Analysis of whole/untreated biological samples using direct thermal desorption combined with gas chromatography

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The research presented in this thesis was sponsored by The Netherlands Science Foundation
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ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor aan de Vrije Universiteit Amsterdam, op gezag van de rector magnificus prof.dr. L.M. Bouter, in het openbaar te verdedigen ten overstaan van de promotiecommissie van de faculteit der Exacte Wetenschappen op maandag 17 december 2007 om 13.45 uur in de aula van de universiteit, De Boelelaan 1105

door
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gleboren te Accra, Ghana
“To every thing there is a season, and a time to every purpose under the heaven”. (Eccl. 3:1)

When upon life's billows
You are tempest tossed
When you are discouraged
Thinking all is lost
Count your many blessings
Name them one by one
And it will surprise you
What the Lord has done

Count your blessings
Name them one by one
Count your blessings
See what God has done
Count your blessings
Name them one by one
Count your many blessings
See what God has done

Are you ever burdened
With a load of care
Does the cross seem heavy
You are called to bear
Count your many blessings
Every doubt will fly
And you will be singing
As the days go by

Refrain

So, amid the conflict
Whether great or small
Do not be discouraged
God is over all
Count your many blessings
Angels will attend
Help and comfort give you
To your journey's end

Refrain

Words of Johnson Oatman, 1897, in his famous soul uplifting song “Count your blessings”. 
Dedicated to my dear parents
Analysis of whole/untreated biological samples using direct thermal desorption combined with gas chromatography

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**Scope of thesis**

This research investigated gas chromatographic analysis of fatty acids of whole/intact biological samples using a direct thermal desorption interface (DTD) inlet system in a fully automated manner using, mainly, trimethylsulfonium hydroxide as methylating reagent. After the general introduction which discusses various methods used in the characterization of fatty acids (Chapter 1), the development of a novel procedure for the analysis of fatty acids of various biological specimens is discussed in Chapters 2&3. Finally, exploitation of the new inlet system for comprehensive two dimensional DTD-GCxGC-ToF-MS analysis of aquatic mesofauna species is described in Chapter 4.

Traditionally, fatty acid analysis is carried out using the tedious and lengthy manual offline Bligh-Dyer method. **Chapter 2** takes a look at the development of a new procedure for the analysis of fatty acids of whole/intact biological specimens with little or no samples pre-treatment.

In **Chapter 2.1**, the foundation for this novel technique is laid in which the problems associated with the traditional extraction procedure such as lengthy and tedious extraction procedures, exposure to contamination, isomerization/degradation of fatty acids and limitations for automation are addressed. To this end, a system was developed that allowed automated exchange of liners (with or without an insert) into a programmed-temperature vaporiser (PTV) injector. The so-called direct thermal desorption interface was used as a reaction chamber for transmethylation of fatty acids. The novel system was optimised using various oil and pure micro-algae samples.

**Chapter 2.2** reports on further optimisation of the novel procedure for application to real life aquatic micro-algae and mesofauna specimens. At-line fatty acid analysis of flow-cytometric sorted green micro-algae and cyanobacteria and hand-picked freshwater mesofauna species were performed, and the results were compared with conventional off-line procedures. It was shown that the at-line procedure was suitable for direct thermally assisted hydrolysis and methylation (THM) of the cellular fatty acids of whole/intact cells and animals. The FAME profiles were the same as those obtained using the conventional off-line lipid extraction procedure followed by hydrolysis and
methylation with BF$_3$ in methanol. The good performance with respect to fatty acids profiling, automation and the absence of side-reactions/isomerization of PUFAs, opened the way to use DTD-THM as an inlet technique in compound-specific stable-isotope ratio ($^{13}$C/$^{12}$C) measurements, as discussed in Chapter 2.3. The DTD in-let system was coupled to a gas chromatograph linked on-line to an isotope-ratio mass spectrometer (GC-IRMS), and the results thus obtained compared to the performance of at-line flash pyrolysis-GC-IRMS in analysis of phyto- and zooplankton specimens. Fatty acid profiles and their isotopic signatures were shown to be very similar in both techniques.

Chapter 3 discusses the application of the novel technique to analysis of pollen, crude soil lipid extracts and human blood/plasma samples. Chapter 3.1 investigates the possibility of using DTD-THM to analyse p-coumaric and ferulic acids, the monomers of the UV-absorbing sporopollenin. Here, thermally assisted hydrolysis and methylation was realized with tetramethyl ammonium hydroxide as methylation reagent. It is shown that very low amounts of pollen (i.e. down to 6 grains) are required to obtain quantitative results, thus enabling sporopollenin analysis on pollen and spores retrieved from sources/environments where sampling of such specimens in high numbers are difficult (e.g. soil, sediments etc.).

In Chapter 3.2, the novel procedure was used to profile fatty acids in crude soil lipid extracts and the results were compared to those of the traditional analytical protocol involving several subsequent fractionation steps. Yields and profiles of fatty acids were observed to be very similar in both procedures. Additionally, it was demonstrated that the at-line method could be used to monitor and quantify population dynamics of environmentally relevant bacteria in complex environmental matrices. The major advantage of the novel method is the substantial reduction in the processing time of soil samples as the traditional manual fractionation of lipid classes and off-line methylation is eliminated from the sample processing scheme. Furthermore, substantially fewer amount of solvent is required compared to the traditional off-line procedure.

Chapter 3.3 discusses the application of the DTD-THM technique in the clinical field, that is, fatty acid profiling of human blood and plasma. Thus far, standard clinical procedures involved a Bligh and Dyer off-line lipid extraction of the samples (or a modification of it). The goal of this research was to investigate the possibility of a fast
and reliable FA-profiling of human blood/plasma omitting the Bligh and Dyer extraction step. Yields of blood/plasma fatty acids turned out to be similar for saturated fatty acids or even higher in the case of polyunsaturated fatty acids compared to the traditional off-line procedure. In addition to its advantage of being operated in a fully automated manner, and thus allowing the analysis of large sample series, another advantage of this procedure is that very low sample amounts (2-3 µl) are required compared to the 50-100 µl usually applied in the conventional off-line procedures.

In Chapter 4 the coupling of the novel inlet system to a comprehensive gas chromatography combined with time of flight mass spectrometer (GCxGC-ToF-MS) is discussed. Chapter 4.1 investigates the possibility of using the high resolution of the GCxGC technique to separate peaks that often co-elute in one dimension (1D) GC analysis of many phyto- and zooplankton species. It was shown that the technique can be useful in the identification of fatty acids that may serve as lipid biomarkers in food-web and/or ecological studies. Several new mono-, di- and tri-unsaturated fatty acids were found in the C_{16}, C_{18}, C_{20} and C_{22} regions of common freshwater plankton representatives belonging to green algae, cyanobacteria and copepods. These fatty acids were not detected in the conventional one dimensional (1D) GC analysis due to either co-elution and/or their presence in low amounts in the sample matrix. Additionally, in the GCxGC technique all congeners of the fatty acids in these micro-organisms could be detected and identified due to the increased analyte detectability and ordered structures in the separation space.
Chapter 1

General overview of Analytical approaches for FAME analysis
A short review of analytical techniques for the analysis of fatty acids methyl esters of biological specimens

1. Introduction

Fatty acid analysis continues to be an important aspect of many research directions such as microbiology, ecology, food-web and clinical studies etc. Fatty acids are the principal components of most natural lipids. They show a great degree of diversity in terms of chain-length, geometry, degree of unsaturation, position of double bonds as well as substitution pattern. The specificity and definitiveness of the fatty acid composition, often referred to as the fatty acid profile or fingerprint/pattern, is particularly characteristic of the lipid and their provenient organisms. The numerous publications made on this subject [1] is a testimony to the fact that, in life sciences, there is a distinct need profiling fatty acids of various natural sources. In order to determine the fatty acid composition of a biological sample, the lipids must first be isolated from the tissue sample (and in many cases further separated into various lipid classes), derivatized and then quantified. The procedure most used for the extraction of lipids from the tissue matrix are those proposed by Folch et al [2] or its variant as reported by Bligh and Dyer [3]. In both procedures, a 1:2 chloroform-methanol mixture is applied to remove the lipids from the sample matrix, whilst the water derived from the sample matrix generates a ternary solvent mixture.

In order to remove contaminants, aqueous potassium chloride (KCI) solution is used to wash the extracted lipids. Derivatization involves the conversion of fatty acids into their corresponding methyl esters (as well as other derivatives) which are volatile compounds, have improved thermal stability and peak shape (i.e. better resolution) in subsequent analysis. This step may be carried out by means of acid catalysis using hydrochloric acid [4,5], sulfuric acid [6] or boron trifluoride (BF₃) [7,8], all in methanol, by alkaline catalysis using sodium methoxide in anhydrous methanol [9] or by using a combination of the two methods [10]. The application of diazomethane as a methylation reagent has also found favor in other derivatization procedures [11]. Details and other significant considerations in lipid isolation from the sample matrix are well documented [12].

A number of chromatographic procedures have been used for the separation of lipids into various classes. Of the lot, normal-phase systems in which the solutes are retained based on their
relative polarity, are the most used. This is normally achieved with the aid of silica supported in a simple glass column, a thin layer chromatography (TLC) plate, a solid-phase extraction column or a high-performance liquid chromatography (HPLC) column. After sample application, solvent mixtures of increasing polarity are then used to elute lipids off the bed or column in an increasing order of polarity. In recent times, a reversed-phase (RP) system with low-wavelength UV detection [13] and a cyanopropyl stationary phase with evaporative light scattering detection [14] have been used instead of silica.

