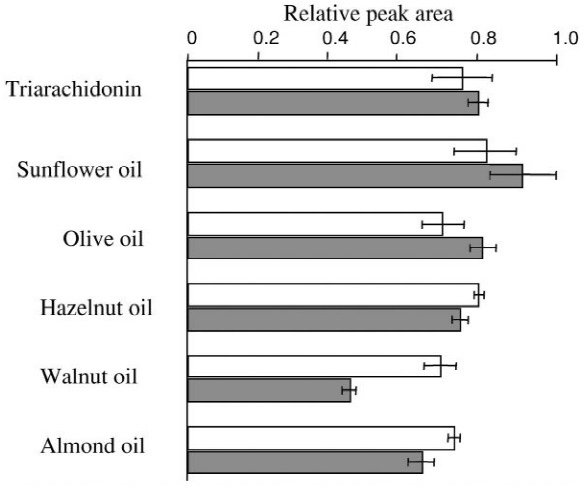

Fig. 3. Comparison of the summed relative peak areas of the C_{16}, $C_{18:2}$, $C_{18:1}$ and C_{18} FAMEs upon THM–GC–MS of different triglyceride samples when using the off-line method (grey) or at-line (white) procedure ($n=6$). Internal standard, C_{22} *n*-alkane.

were introduced into the 40-μl vial referred to earlier, which enabled better mixing of the microbial organism and TMSH. Utilising deactivated liner inserts resulted in the intended improvement and moreover, the hydrophobic properties of the deactivated glass further concentrated the sample and reagent at the bottom of the insert. To evaluate the effect of at-line drying, 1.8 μg of lyophilised microalgal cells in 2 μl methanol or 2 μl water were injected into the liner 40-μl vial. Next, drying was effected by applying vacuum for 3–5 min.

When the samples were completely dry before introduction of the reagent, 0.05 *M* TMSH in MeOH, no differences were observed in the chromatograms of an aqueous and a methanolic cell suspension. On the other hand, the presence of small amounts of water caused additional peaks to occur in the chromatograms. These can be attributed to chemolysis products of proteins, sugars and/or other cell constituents formed due to a strong hydrolysing action of the reaction mixture during incubation. By varying the time prior to evaporation of the methanol in the reaction mixture, it was found that, at room temperature, an incubation time of 30 min is essential to allow the reagent to enter the cells and hydrolyse the ester-bound fatty acids. Furthermore, other cell constituents scavenge part of the excess reagent during incubation, which causes isomerisation of unsaturated fatty acids to be more limited. Consequently, after 30 min the liner was placed inside the vacuum system and dried for 5 min. According to the literature, the temperature for THM reactions involving TMSH should be 350 °C. The results of a brief optimisation study are shown in Fig. 4. According to these experiments, the optimum temperature for *S. chaetoceras* was about 275 °C and that for *T. minimum* around 350 °C. Next to the higher optimum temperature for *T. minimum*, one should note that the FAME yields for this species were very low at temperatures below 350 °C. Both effects are probably caused by the presence of an inaccessible aliphatic-polymeric cell wall.

This so-called algaenan, which is not present in *S. cheatoceras*, physically blocks the release of the FAMEs from within the cell at lower temperatures. In general, the lower temperature limit in studies such as are presented here, is determined by the thermal energy needed to complete the methylation

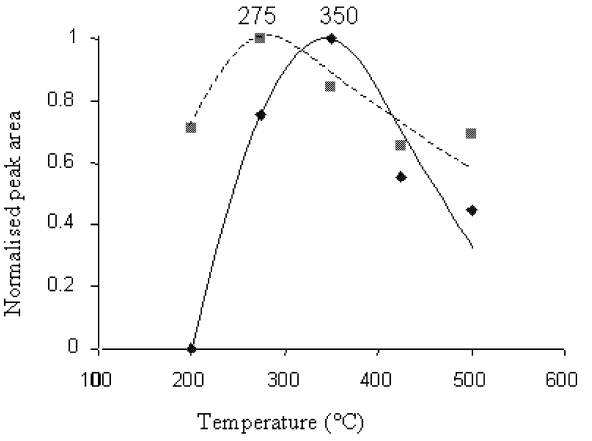

Fig. 4. Normalised peak areas of the summed FAMEs released upon TMSH-based THM of lyophilised cultures of *S. chaetoceras* (♦) and *T. minimum* (■) as a function of temperature.