In the case of complex lipid samples which contain a lot of unsaturated fatty acids, separation of the fatty acids into classes are frequently followed by further fractionation usually with the aid of silver ion chromatography in the thin-layer or column mode, often after their conversion into their methyl esters. In such argentation chromatography [15], as they are commonly known, FAME separation depends on the number, configuration and to some extent the position of the double bonds. The number of double bonds is the dominant/governing factor, but the cis isomers tend to be retained distinctly more strongly than their trans counterparts. For fractionation of samples according to the geometric isomers and number of double bonds, various strategies have been developed. Undoubtedly, gas chromatography (GC) and high performance liquid chromatography (HPLC) are the most used separation techniques in fatty acid (FA) profiling of lipids.

2.0 Gas chromatography
2.1 General

Compared to HPLC, GC remains the favorite of most analysts when it comes to fatty acids (FAs) analysis. A requirement for GC analysis is that FAs must be derivatized to volatile derivatives to be transported in the gas phase. The majority of FAs in lipids are bound as esters or amides, with only a small number present as non-esterified or the so-called free fatty acids (FFAs). Depending on the information needed, FFAs which may be present in the sample matrix in low quantities are converted into FAMEs together with the lipid-bound fatty acids.

In some instances, they are measured separately after preliminary fractionation into lipid classes. Thus, the procedure that may be used for derivatization may well depend on the sort of information being sought.

For GC analysis of lipid-bound fatty acids, transesterification of the FAs to their
corresponding FAMEs is the method of choice. Presently, a lot of different techniques are in use and this includes the time-tested acid- and base-catalyzed reactions mentioned earlier, on-column pyrolytic methods and derivatization using other reagents. Each of these procedures have their advantages and disadvantages and they have been well reviewed [16 –22]

2.2 Derivatization

2.2.1 Carboxyl group

The main advantage of acid catalysis is its capability to convert both bound and free fatty acids to their corresponding FAMEs at the same time. BF₃, HCl and H₂SO₄ are the most extensively used acid catalysts, usually as 14%, 5% and 2% solutions respectively. The most commonly used among the trio is BF₃. Using BF₃, FFAs and FA’s present in phospholipids, triacylglycerols (TAGs) and cholesteryl esters are converted to their corresponding FAMEs in 2, 5–10, 25 and 60 min. respectively at 100 °C [23]. Nearly twice as much time is needed with the application of HCl and H₂SO₄ [24]. Perchloric acid, which is not liked very much by analyst due to its putative explosive hazards, may be used as a substitute for of HCl and H₂SO₄ in similar concentrations. The shorter reaction times realized when BF₃ is applied may be explained by the higher concentration of BF₃ used compared to the other acids [25]. This same factor (i.e. higher concentration) may also be responsible for the frequent observation of artifacts [26,27].

These short-comings can, however, be reduced to the barest minimum if a preliminary saponification with methanolic KOH is performed followed by re-esterification of the FFAs under mild conditions [28 –30]. It has been shown that with 14% BF₃, the reactions times for FFAs, phospholipids and TAGs given above could be drastically reduced to 20, 30 and 60 s respectively if the conventional heating system is replaced with microwave irradiation, although the power and reaction volume may affect the duration of irradiation [31,32]. Comparable reductions have been found with the application of 2% H₂SO₄ catalyst [33]. In addition, oxidation of unsaturated acids is greatly reduced by the irradiation procedure compared to the ordinary heating in glass tubes [34]. BCl₃ and AlCl₃ are two other important acid catalysts. BCl₃ has been shown to be a much milder methylation reagent than its fluorine analog [35,36]. The use of AlCl₃, on the other hand, results in transesterification of lipids. It is however, unable to methylate FFAs to their corresponding methyl esters [37]. The strong point of acid catalysis is its ability to concurrently methylate both bound and free fatty acid.
However, if the amount of FFAs is not significantly large in the sample matrix, then alkaline methylation reagents should be the preferred choice. In addition to their faster reaction rate, base-catalyzed transesterification reactions do not cause labile degradation and/or isomerization of double bonds of fatty acids. For example, 1M methanolic NaOCH$_3$ solution has been shown to require 1.5 and 60 min. to completely convert phospholipids, TAGs and cholesteryl esters into their corresponding fatty acid methyl esters respectively at room temperature [19].

Methanolic solutions of sodium or potassium hydroxide are the other commonly used base-catalysts. Due to the fact that methanol is more acidic than water, (pH$_{water}$ < pH$_{methanol}$), the hydroxide ion (OH$^-$) exists predominantly as methoxide ion (CH$_3$O$^-$) in methanol [38]. A brief description of the base-catalyst reaction is as follows: A reversible transmetylation reaction and an irreversible saponification reaction occur simultaneously in the presence of OH$^-$ and CH$_3$O$^-$ ions. As a result of the fact that transmethylolation reaction proceeds faster than saponification reaction, maximum FAME yield is rapidly achieved. This is, however, followed by a slow but steady decline in the FAME yield as a result of the saponification reaction (of the FAMEs formed) which slowly continues. In order to prevent saponification of the FAMEs formed, the solution should be quenched soon after the transmethylolation reaction is finished, particularly with hydroxide reagents [17,38–40]. The mechanism of base-catalyzed transesterification is well documented [17,40,41]. The transesterification reaction can be made to proceed faster by increasing the concentration and base strength of the methoxy ion (e.g. replacing methanol with less acidic or aprotic solvent) [42–44].

Another group of lipid methylation reagents are the so-called strong organic bases. Examples are such compounds as tetramethyl ammonium hydroxide (TMAH), tetramethyl phenyl ammonium hydroxide, (TMPAH), trimethylsulfonium hydroxide (TMSH) etc. These compound mainly convert/transesterify lipids into FAMEs and FFAs to their salts. However, unlike their inorganic counterparts, the FA salts of the organic bases breakup at high temperatures of the GC injection port thereby forming FAMEs. Thus, this allows for a simple one-step determination of both esterified and free fatty acids. This procedure has found profound application in pyrolytic FAME profiling of various biological samples [43,45–57]. For selective methylation of free fatty acids in the presence of bound fatty acids, tetrametylammonium acetate (TMAAc) has been shown to do the job [58]. Trimethylsulfonium hydroxide has distinct advantages such as a low pyrolysis temperature and insignificant
degradation/isomerization reactions over other quaternary ammonium hydroxides [52,53,55,]. As a result, it is commonly used in the fatty acid profiling of lipids from plant and animal sources [59,60] as well as various bacteria [61–64]. It has, however, been shown that the solution should be acidified before injection in order to prevent/reduce losses in polyunsaturated fatty acids (PUFAs) [65]. Schulte et al. have recently recommended the adaptation of a rapid procedure as a standard method [66]. Despite its toxic and explosive nature, diazomethane has been used for rapid esterification of FFAs for a very long time after adaptation of special procedures, reagents and apparatus which allow for the use of this compound in a relatively safe manner [67].

2.2.2. Other functional groups

The derivatization procedures mentioned earlier (section 2.2.1.) are mainly used to first convert carboxyl functional groups into their corresponding methyl esters. If other functional groups (such as the hydroxyl group, OH) are present, they may also be derivatized. For the conversion of hydroxyl groups into their more volatile forms, trimethylsilylimidazole (TMSIM) and N, O-bis-(trimethylsilyl) trifluoroacetamide are generally used resulting in the formation of trimethylsilyl (TMS) ethers [68,69]. Even though tert.-butyldimethylsilyl (tert.-BDMS) ethers are more hydrolytically stable, their formation requires far longer reaction times [70]. Hydroxyl groups can also be converted to trifluoroacetates and acetates [71–74].

2.3 Resolution

Separation of positional and configurational isomers which co-elute is a major challenge in the profiling of fatty acids. Realization of high peak resolution often suffices to overcome this problem [75,76]. Packed columns have now been largely relegated to the background as a result of their inability to separate positional and/or configurational isomers. They may, however, be used for routine samples containing limited amounts of fatty acids or for rapid scouting runs. The merits and drawbacks of packed and capillary columns for supercritical fluid chromatography (SFC) have been recently reviewed [77–79]. As a result of its relatively higher resolving powers or efficiency, fused-silica capillary columns, that is wall coated open tubular (WCOT) columns have replaced packed columns in fatty acid profiling of lipids. As a result of the high efficiency of WCOT, minimal reliance is placed on their selectivity to achieve high resolution.
Furthermore, column temperature and/or flow velocity of the carrier gas can be controlled to maximize resolution or reduce analysis time. Carrier gas type also affects the resolution and analysis time of a particular chromatographic system. For a given resolution, the shortest analysis time is achieved with hydrogen as carrier gas followed by helium gas, provided the operating flow-rates are above the optimum linear velocity [80,81].

Another important factor to consider is the purity of the carrier gas, as the presence of oxygen and water impurities tend to degrade stationary phases and this is made even worse at higher temperatures. For example, this effect has been observed on polyethylene glycol (PEG) stationary phases at temperatures that are substantially lower than that specified by the manufacturers by Conder et al [82]. To overcome this problem, oxygen and water traps are normally installed in the carrier gas line. Typically, WCOT columns of 25-30 m with an inner diameter of 0.25 mm are used for fatty acid analysis. Longer columns (up to 100 m) may be used for challenging separations (such as separation of positional and geometrical isomers of unsaturated fatty acids) where the highest of resolutions are required. With the exception of moderately polar polyethylene glycols, stationary phases mostly used for fatty acid methyl ester analysis are made of non-polar methylpolysiloxanes and the very polar cyanoalkylpolysiloxanes in various proportions.

Some modifications have the methyl groups replaced with somewhat polar phenyl or vinyl groups. A list and description of the most commonly used stationary phases for capillary GC has recently been compiled [21]. On non-polar (mainly methylpolysiloxanes) stationary phases, unsaturated FAMEs generally elute before their saturated analogues depending on the number of double bonds. As a result of this, clustering of peaks may occur around the even chain lengths without, however, overlapping or crossing over into the adjacent group of even chain length FAMEs [83]. The fact that separation is primarily based on volatility accounts for the poor resolution of unsaturated fatty acid on non-polar stationary phases. The utilization of polar phases such as polyethylene glycol, allows for resolution according to carbon number, and unsaturated FAMEs are eluted after their saturated analogue with minimal overlap of different chain lengths [84,85].

Very polar stationary phases, however, show a greater affinity to the polar double bonds and thus strongly retain them. The higher the degree of unsaturation, the more they are retained and are thus eluted progressively later than their saturated counterparts. Polar stationary phases are
also, for obvious reasons, the most suitable for the separation of cis, trans isomers [86]. Inevitably, overlapping of FAME peaks occur as a result of the multiplicity of isomers and this may require the use of two columns [87]. Columns of intermediate polarity are usually used for the separation of PUFAs and preference for PEG columns has been expressed by two authorities in the field of lipids study [19,89].

Non-polar stationary phases are more thermally stable and thus show minimal column bleeding. They are the preferred choice, good resolution permitting, for routine FAME analysis where positional and geometric isomers are not present. For the separation of wide range of PUFAs where cis, trans isomers may be present, polar columns are used. Satisfactory results have been obtained in the separation of majority of lipid samples encountered in various fields using WCOT columns.

A notable exception, however, is the partially hydrogenated oils (margarines and shortenings) whose trans fatty acid content have generated a lot of interest in the health and nutritional fields [88]. Samples such as these tend to be very complex, containing various mixtures of positional and geometric isomers which cannot be completely resolved even with very long (100 m) highly polar (cyanosilicone) capillary columns. Preliminary semi-preparative separations are normally performed on them using argentation thin-layer chromatography (TLC) according to the number of olefinic linkages and their geometric isomers [89]. GC combined with infrared (IR) methods, after preliminary fractionation using methoxybromomercuroic adducts by TLC based on the degree of unsaturation, followed by AgNO₃–TLC for the separation of configurational isomers is another procedure that has been applied in margarine analysis [90].

2.4 Identification
2.4.1 Structural information

Identification of commonly encountered fatty acids is realized with the aid of the retention times of authentic FAME standards (C₄–C₂₄; both saturated and unsaturated) that are commercially available. It has also been shown that less stable PUFAs could be identified with the aid of chromatograms of well characterized samples such as Cod liver and canola oils [19,89]. Retention data in literature are generally presented in several ways such as relative retention times [91,92], and equivalent chain length (ECL) values [93]. The ECL procedure is like the Kovats retention indices and are based on a near linear relationship between the
logarithms of the adjusted retention times (log $t'_R$) of a homologous series of compounds (saturated straight-chain FAMEs) and their carbon chain lengths. A plot of log $t'_R$ of saturated, straight-chain FAMEs against their integer chain lengths is used to establish the correlation. For an unsaturated (or substituted) FAME, the ECL is determined from its log $t'_R$, and it corresponds to the non-integer chain length of a hypothetical saturated FAME. ECL values vary from stationary phase to stationary phase when measured isothermally and under the same conditions. Extensive tabulation of ECL values can be found in literature for packed and capillary columns [88,90,94]. ECL values obtained on a capillary column have been shown to be temperature dependent, and that they increase with the polarity of the stationary phase as well as the polarity of the analyte (i.e. degree of unsaturation) [95].

2.4.2 Determination of double bond position

Determination of double bond position and the geometric configuration of olefinic linkages remain one of the main challenges in fatty acid analysis. To this end, many methods such as the production of epoxides of monoenoic and dienoic acids after reaction with m-chloroperbenzoic acid in CHCl$_3$ at 25 °C have been applied [33,96]. Epoxy derivatives can be directly analyzed or may be further converted to their corresponding diols by using reagents such as KMnO$_4$ or OsO$_4$ which results in cis and trans isomers being converted to cythro and threo diols respectively [97,98]. Dimethyloxazoline (DMOX) reagent has been shown to be useful in determining the location of a double bond position in lipid analysis of marine flagellates [99]. Mercury adducts (usually the methoxybromomeric derivatives) may also be prepared from complex mixtures of unsaturated fatty acids, fractionated by thin layer chromatography (TLC) according to the degree of unsaturation and then analysed by capillary GC [100].

Authentic FAME standards and literature ECL values have been applied to identify fatty acids of a host of biological samples in a rapid and simple manner. For the confirmation of analyte fatty acids, the application of gas chromatography coupled to mass spectrometer (GC-MS) has become the method of choice in recent times as it offers the possibility of comparing the mass spectrum of the fatty acid of interest to a reference spectrum stored in a computer data bank. In cases where no match is found, other means such as isolation and derivatization followed by GC-MS analysis may be utilized. Such procedures have been extensively reviewed [101 –106].
Retention parameters and structural information provided by GC-MS analysis of organic samples play a major role in the identification of complex organic compounds, not at least in the location of double bond position and geometry, cyclopropyl rings, branched chains as well as other substituents. For the location of double bond positions, two major approaches have been developed, namely: (i) the “On-site” procedure in which the double bond position is determined or located with the help of chemical modification and (ii) the “remote group” derivatization procedure in which the carboxyl group reacts with the appropriate reactant to produce a nitrogen-containing substituent which stabilizes the positive charge formed from electron bombardment and subsequently reducing double-bond migration. The latter procedure is far more convenient and versatile than the former. Double bonds of unsaturated fatty acids may be reacted to form various addition compounds as a means to isolate individual fatty acids or as parts of a method for establishing the configuration or location of the double bond in the aliphatic chain. Conversion of double bond or olefinic linkages to epoxides followed by GC separation of the resulting cis-trans isomers is one of the most used on-site methods in double bond location in the aliphatic [107]. Other derivatives can also be prepared from epoxides, or alternatively, the cis-trans isomers can be pre-fractionated by argentation chromatography and then further resolved by GC.

\[
\text{CH}_3(\text{CH}_2)_n\text{CH}==\text{CH}(\text{CH}_2)_m\text{COOCH}_3 \xrightarrow{\text{I}_2} \text{CH}_3(\text{CH}_2)_n\text{CH}==\text{CH}(\text{CH}_2)_m\text{COOCH}_3
\]

**Fig. 1:** Reaction scheme for the preparation of dimethyl disulfide adducts

Another equally useful procedure for double bond location employs the conversion of polyenoic fatty acids to their polyhydroxy derivatives with OsO\textsubscript{4} and then to their TMS ethers for GC-MS analysis. This procedure has been successfully applied to fatty acids with up to six double bonds and was applicable to both isolated and conjugated unsaturated systems [108]. Addition of dimethyl disulfide [109] (see fig. 1) or deuterium [110] across the double bond are other methods that are widely used to locate double bond positions.

Unlike the on-site method, the remote group procedure does not distinguish between
geometrical isomers. The main advantage of the remote group procedure is its generality, being potentially applicable to all types of structures with the exception of methyl esters which tend to be unsuitable derivatives as they tend to undergo double bond migration. Some of the appropriate derivatives include amides and esters of nitrogen-containing alcohols such as ozazolines [111–113], diethylamide [114], picolinyl esters [115,116] and pyrrolidides [117,118]. A reaction for the preparation of picolinyl esters is shown in figure 2. It is simple and rapid, involving brief reactions with first carbonyldiimidazole then 3-(hydroxymethyl) pyridine and a catalyst.

![Reaction scheme for the preparation of picolinyl esters](image)

Fig. 2: Reaction scheme for the preparation of picolinyl esters

Picolinys esters can also be prepared via the acid chloride by reaction with oxalyl chloride overnight, and reacting this with 3-hydroxymethylpyridine in dichloromethane to form the picolinyl ester. Dry solvents and fresh reagents are required because the reactions are sensitive to moisture. GC separation of picolinyl esters and pyrrolidides derivatives are carried out at temperatures that are about 50° C higher than that required for the separation of FAMEs. For this reason, their GC analysis is carried out using non-polar phases to reduce column bleed. On the other hand, because ozazolines and diethylamide derivatives are more volatile, their GC analysis is normally performed on polar GC columns. Besides molecular mass, fragment patterns in mass spectra provide immense assistance in the location of double bonds. For instance, most mass spectra reveal a pattern of fragments that decreases by 14 mass units as a result of successive methylene group cleavage and by 12 mass units where a double bond is located. For highly
unsaturated fatty acids, the picture is not that simple. Procedures for location of double bonds in fatty acids have been comprehensively reviewed [119,120].

2.4.3 Isotope characterization

Gas isotope ratio mass spectrometry (GIRMS) or simply IRMS is probably the oldest type of MS used in analytical chemistry [121]. IRMS has been a standard tool in areas, such as geochemistry, microbial ecology, environmental sciences etc. [118,122,123]. IRMS has received the attention of other areas of applied analytical chemistry such GC-MS and LC-MS since the introduction of commercially available IRMS instruments coupled to a gas chromatograph via a combustion interface. GC/combustion-IRMS (GC/C-IRMS) technique has traditionally been used to measure the natural variation in isotope ratios of single compounds due to isotope fractionation during primary production, respiration and assimilation [124]. This natural abundance approach has been used to study the source of carbon assimilation by microorganisms [125,126] and to identify microbial populations involved in specific processes [127 –131].