reaction and release the FAMEs from their natural matrix. On the other hand, at high temperatures, charring of the matrix occurs, which traps the reaction products before they can be released. For all further work, a temperature of 350 °C was preferred to ensure compatibility of the results obtained for both organisms. As in the case of the triglycerides, there is a distinct optimum of the reagent/analyte ratio for THM of the green microalgae (Fig. 5). The 90% yield window is in the range 0.02–0.05 (mol reagent/g sample). These results suggest that in order to perform methylation under optimum con-

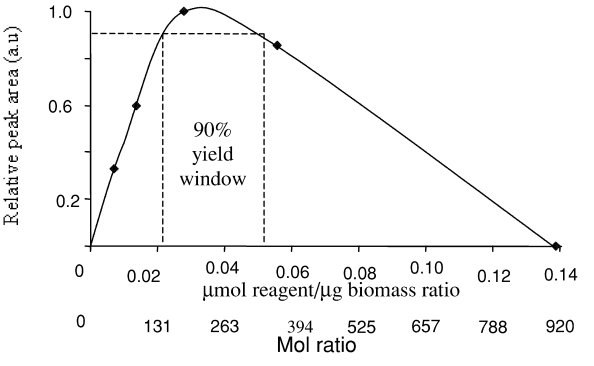

Fig. 5. Normalised peak area of the $C_{18:2}$ FAME released upon TMSH-based THM of lyophilised culture of *S. chaetoceras* as a function of reagent/biomass ratio (μmol:μg) converted into mol ratio for a calculated triglyceride content of 13% (cf. Fig. 6). Similar results were obtained for *T. minimum*.

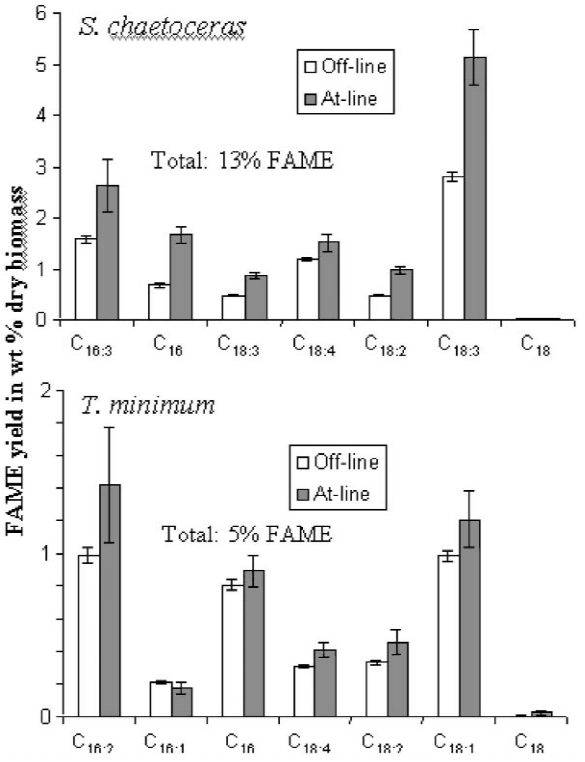

Fig. 6. FAME yield in % (w/w) of dry biomass upon THM with TMSH of lyophilized *S. cheatoceras* and *T. minimum* cells.

ditions, one has to know the approximate amount of fatty acids in a sample.