In isotope measurements, analytes are converted into a simple gas, isotopically representative of the original sample, before entering the ion source of an IRMS. Modern GC/C-IRMS instrument comprises of a GC equipped with a capillary column that is used to separate the compounds of interest at high resolution. The outlet of the column is attached to a miniature oxidation reactor where the organic molecules are combusted to CO₂, N₂, and H₂O gas. A reduction reactor is included for ¹⁵N analysis to convert oxidized nitrogen species to N₂ gas.

Water is removed on-line and the purified CO₂ and N₂ are led into an isotope ratio mass spectrometer. In the case of CO₂ (i.e.¹³C/¹²C), the data comprises of three ion traces for the different isotopomers ¹²C¹⁶O₂, ¹³C¹⁶O₂ and ¹²C¹⁸O¹⁶O with their corresponding masses at m/z 44, 45 and 46, respectively. These three ion beams are simultaneously registered by a multiple Faraday cup (FC) arrangement in which each isotopomer is assigned to a dedicated FC. The resulting ion currents are monitored, subsequently digitized and the resulting peak data transferred to the host computer where the peak area of each isotopomer is quantitatively integrated and the corresponding ratios are calculated. As a rule results obtained in isotope ratio measurements are calibrated against an international standard or derived reference material. The isotopic swing of the 44/45 ratio also provides additional information on peak separation.

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Many compounds have to be derivatized before GC analysis and the isotope data should be corrected for the carbon atoms added during derivatization [131,132–134]. Detailed description of the design and operations of GC/C-IRMS instrument has received extensive publications [135–138].

2.5 Inlet techniques and at-line derivatization

In order to by-pass the laborious work-up of the conventional extraction procedure, methods such as pyrolysis gas chromatography (Py-GC), pyrolytic methylation or the so-called thermal hydrolysis and methylation combined with gas chromatography (Py-THM-GC-MS) [48,50,51,58,139–142] and in more recent times, direct thermal desorption (DTD-GC-MS) [47,53] and comprehensive GCxGC techniques [143–148] have all been applied in lipids characterization. These techniques, with the exception of GCxGC, have also found application in stable isotope analysis of fatty acids [149–152].

Analysis of lipids by GC is usually performed using the traditional Bligh-Dyer off line extraction procedure. This method has been used in analyzing fatty acids of various samples such as waxes etc [153,154]. The main disadvantage of this procedure is the rather lengthy and tedious sample preparation step. To overcome this disadvantage, a novel technique, now referred to as thermally assisted hydrolysis and methylation (THM), was introduced by Challinor by using TMAH as an alkaline reagent for the analysis of polyesters [54]. THM was carried out on the flash-heated filament of a pyrolyzer directly coupled to a gas chromatograph. Even though some THM products remained unidentified, the potential of using THM-GC for lipid analysis was clearly demonstrated. Since its development, this technique has been applied to analyze a variety of fatty acids in several glycerol-based samples, e.g. fats, oils and even whole microorganisms and the resulting patterns of FAMEs have been claimed to be suitable fingerprints for discrimination purposes [48,50,55–57,107,108,110].

The mechanism of the THM reaction of esters has been investigated and reported in a number of papers [155–158]. THM of esters simply means saponification of the ester bond as a first step and this is supported by the alkalinity of TMAH. This initial reaction results in the formation of tetramethylammonium salts (soaps) of the ester constituents as intermediates, which are then thermally transformed to the corresponding methyl derivatives (methyl esters and methyl ethers) via a thermally induced nucleophilic reaction of the soap anions with one of the
methyl groups of the TMA cations.

Both ester-linked and free fatty acids are converted to their respective methyl esters by TMAH as a result of its alkalinity. In cases where there is a need to selectively derivatize free fatty acids in the presence of ester-bound ones, TMAAc, a less alkaline reagent, has been shown to do the job. Here, ester hydrolysis is avoided and this allows for the selective analysis of free fatty acid [58].

One major drawback of the THM-TMAH-GC procedure was the occurrence of additional isomerization products of PUFAs [50,108]. To overcome this drawback, TMSH was applied as methylation reagent in THM reaction of lipids to saponify and methylate oils and fats [51]. It was shown to be particularly suitable for the analysis of PUFA-containing lipids as any appreciable level of isomerization or degradation is avoided as a result of the lower pyrolysis temperature (350 °C) in spite of the similar alkalinity of TMSH and TMAH (pKb=12).

In order to obtain more structural information of analytes in lipid analysis of samples such as waxes, [159–163] pyrolysis combined with gas chromatography (Py-GC) was introduced. Even though Py-GC is a suitable fingerprinting technique which allowed rapid identification of the resulting pyrolysate patterns which were characteristic of the different wax species, some limitations still remained. Firstly, Py-GC of waxes showed that even though some of the wax components were volatile enough to survive pyrolysis, they were not volatile enough to be transferred from the pyrolyzer to the GC and were, therefore, excluded from the subsequent chromatographic analysis.

Secondly, wax pyrolysis yielded fragments, especially fatty acids, which were hampered in their transfer to the analytical system as a result of their high polarity and/or low volatility. Finally, pyrolytic fragmentation may lead to pyrolysis products that are structurally non-specific. For instance, hydrocarbon patterns caused by the pyrolysis of long chain aliphatic wax components via a free radical mechanism. The amount of structural information that can be obtained from the interpretation of such programs has been extensively discussed [164].

2.6 DTD-GC-MS

Even though the development of the Py-THM-GC-MS technique eliminated most of the manual work-up associated with the traditional extraction procedure, still the manual application of the sample to the pyrolysis wire makes it a little cumbersome. Furthermore, it makes
automation very difficult. To overcome these short-comings, a system that allows for automated exchange of the liner (with or without an insert) of a programmed-temperature vaporizer (PTV) injector was developed to analyse whole/intact biological samples [47,53]. The so-called direct thermal desorption interface was used as a reaction chamber for the transmethylation of microbial fatty acids in their natural matrix. In combination with an automated sample processor, this novel procedure enables a fully automated analysis sequence to be carried out without any manual steps.

Even though the DTD set-up does not allow hot injection, the rapid heating of the PTV after introduction of the sample and hydrolysis/methylation reagent creates THM conditions in an on-line fashion. A summary of the procedure is as follows: (i) Injection of the aqueous cell suspension into a special micro-vial placed inside a DTD liner, (ii) at-line drying of water by the application of vacuum, (iii) addition of a THM reagent in MeOH and incubation, followed by evaporation of the organic solvent and (iv) THM-GC-MS analysis. DTD-GC-MS has been successfully used to analyze various biological samples without any sample treatment [47, 53, 165 –168].

2.7 Comprehensive GCxGC

In recent times, comprehensive two-dimensional gas chromatography (GCxGC) is increasingly becoming the technique of choice in lipid analysis of complex samples where higher resolution power is required (e.g. Fats, vegetable and fish oils etc) [111-116]. GCxGC provides unsurpassed peak capacity as well as providing an improved potential for group type separation. GCxGC is a multidimensional separation where the initial sample is separated on two different GC columns connected in series with a cryogenic modulator between them [169]. Peaks eluting from the first dimension GC column enter a cryogenic modulator, which traps each subsequent small portion of the eluate, focuses it and introduces it onto the second GC column where additional separation takes place. This novel technique achieves a near complete characterization of the initial sample.

The second separation is made sufficiently fast (e.g. 5-10 s) so as to allow continuous introduction of small fractions from the first column without mutual interference and thus enabling the subjection of the whole sample to two-dimensional (2D) separation in a single run. The peak capacity realized during 2D GCxGC is close to the product of those of the two
individual GC separations [149,170]. Since its development, various types of modulators ranging from the earlier thermal sweeper type to the more popular longitudinally modulated cryogenic system that exploits the freezing effect of a CO₂ stream passing through a moving modulator has been used [171,172].

Usually, non-polar stationary phase GC columns (e.g. 5% Phenyl + 95% Dimethyl Polysiloxane) are used in the first dimension and polar stationary phases (e.g. 50% Phenyl Polysilphenylene-siloxane) are used as second GC columns. This column combination results in a first-dimension separation which is mainly based on vapor pressure and as a consequence, compounds contained within one portion of the eluate that is introduced into the second column essentially have the same vapour pressure. As a result, the separation on the second column depends on the specific interactions with the stationary phase and not on temperature. Consequently, every compound acquires two characteristic retention times, thus making identification of an analyte in a sample matrix more reliable [149,173]. Until now, Comprehensive GCxGC analysis of lipids is normally preceded by off-line extraction and methylation of fatty acids using the traditional Bligh and Dyer procedure or a modification of it. Recently, however, Akoto et al have carried out fatty acid analysis of micro-algae and aquatic mesofauna species using a novel DTD-THM-GCxGC-ToF-MS system without any sample preparation [165]. Comprehensive two-dimensional GC has been extensively reviewed [174,175].

3.0 High-performance liquid chromatographic methods
3.1 General

Unlike GC where FAMEs are the sole derivatives on which profiling of fatty acids is based, in HPLC, a large number of different derivatives including those containing various chromophores and fluorophores, underivatized fatty acids or their methyl esters [12,176–178]. Separation of free fatty acids by HPLC is mainly performed with reverse-phase systems which consist of alkyl chains of various lengths bonded onto a silica base. Here, retention and selectivity are both directly related to the alkyl chain length of the bonded phase. They both increase with an increase in the length of the alkyl chain of the bonded phase [179–181]. Octadecysilyl (ODS) phases as well as Octysilyl ones are widely used in fatty acids separations as a result of their commercial availability. The order of elution of fatty acids on these non-polar
phases are similar to that of a GC fatty acid analysis performed on non-polar phase columns, except that unsaturated fatty acids are eluted considerably ahead of their saturated analogs. As a matter of fact, a reduction in retention time approximating that of two fewer methylene groups is produced by each double bond resulting in close elution of fatty acids. For instance, 16:1 and 20:4, cis and trans 18:1, 20:0 and 22:1 as well as 22:0 and 24:1 are among some of the difficult-to-separate pairs or combinations, the so-called "critical pairs" [173,175,182].

Unlike in GC where the task of the carrier gas is to transport the analytes to the detector and therefore has to be inert, in RP-HPLC, the eluent can be modified by varying the proportions of water and the nonaqueous components (mostly methanol and/or acetonitrile) to effect changes in retention and hence in resolution. In case of fatty acids, it has been shown that relative to methanol, retention of carbon chains longer than C₈ was increased by acetonitrile. Methanol was found to be more effective for the shorter chain lengths [176,183]. Acetonitrile has been shown to produce poor resolution for certain critical pairs such as 16:0 and 18:1 which are the major FAs in animal lipids [184]. A mixture of acetonitrile and methanol with water usually suffices to achieve optimum peak resolution and the ability to modify retention with the addition of other organic solvents and various buffers to the eluent is what gives more flexibility to HPLC than GC.

3.2 Derivatization

With the exception of FAMEs that may be obtained by transesterification, almost all the methods for the preparation of derivatives for fatty acid profiling using HPLC begins with saponification of the sample lipids. The saponification procedure involves refluxing of the specimen with 1 M KOH in 95% ethanol for 1 h or leaving the mixture at room temperature overnight. For cholesteryl esters, a longer reaction time may be required. The mixture is then acidified and subsequently, the free fatty acids extracted with ether. [12].

Phenacyl esters and their substituted analogs are the most widely used derivatives for HPLC fatty acid analysis. The procedures for the preparation of these derivatives are similar, differing mainly in catalyst used. Typically, the procedure involves heating the fatty acids, the phenacyl bromide as well as the catalyst in a solvent for 15-30 min. at 100 °C or at 50 °C for 2 h or leaving the mixture overnight at room temperature. Mostly KHCO₃, triethylamine, lithium carbonate and N,N-diisopropylethylamine in dimethylformamide are used as catalysts [185–
Conduction of this reaction in a two-phase system in which the fatty acids are extracted as an ion-pair with tetraalkylammonium ions from an aqueous buffer into an organic solvent where derivatization is subsequently carried out has been reported [188]. This general procedure may be used with different derivatizing reagents with the reaction time and temperature depending on the specific reagent being used.

3.3 Resolution

Ideally, the desire of every separation scientist is to have a system with high speed, sensitivity and efficiency but these parameters/characteristics are not always mutually compatible. At the expense of resolution, speed can be gained; the use of larger and highly conjugated derivatizing agents often results in increase in sensitivity, but usually at the expense of resolution. This is because RP selectivity is derived from the structural differences in the carbon backbone of the analyte fatty acids and so, enlarging the chromophoric label results in an increase in the proportion of the non-selective RP interaction and this in tend diminishes or reduces the resolution observed for the derivative as a whole.

On the other hand, increase in the polarity of the chromophoric tag results in a reduction of the non-selective interaction thereby improving resolution. Thus, enhanced sensitivity frequently results in resolution loss.

3.4 Detection

3.4.1 UV absorption

Inspite of the fact that most lipids lack chromophores of value in spectrophotometric detection, underivatized fatty acids and their methyl esters have been separated by HPLC and monitored by refractive index or more sensitively, by low-wavelength UV detection [189,190]. Unfortunately, not many solvents are transparent in this region with water and acetonitrile being the most suitable eluent components. Even though a good resolution is observed, the sensitivity is rather modest and depends strongly on the degree of unsaturation of the analyte. To this end, an alternative derivatization procedure for RP-HPLC of fatty acids has been developed, in which fatty acid esters containing strongly UV-absorbing substituents in the alcohol moiety are prepared, so that components emerging from the columns can be detected by means of UV spectrophotometry [181].
Among the many derivatives reported, phenacyl esters or their substituted analogs are by far the most widely used. In most respects, the substituted reagents behave just like the parent and the general greater sensitivity achieved accounts for its preference. Fatty acid derivatives with maximum absorbance at 242 nm and a molar absorptivity of 14 000 [181] or 12 100 [183] l mol⁻¹ cm⁻¹ have been formed from the parent reagent.

However, at 254 nm where analytes are often monitored (especially in the past when fixed wavelength detectors were employed), a considerably reduced molar absorptivity value of 6000 l mol⁻¹ cm⁻¹ is observed. Using step gradients of acetonitrile-water, 24 fatty acids ranging from C₁₂ to C₂₄, including saturated, unsaturated and configurational isomers, has been successfully separated on a 90 cm (longer than conventional) C₁₈ RP column packed with 10-μm particles. Whereas the common critical pairs were well resolved, oleic and vaccenic acids coeluted. They were, however, separated from petroselinic acid. The down side of this analysis is that it took 4 hrs. A 70–100% linear acetonitrile–water gradient on a C₈ RP containing 5-μm particles was found to be the optimal resolution conditions for phenacyl esters when separation of fatty acids from butter was performed [176].

Phenacyl derivatives have also found application in elution order studies [191,192], analysis of free fatty acids in human blood plasma [193], separation of cis and trans isomers [194,195], resolution of retinal fatty acids [196] as well as the determination of cellular fatty acids of microorganisms [197] and amniotic fluid [198]. Phenacyl and other derivatives have found profound application in HPLC analysis of fatty acids [199–206].

3.4.2. Fluorescence

Unlike UV detection, enhanced sensitivity (could as high as two orders of magnitude) as well as selectivity is realized when fluorometric detection is applied in HPLC analysis of derivatives of fatty acids, whereas resolution is generally diminished. Most of the widely used derivatives are either based on anthracene, or another polynuclear aromatic system or on coumarin. The use of fluorescence detection in HPLC has been comprehensively reviewed [207]. In recent times, fluorescent derivatives have found profound application in biomedical research, especially in the analysis of free fatty acids in blood, thanks to their very high sensitivity. As a result of this, small sample volumes of low free fatty acid concentrations have been analyzed after undergoing simple derivatization procedures without prior extraction.
Many reagents have been used in the formation of the appropriate fatty acid derivatives. For instance, 9-Diazaanthracene has been used in the formation of anthrylmethyl esters of fatty acids by reaction in an inert solvent [208,209]. Excitation was performed at 360 nm and emission detected at 440 nm. A detection limit of 15 pg/µl was achieved, which was ten times lower than obtained by UV absorption of the same derivative at 254 nm. Other reagents that have found application in fluorescence detection of fatty acid derivatives include, 9-(Hydroxymethyl)anthracene [210,211], 9-(Chloromethyl)anthracene [212], Dansyl-semi-piperazide [213], 4-bromomethyl-7 Methoxycoumarin [214].

4. Other methods

In recent years, NMR spectroscopy (both 1H NMR and 13C NMR) has increasingly found application in the identification of lipid structures, especially in the detection and location of double bond systems in fatty acid chains (in the form of methyl ester derivative) [215]. The characteristic absorption bands of functional groups is well exploited in infrared (IR) and Raman spectroscopy [216–218]. More detailed general information can be found in a number of reviews [219,220].

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Chapter 2

Development of Direct Thermal Desorption Methodology
2.1 At-line gas chromatography–mass spectrometry analysis of fatty acid profiles of green microalgae using a direct thermal desorption interface

Abstract

Thermally assisted hydrolysis and methylation-gas chromatography (THM–GC) is an important tool to analyze fatty acid in complex matrices. Since THM–GC has major drawbacks such as isomerization when applied to fatty acids in natural matrices, a direct thermal desorption (DTD) interface and an incubation time of 30 minutes were used to circumvent these problems. Using vegetable oils such as sunflower oil and triarachidonin, the conversion of triglycerides into their fatty acid methylesters (FAMEs) was investigated. The yields using a DTD (and TSMH 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 analyze 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 analyze fatty acids in microbial cells in a fully automated fashion, which will allow high sample throughput.

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 like hydrolysis and derivatization 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 BF₃/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 isomerization 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\textsubscript{20:4} (5,8,11,14 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\textsubscript{20:4} (5,8,11,14 all cis) 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 PTV injector. The so-called direct thermal desorption interface (DTD) 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 μ-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 BF₃/methanol transesterification).

2 Experimental

2.1 Instrumentation

A 3800 GC (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 was operated at a constant column pressure of 70 kPa using a CP-SIL 8CB/MS (Varian) fused silica capillary column (15 m x 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 x 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 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 BF₃–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 isomerization 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 were 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 n° sample.