However, the optimum reagent/sample-amount ratio was found to be essentially the same for both *T. minimum* and *S. chaetoceras*, even though the total FAME concentration in the latter algae is considerably higher (13 vs. 5%) (Fig. 6). Since the same amount of dry biomass was used for both micro-organisms, the excess of TMSH—which is higher in the case of *T. minimum* with respect to the fatty acids—is probably neutralised by the higher amount of other cell materials, which confirms our earlier suggestion (cf. Section 3.4). Admittedly, this effect has to be further investigated but, at this stage, one may suggest that it can help to circumvent problems with microbial samples containing an unknown amount of fatty acids. When performing THM of microbial cells using the conditions described above, the excess of TMSH required for optimum conditions is much lower than observed for the triglycerides. Whereas the 90%-yield window was found for a reagent/analyte (μmol/μg) ratio of 1800–3800, in the latter case (cf. above), the optimum value with *S. chaetoceras* and *T. minimum* was in the 130–330 range. This difference is probably due, in part, to the longer time of hydrolysis inside the liner insert (viz. 30 min instead of direct heating in the injector). Furthermore, phospholipids, which are the predominant class of ester-bound fatty acids, will most likely have reaction kinetic which differ from those of triglycerides. Moreover, unlike the triglycerides, they easily dissolve in methanol, which will make them more accessible to the reagent during incubation.

A comparison of the yields of the dominant fatty acids obtained by means of the off-line and at-line procedure showed that the latter is just as effective, or even slightly better, in releasing FAMEs from a

Table 1
Concentrations of dominant FAMEs in lyophilised microbial biomass of *S. chaetoceras* and *T. minimum* and their RSDs (n=6) upon THM–GC–ITD-MS

S. chaetoceras				*T. minimum*			
Compound	% (w/w)	RSD (%)	RSD[a] (%)	Compound	% (w/w)	RSD (%)	RSD[a] (%)
$C_{16:2}$	2.63	19	14	$C_{16:n}$	1.42	25	9
C_{16}	1.67	9	6	$C_{16:1}$	0.18	21	13
$C_{18:3}$	0.87	6	3	C_{16}	0.89	10	4
$C_{18:4}$	1.52	11	5	$C_{18:4}$	0.40	12	9
$C_{18:2}$	0.99	7	3	$C_{18:2}$	0.45	15	13
$C_{18:3}$	5.15	10	7	$C_{18:1}$	1.21	14	3
C_{18}	0.04	4	7	C_{18}	0.02	16	12

[a] RSD value obtained by determination of standard deviation of peak areas relative to total peak area, which represents the RSD of the FAME profile.

Table 2
Concentrations of dorminant fatty acids in the lyophilised microbial biomass of *S. chaetoceras* and *T. mimimum* as determined by using off-line BF_3–MeOH and their RSD values ($n=6$)

S. chaetoceras			*T. minimum*		
FAME	% (w/w)	RSD (%)	FAME	% (w/w)	RSD (%)
$C_{16:2}$	1.58	5	$C_{16:n}$	0.99	5
C_{16}	0.69	7	$C_{16:1}$	0.21	4
$C_{18:3}$	0.50	3	C_{16}	0.81	4
$C_{18:4}$	1.21	2	$C_{18:4}$	0.31	3
$C_{18:2}$	0.50	4	$C_{18:2}$	0.33	4
$C_{18:3}$	2.82	3	$C_{18:1}$	0.99	3
C_{18}	0.04	13	C_{18}	0.05	12