2.5 Off-line transesterification

0.5 ml of a sample solution or aqueous suspension (cf. Section 2.4) was dried under N₂ and 0.5 ml of BF₃/MeOH (12%, 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 (3x) and the combined organic extracts were concentrated to 500 μl. 2 μl of the final solutions were 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, St. Louis, MO, USA) and finally washed with toluene (3x), ethyl acetate (3x) 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 analyzed, 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 C14:0 to C24:0 and unsaturated ones from C14:1 to C24:1. 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 isomerization was found to occur, as was also reported by Ishida et al. [7] and Jun-Kai et al. [14]. The high degree of isomerization (>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. Figure 1a illustrates the undesired effect of isomerization; all asterix (*) labeled 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 optimize 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 isomerization 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 isomerization. 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 isomerization, but also yielded unacceptably low recoveries of the FAMEs: there simply was not sufficient reagent to complete both hydrolysis and methylation (Figure 1b).

Attempts to use different heating regimes (2–16°/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 C20:4 FAME (all cis). *: C20:4 internal standard (is) = C22 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\, \text{all\,cis})} FAME was significantly higher and the degree of isomerization much lower (Figure 1c). Despite this significant improvement of the quality of the analysis, isomerization at high reagent concentrations made optimization 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 isomerization. On the other hand, isomerization was less than when a premixing step was applied. Evaluation of the reagent/analyte ratio resulted in a distinct optimum.

Figure 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, isomerization of double bonds decreases the yield of the methylesters of the original unsaturated fatty acids. When the same approach was used to analyze 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 Figure 3.
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 optimize 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 were introduced into the 40-μl vial referred to earlier, which enabled better mixing of the microbial organism and TMSH. Utilizing 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 lyophilized 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.

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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 is) = C22 n-Alkane
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 hydrolyzing 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 hydrolyze the ester-bound fatty acids. Furthermore, other cell constituents scavenge part of the excess reagent during incubation, which causes isomerization 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.

![Normalized peak areas of the summed FAMEs released upon TMSH-based THM of lyophilized cultures of S. chaetoceras (•) and T. minimum (●) as a function of temperature.](image)

The results of a brief optimization study are shown in Figure 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 [26]. This so-called algaenan, which is not present in S. chaetoceras, 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 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 microalga (Fig. 5). The 90% yield window is in the range 0.02 – 0.05 (mol reagent/gram sample). These results suggest that in order to perform methylation under optimum conditions, one has to know the approximate amount of fatty acids in a sample.

![Fig. 5: Normalized peak area of the C18:2 FAME released upon TMSH-based THM of lyophilized 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](image)

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 neutralized 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 kinetic reaction(s) 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 natural matrix although the repeatability was worse (Tables 1 and 2).

**Fig. 6**: FAME-yield in w% of dry biomass upon THM with TMSH of lyophilized *S. chaetoceras* and *T. minimum* cells.
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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>wt%</th>
<th>RSD</th>
<th>RSD^n</th>
<th>Compound</th>
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Table 1: Concentrations of dominant FAMEs in lyophilized microbial biomass of *S. chaetoceras* and *T. minimum* and their RSDs (n=6) upon THM-GC–ITD-MS.

1RSD value obtained by determination of standard deviation of peak areas relative to total peak area, which represents the RSD of the FAME profile.

<table>
<thead>
<tr>
<th>Compound</th>
<th>wt%</th>
<th>RSD</th>
<th>FAME</th>
<th>wt%</th>
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<td>12</td>
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Table 2: Concentrations of dominant fatty acids in the lyophilized microbial biomass of *S. chaetoceras* and *T. minimum* as determined by using off-line BF$_3$/MeOH and their RSD values (n=6).

Figure 7 shows the C$_{14}$–C$_{18}$ FAME window of the 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. Figure 8 shows the ion trace of m/z 103, which is the most abundant ion in fatty acid profiles of *E. coli*. The peak eluting at 12.25 min could be identified as a 3-hydroxy-C₁₄ acid. Its identity was confirmed by means of the masses m/z 259, 241 and 103, which correspond to [M + H]⁺, [M + H - H₂O]⁺, and [CH(OH)-CH₂-COOCH₃]⁺ ions, respectively. Due to the well-known self-ionization effect occurring in ion-trap MS systems, m/z 259, [M + H]⁺, rather than m/z 258 is observed.
**Fig. 8**: GC–ITD-MS (m/z 74 & 79) 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.
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 analyzed by GC–MS. The fatty acid profiling of triglycerides by THM-based methods is well documented, but the distinct advantage of the DTD approach is the possibility to directly analyze 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.

4.1 References

2.2 Automated gas chromatography–mass spectrometry
Analysis of Raw Biological Samples: Application to fatty acid profiling of aquatic micro-organisms

Abstract
A Direct Thermal Desorption (DTD) interface was applied to profile the fatty acid composition of whole/intact aquatic micro-organisms using trimethylsulfonium hydroxide (TMSH) as derivatization reagent in a fully automated fashion without any sample treatment.

DTD was used to release cellular free and esterified fatty acids, after conversion into their methyl esters, from the very limited amounts of cells available in plankton community analysis using fluorescence-activated cell sorting of mixed (natural) microbial/algal populations. The liner of the GC injector is used as a sample-and-reaction container with the aid of the DTD interface. The fatty acids are converted into their methyl esters after an incubation (hydrolysis) time of 30 min with TMSH. After transportation into the injector, the liner is heated to effect thermally assisted methylation. Simultaneously, the fatty acid methyl esters (FAMEs) are introduced from the liner into the capillary GC column.

On the basis of peak intensities, the fatty acid compositions thus obtained, were shown to be closely similar to those of a conventional lipid extraction and methylation procedure. The method was applied to some common freshwater algae, the green algae Scenedesmus acutus, the diatom Asterionella formosa, and the filamentous cyanobacterium, Limnothrix sp. strain MRI. Using this novel method, poly-unsaturated fatty acids were methylated into their corresponding methyl esters without isomerization/pyrolytic side-reactions.

The present method has been used to perform large of series analyses of algal and microbial cells. Up to eighteen samples could be analysed per day. In order to reach this sample throughput, preparation of the \((n+1)\)th sample was performed simultaneously with GC analysis of the \(n\)th sample.
1. Introduction

Analysis of microbial fatty acid is traditionally carried out by extraction of the lipid from (isolated) microorganisms, hydrolysis, methylation and, finally, analysing and quantifying with gas chromatography (GC). The resulting FAME profiles can be used for their identification and differentiation, sometimes down to the species level [1–3]. Some of the disadvantages of fatty acid profiling using such a traditional method, are the rather lengthy steps required to extract and derivatize the fatty acids. In addition, enough microorganisms must either be originally present in the crude sample or be (selectively) cultured or enriched (approx. 10-15 mg wet weight of cells (~10^{10} CFU/ml)) to provide adequate amounts of fatty acids for the extraction and derivatization [4, 5], since in these traditional procedures, only an aliquot of the final extract is injected into the GC system.

To reduce the amount of sample and tedious traditional work-up procedures, as well as to speed up analysis, thermally assisted hydrolysis and methylation (THM) effected by Curie-point pyrolysis in the presence of methylating reagents has been introduced for the rapid profiling of microorganisms [6,7]. THM has also become the tool of choice for the determination of the chemical composition of, for example, natural waxes, esters of animal fats, vegetable oils and other classes of lipids in their natural matrices [8,9].

Pyrolysis GC is a technique that can be used to provide GC data from involatile/solid materials. When Pyrolysis is applied to perform the different functions in fatty acid profiling, the raw sample is placed onto the pyrolysis wire/support and, next, the derivatisation reagent is applied. Even though the application of a pyrolysis unit provides adequate volatile fragments and eliminates the problems of difficult sample work-up, the manual application of sample and reagent as well as the placement of the pyrolysis wire in the injector still hamper the analysis of large sample series.

Direct analysis of polar compounds on stationary phases is hampered by their high involatility, which can result in peak tailing, etc. In order to produce less polar and more volatile compounds, reagents that enable a one-step hydrolysis/methylation, so-called transesterification, have been selected, e.g. reagents such as tetramethyl ammonium hydroxide (TMAH) and trimethyl phenyl ammonium hydroxide (TMPAH) [10–13]. This one-step approach avoids decarboxylation and produces methyl esters of carboxylic acids as well as methyl ethers of hydroxyl groups, thus making many polar products volatile enough for GC analysis.
Pyrolytic methylation has been applied in various compositional studies such as the analysis of membrane-derived fatty acids, biopolymers, humic substances and even whole soils [6,7,13 –17]. One major drawback in the use of TMAH and TMPAH as methylating reagents is their ability to cause degradation/isomerisation side-reaction of PUFAs [18–21]. The degradation/isomerisation effects of TMAH are due to its strong alkalinity [20]. TMSH has been reported to be a better reagent for on-column methylation because methylation with TMSH is achieved at lower temperatures. This causes less pyrolytic side-reactions to occur, even though both reagents have similar alkalinity (pKb=12) [21].

Many of the problems associated with the traditional pyrolysis procedure, such as manual sampling handling in the extraction step etc., were solved when THM was performed in the liner of a PTV using a DTD interface [22]. Its development to perform all steps required by means of an automated exchange of the liner(s) of a programmed-temperature-vaporiser (PTV) injector and some capabilities of a robotic auto-sampler has been described earlier [22]. A short description is as follows: The sep-liner with a stainless-steel cap was used as reaction chamber. The auto-sampler was programmed to inject the raw sample in aqueous medium (2μl), to add reagent to the dry sample, after evaporation of water, and to place the reagent-added sample into the programmed-temperature-vaporiser (PTV) injector after a 30 min incubation time. In the injector the methylation reaction, so-called transesterification reaction, takes place after heating up to 350°C.

After analysis and cooling of the oven, the injector head opens automatically and the liner is replaced with a fresh one containing the next prepared sample. The DTD set-up allows solid and/or difficult sample matrices to be analyzed without any prior solvent extraction or other sample preparation. Samples are placed directly into glass-lined/stainless-steel desorption tubes or glass liners with or without insert, which are then subjected to controlled heating in a flow of inert gas. The desorbed volatiles are transferred directly to the GC for analysis in a one-step process.

In the present paper, we discuss the application of a fully automated DTD-GC-MS system for THM/FAME profiling of whole aquatic microorganisms in their natural matrix. The main objectives were the quantification of total FAME yields and the preservation of PUFAs. The n-alkane C20 was used as internal standard. The most beneficial effects were that the total procedure could be automated, that very low amounts of sample, down to 100 ng total cell carbon were required. Another major advantage of our approach is that it allows for
a much higher throughput of the small-sized samples (amounts down to 100 ng total-cell carbon), as are often obtained in microbial and ecophysiological-oriented studies [7,17,23]

2 Experimental

2.1 Instrumentation

A 3800 GC (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 mass spectrometer (ITD-MS) (Saturn 2000, Varian) were used. A DTD interface (ATAS) was mounted on top of an Optic 2/200 injector (a photograph and schemes of the DTD are presented in Fig. 1) which actually replaces the septum nut of the Optic 2/200.

![Fig. 1: Photograph (scheme) of the total (PTV, Sepliner, & DTD) system in analysis position (A) and capped PTV/sepliner with glass insert (B).](image)

A special pneumatic opening/closing device facilitates the opening/closing of the DTD injector head. In order to operate in a fully automated manner, the upper part of the PTV liner and the “base tip” of the FOCUS had to be adapted. The liner should be sealed with a stainless-less steel cap and the “base tip” of the auto-sampler arm should be magnetic, such that the auto-sampler arm can lift and transport a capped liner. With this set-up, the liner of the GC injection interface can be used as a sample container and/or reaction chamber. The GC was operated at a constant column pressure of 70 kPa using He as carrier gas. A HP-5MS (Agilent, Little Falls, MN, USA) fused silica capillary column (30 m x 0.25 mm i.d., 0.1 μm
film thickness) was connected to the ion source of the ITD via a 1-m fused silica restriction (0.5 m x 75 μm i.d.). For detection and identification, the ITD settings were: trap temperature, 220ºC; interface temperature, 270ºC; manifold temperature, 50ºC; A/M amplitude, 4.0 V; emission current, 10 μA; AGC target set at 10,000; scan time, 1.7 s and scan range, m/z 40–650.

2.2 Materials

For THM, TMSH (0.25 M in MeOH, Fluka) was used. For reference purposes, transesterification with BF₃ (12%) in MeOH (Acros) was used. As THM samples, batch-grown cultures of the green algae Scenedesmus acutus, the diatom Asterionella formosa, and the filamentous cyanobacterium Limnothrix sp. strain MR1, were chosen; all were obtained from the Centre for Limnology, Nieuwersluis (Netherlands Institute of Ecology, The Netherlands).

2.3 THM of monoalgal cultures

To study the FAME profile as well as possible isomerization of unsaturated fatty acids upon THM of whole algal cells, the Focus was programmed to inject 2 μl of a (0.90 mg cell carbon/ml) aqueous suspension into a 40-μl vial placed inside a capped liner. The liner was then placed in a built-in vacuum system in the tray for sep-liners, using the magnetic tip of the autosampler in combination with a stainless-steel cap for the sep-liner, and allowed to dry for 3 min. The at-line vacuum system was built by connecting the right hand upper corner hole of the sep-liner holder to a vacuum source, which can be turned on/off by a valve fitted to it. A 1.7 m³/h vacuum pump (Dijkstra Vereenigde BV, Lelystad, The Netherlands) served as the vacuum source.

To improve evaporation as well as desorption of analytes, a glass vial/insert of the same length as the sep-liner and with a ‘pinched’ hole just above its midpoint (to provide an additional outlet for analytes/water into the sep-liner) has been developed into which samples can be placed for analysis (see picture 1C).

Furthermore, in difficult matrix sample analysis, the use of glass vial/insert enables analytes of interest to be desorbed from the sample and transferred to the analytical column, while the potentially interfering matrix constituents remain in the insert, which can be removed and cleaned after the liner has been changed.

After complete drying, 1 μl of a 0.005 M TMSH solution in methanol was injected and the reaction mixture allowed to incubate for 30 min. Next, the contents of the inserts
were dried by applying vacuum for 45 sec. They were transferred to the DTD interface and, finally, THM was performed at 350 °C by increasing the injector temperature from 40 °C to 350 °C at 16 °C/s under splitless conditions. The GC was programmed from 35 °C (3 min hold time) to 120 °C at 30 °C/min, followed by an 8°C/min gradient to 280 °C (5 min hold time). At the start of the first sample preparation procedure, the GC was programmed to perform a dummy run while the above-mentioned steps took place. At the start of the next procedure, the prepared liner is placed into the injector for analysis, thus, while the GC is running, the next sample is being prepared simultaneously.

2.4 Off-line transesterification

0.5 ml of a sample solution or algal cell suspension (cf. Section 2.3) was dried under N₂ and 0.5 ml of BF₃ (12 vol. %) in MeOH (Fluka) was added. The reaction mixture was heated to 80°C for 30 min. Next, 0.5 ml water was added. The aqueous layer was extracted three times (3x) with 1 ml hexane and the combined organic extracts were concentrated to 500 μl. 2 μl of the final solutions were injected in the splitless mode at an injector temperature of 40 °C; that is, the exact amount as in the at-line approach should reach the column. GC–MS conditions were the same as those reported above.

2.5 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, St. Louis, MO, USA) and finally washed with toluene (3x), ethyl acetate (3x) and methanol, followed by 30 min drying at 100 °C.

3 Results and discussion

The final procedure described earlier [22] was evaluated and optimised for three freshwater microorganisms. TMSH was again the best choice for methylation, since it produced no significant amount of transesterification isomers. Maximum response for FAMEs was already reached at a PTV temperature of 300 °C rather than at 350 °C as in the earlier study. Since no losses were observed at 350 °C, this temperature was selected in order to enable a single procedure to be applied to as many different microorganisms as possible. An incubation time of 30 min and a concentration of 0.005M TSMH, was again the same as in the earlier study.
The yields of the FAMEs were calculated according to the amount of the n-alkane C20 internal standard. In 1μl of cell culture 2.5 ng of C20 was added. Both the off-line and DTD procedures were carried out six fold for the batches of Asterionella formosa, Limnothrix sp. Strain MRI and Scenedesmus acutus species. A typical selection of the data is presented in table 1. (six FAMEs for Limnothrix sp. Strain MRI and 8 FAMEs for Scenedesmus acutus).

<table>
<thead>
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<th>FAME</th>
<th>S. acutus (%)</th>
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<tr>
<td>C16:4</td>
<td>8.9</td>
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<td>2.1</td>
</tr>
<tr>
<td>C18:0</td>
<td>7.3</td>
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<td>5.7</td>
</tr>
<tr>
<td>C18:1</td>
<td>39.0</td>
<td>38.0</td>
<td>20</td>
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<tr>
<td>C18:2</td>
<td>19.0</td>
<td>19.0</td>
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<tr>
<td>C18:3</td>
<td>3.0</td>
<td>2.7</td>
<td>3.2</td>
</tr>
</tbody>
</table>

Repeatability values (RSD%) were 1.2-4.8. The most striking result is that profiles, i.e. individual percentages of FAMEs with respect to the total FAME yield, were generally the same.

Data on Asterionella formosa are presented in Figure 2. The relative yields (i.e. relative to the internal standard) of the FAMEs of the diatom, Asterionella formosa, in the at-line procedure are comparable to those of the traditional off-line method, (Figure 2). For the eleven FAMEs present in the resulting chromatograms from the two procedures, relative yields were comparable within 2% for ten of them.
Even for the remaining one, C\textsubscript{16:1}, relative yield was within 5%. From these results, it may be concluded that the new method is at least as good as the traditional method. Similar results were obtained for the green algae, Scenedesmus acutus, and the cyanobacterium, Limnothrix sp. strain MRI (table 1). Repeatability for both was satisfactory, with RSDs of typically 1.4 – 4.9% (table 1, n=6). Figure 3 displays the C\textsubscript{14} - C\textsubscript{20} FAME window of As. formosa. Next to the close resemblance of the FAME profile, it clearly illustrates that PUFAs, which show up in the off-line procedure, can also be found with the new procedure. Moreover, compounds due to isomerisation, which are not present in trace (3a) do not appear in trace (3b). The same is true for Figure 4, which presents the chromatographic region for Sc. acutus and Limnothrix sp. Strain MRI, respectively (off-line chromatogram not shown).

Figure 3 shows no isomerisation of PUFAs (i.e. pyrolytic side-reactions) after \textit{in situ} methylation reaction of the algal cells with TMSH. PUFAs play important roles in vital metabolic and physiological functions. For instance, by virtue of being at the base of the trophic ladder of aquatic ecosystems, algae provide energy and essential dietary components for primary consumers and organisms higher up in the food-chain [24]. Furthermore, PUFAs are unique to microorganisms in some cases, and therefore may serve as their biomarkers in ecological and food-chain studies [19]. With the present method, we could identify, for instance, the poly-unsaturated C\textsubscript{20:5} fatty acid by its mass spectrum with the typical m/z 79 &
91 fragments (figs. 3c & 3d) present in many diatoms, including *Asterionella formosa*. It is used as biomarker in stable isotope ecology research [17,25].