natural matrix although the repeatability was worse (Tables 1 and 2). However, if the repeatability of the FAME profiles is considered, the results are much better (Table 1). Most likely, this indicates that the total amount of FAMEs released upon THM fluctuates more in the at-line operation, but without affecting the overall profile. In at-line THM, several additional compounds were generated next to the FAMEs, which were absent in the off-line method. Fig. 7 shows the C_{14}–C_{18} FAME window of the total ion current (TIC) traces, which reveals the presence of two phytols. Phytol is the ester-bound isoprenoid alcohol bound to chlorophyll, which is obviously more effectively released from the cellular matrix when THM is applied. Further more small amounts of several methoxylated sterols (results not shown) are observed. Further study is required to find out if the quantities of these compounds released by the THM reaction are representative for their actual amount in the organism and whether they reflect a species-specific distribution, which can be used for identification purposes. Finally, the presence of hydroxylated fatty acids, compounds that do not show up in the conventional off-line procedure, could be confirmed in the present approach. Fig. 8 shows the ion trace of m/z 103, which is the most abundant ion in fatty acid profiles of *Escherichia coli*. The peak eluting at 12.25 min could be identified as a 3-hydroxy-C_{14} acid. Its identity was confirmed by means of the masses m/z 259, 241 and 103, which correspond to $[M+H]^+$, $[M+H-H_2O]^+$, and $[CH(OH)-CH_2-COOCH_3]^+$ ions, respectively. Due to the well-known self-ionisation effect occurring in ion-trap MS systems, m/z 259, $[M+H]^+$, rather than m/z 258 is observed.

4. Conclusions

The present paper illustrates that it is possible to perform transmethylation reactions in the liner of a DTD interface. The method allows a rapid and sufficiently precise conversion of triglycerides into the corresponding FAMEs, which can be analysed by GC–MS. The fatty acid profiling of triglycerides by

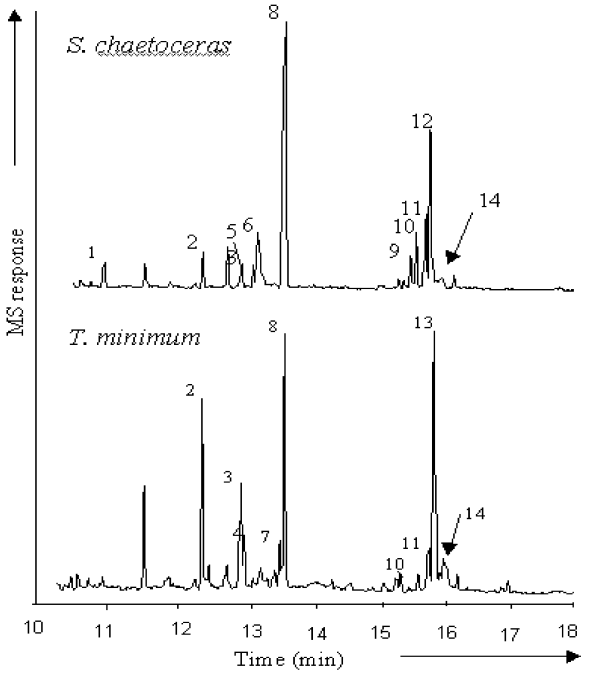

Fig. 7. TIC traces of C_{14}–C_{18} FAME window as observed after THM with TMSH of lyophylised cells of *S. chaetoceras* and *T. minimum*. Peak assignment of FAMEs: (1) C_{14}; (4) $C_{16:n}$; (5) $C_{16:2}$; (6) $C_{16:3}$; (7) $C_{16:1}$; (8) C_{16}; (9) $C_{18:3}$; (10) $C_{18:4}$; (11) $C_{18:2}$; (12) $C_{18:3}$; (13) $C_{18:1}$; (14) C_{18}; phytols: (2) and (3).

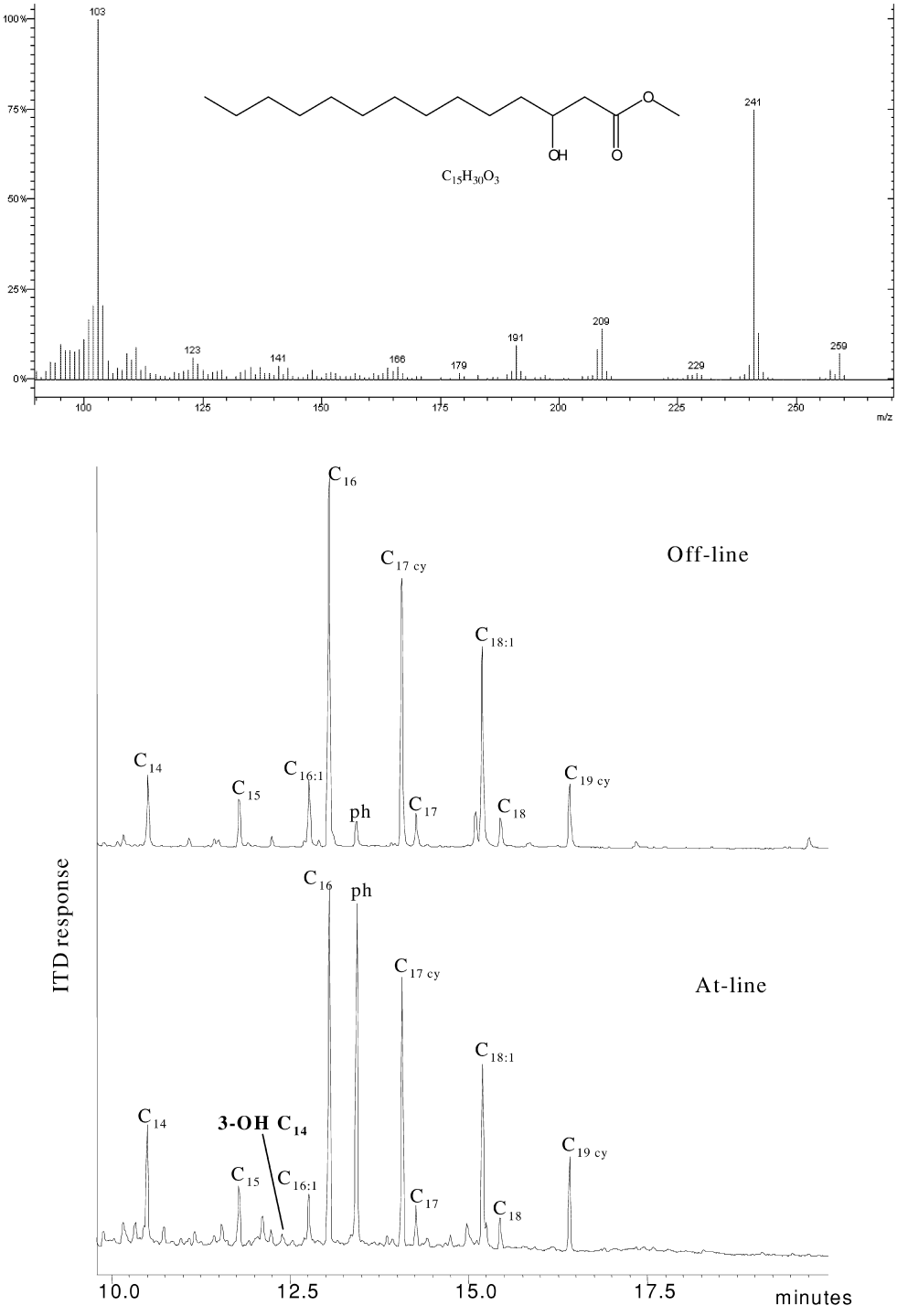

Fig. 8. GC–ITD-MS (m/z 103): comparison of off-line and at-line FAME profile of *E. coli*. The at-line method shows the presence of 3-hydroxy C_{14} FAME; its mass spectrum is displayed in the top frame. ph: Pthalate; cy: cyclopentane.

THM-based methods is well documented, but the distinct advantage of the DTD approach is the possibility to directly analyse fatty acids in complex matrices which leave behind a residue in the injector. In combination with an automated sample processor, one can now exchange the liner after every analysis. In addition, sample preparation and at-line drying of aqueous samples can be included. This unique combination offers the opportunity to (i) directly inject an aqueous cell suspension of, in this case, microalgae, (ii) perform THM inside the liner, and (iii) transfer the released compounds to the GC column. First results regarding analytical performance data and robustness of the novel approach are promising, and also with regard to FAME yields and profiles as compared with current off-line procedures. Full automation will require the implementation of a vacuum source as an add-on to the Focus autosampler for sample drying.

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