**Fig. 3** Total ion current (TIC) GC–MS traces of C_{14}–C_{20} FAME window after off-line (b) and at-line (b) analyses of *Asterionella formosa*. The experimental (d) and database (NIST) (e) spectra of C_{20:5} are shown in the top frames. Peak assignment of FAMES: (1) C_{14:0}; (3) C_{16:2}; (4) C_{16:1}; (5) C_{16:0}; (6) C_{18:3}; (7) C_{18:2}; (8) C_{18:1}; (9) C_{18:0}; (10) C_{20:5}; (11) C_{20:4}; (12) C_{20:0}. Internal standard, C_{20} n-alkane.
Similarly, C_{16:4} and C_{18:3} are important PUFAs (biomarkers) for many green algae including *Scenedesmus acutus* and cyanobacteria respectively (fig. 4A and 4B). On the other hand, saturated fatty acids such as C_{14:0}, C_{16:0}, and C_{18:0} are of little chemotaxonomic value as biomarkers, because they are synthesised by most organisms and occur at various levels of concentration in all algal groups.

**Fig. 4** Total ion current (TIC) GC–MS traces of C_{12–18} FAME window after THM with TMSH of cells of *Limnothrix sp.* strain MRI (A) and *Scenedesmus acutus* (B). Peak assignment of FAMEs: (1) C_{14:0}; (2) C_{16:4}; (3) C_{16:1}; (4) C_{16:0}; (5) C_{18:3}; (6) C_{18:2}; (7) C_{18:1} (8) C_{18:0}. Internal standard, C_{20} n-alkane.

### 4. CONCLUSIONS

The proposed DTD-based procedure is suitable for direct THM analysis of the small amounts of cells available in flow cytometric cell sorting. The FAME profiles are the same as those obtained using the conventional off-line lipid extraction procedure. TMSH is the reagent of choice, since it produces no significant isomerisation products.

The DTD approach allows large numbers of analyses of microbial cells to be performed rapidly and precisely, up to eighteen samples could be analysed per day in a fully automated manner without any maintenance problems for months. Furthermore, very low amounts of sample, amounts down to 100 ng total-cell carbon, as are often obtained in microbial and ecophysiological-oriented studies, can be analysed. In order to reach this
sample throughput, preparation of the 
(n+1)th sample was performed simultaneously with GC analysis of the nth sample. Control is through a local module or PC with software running under Windows.

The good performance, automation and the absence of side-reactions/isomerisation of PUFAs using the novel approach, is very significant and it opens the way to use the novel method as an inlet system in compound-specific stable isotope analyses involving $^{13}$C/$^{12}$C isotope ratio measurements. In such studies, relatively high amounts of the analytes (≥ 5 ng carbon per compound on column) are a prerequisite for the accurate measurement of isotopic numbers [17].

4.1 References

17 Pel, R., Floris, V., and Hoogveld, H., Freshwater Biol. in press.


2.3 Determination of the carbon isotopic composition of whole/intact biological specimens using at-line direct thermal desorption to effect thermally assisted hydrolysis/methylation

Abstract

In this paper, we discuss the use of a direct thermal desorption (DTD) interface as an alternative to Curie-point flash pyrolysis system as an inlet technique in gas chromatography-combustion isotope-ratio mass spectrometry (GC/C-IRMS) analysis of whole/intact phytoplankton and zooplankton specimens. The DTD in combination with a combipal auto-injector is programmed to perform the injection, evaporation of solvents, transport of capped PTV liners to the PTV injector and chemical derivatisation (thermally assisted hydrolysis/methylation; THM) such that a profile of a cellular fatty acids is obtained. Flow-cytometric sorted microalgae and hand-picked zooplankton are used as samples with trimethylsulphonium hydroxide (TMSH) as methylating reagent.

A major advantage of this novel approach over the Curie-point technique is the automation of the total procedure, which allows unattended analysis of large sample series. The profiles and $\delta^{13}C$ carbon isotopic signatures of the fatty acid methyl esters (FAMEs) produced are very similar to those obtained using the Curie-point flash pyrolysis method. It is shown that algal samples must be kept no longer than 48 hours in the DTD-sample tray prior to the THM-analysis in order to maintain the integrity of their FAME-profile.

1.0 Introduction

The use of stable isotopes in ecology at natural abundance levels has emerged as a powerful tool for the study and understanding of ecophysiological processes, food web interrelationships as well as environmental interactions such as climatologic and/or anthropogenic induced trophic shifts. Stable isotope studies initially begun as a principal application in ecophysiological studies for the measurement of $^{13}C/^{12}C$ ratios to identify the photosynthetic pathway of a species [1,2]. Nowadays, stable isotopes,
at natural abundance levels (or deliberately added at tracers), are used to find answers to a broad range of questions such as water-use efficiency, water-source studies, Nitrogen fixation, food source elucidation, authenticity control, fraud detection in food analysis, identification of populations and many more [3–6]. For instance, differences in isotopic composition can be used to trace the origins and to track the fate of organic compounds in environments in which primary producers have different isotopic compositions such as coastal and estuarine ecosystems, rivers and lakes as well as in terrestrial ecosystems undergoing a transition from C3 to C4 plants [7].

More recently, there has been substantial increase in the application of stable isotope in the field of microbial and aquatic ecology by means of compound-specific isotope-ratio analysis using GC-combustion-IRMS. For example, the use of stable isotope-labelled substrates in combination with biomarker analysis offers a unique opportunity to identify and quantify, in an integrated way, the pathways of substrate utilization and degradation rates of micro-organisms [8,9]. In microbiology as well as microbial and phytoplankton ecology, classification and identification of micro-organisms based on rapid and specific methods have proven to be a valuable approach [10]. The combination of modern chromatographic methods such as gas chromatography (and also liquid chromatography) with mass spectrometry techniques has led to the identification of a large number of chemical constituents showing a degree of specificity suitable for chemotaxonomic and/or diagnostic purposes [11,12].

For the identification (and classification) of micro-organisms, the membrane lipid component of their cell constituent is mostly used [13,14]. Fatty acid profiles have been used successfully in the chemotaxonomic screening of a variety of (pathogenic) bacterial isolates [15], the identification of micro-organisms and micro-algae in ecological studies aimed at defining community structure [14–19], in the composition of depositional environments [20], in measuring soil microbial community diversity and defining differences in the communities of different geologic horizons with differing geochemical and geophysical compositions [21–23], and as specific markers in tracing the origin of organic materials in deposits [24,25]. Furthermore, the resulting FAME profiles can be used for their identification and differentiation, sometimes down to the species level [26–29].

Analysis of cellular fatty acids is traditionally carried out by extraction of the lipid from (isolated) micro-organisms, hydrolysis, methylating and finally by analysing and quantification with gas chromatography. Even though commonly a fraction of the
final extract is injected into the GC system after this rather lengthy and extensive sample preparation steps, still, enough micro-organisms must either be originally present in the crude sample or be (selectively) cultured/enriched to provide adequate amounts of fatty acids for the extraction and derivatization steps [30,31]; approximately 10-15 mg wet weight of cells/tissue is necessary.

To reduce the tedious traditional work up procedures as well as to speed up analysis, THM effected by Curie-point pyrolysis in the presence of a methylating reagents has been introduced for the rapid profiling of micro-organisms [32–,33,34].

Pyrolysis-GC is a technique that can be used to provide chromatographic data from otherwise involatile/solid materials. The technique involves the placing of the material of interest onto the pyrolysis wire and then the application of derivatisation reagent. Next, the wire with its content is placed in the injector and is decomposed by rapid heating to give volatile (methylated) components, which are then separated chromatographically to give a fingerprint chromatogram. Even though the application of a pyrolysis unit eliminates the problems of laborious sample work-up, the manual steps still hamper the analysis of large sample series and restricts automation of the full procedure.

In an earlier paper [35], we discussed the use of DTD interface for the analysis of whole/intact microorganisms without any sample preparation using TMSH as methylating reagent. Even though the undesirable base-catalyzed isomerization of polyunsaturated fatty acids (PUFAs) brought about by THM reactions in the presence of high concentrations of TMAH (25%) has been shown to greatly diminished when volume proportion dilution of 1:50 v/v (which gave a pH of 8.8) is applied [36,37], TMSH has been shown to produce higher yield of PUFAs in a non-discriminative transesterification of green micro-algae at low reagent concentrations (e.g. 0.05 M), [38].

In this paper, we discuss the performance of DTD as an alternative inlet system in GC/C-IRMS isotopic analysis of whole/intact freshwater micro-algae, cyanobacteria and small zooplankton specimens with respect to the Curie-point pyrolysis GC/C-IRMS set-up used by Pel et al. in their studies [39]. A major advantage of this novel approach is that it enables analysis of small-sized samples (amounts down to 100 ng total-cell carbon), as are often obtained in microbial and ecophysiological-oriented studies and the automation of the total procedure allows for a much higher sample throughput [39,40].
2. Experimental

2.1 Instrumentation

A 3600CX GC system (Varian, Walnut Creek, CA, USA) equipped with an Optic 2/200 temperature and pressure programmable injection system (ATAS, Veldhoven, The Netherlands). A DTD interface (ATAS) was mounted on top of an Optic 2/200 injector, which actually replaces the septum nut of the Optic 2/200. A special pneumatic opening/closing device facilitates the opening/closing of the DTD injector head. In order to allow for full automation of the system, the upper part of the PTV liner was adapted. Sep-liners, as the adapted PTV liners are referred to, were sealed with a stainless-steel cap. This way, capped liners could be lifted and transported by the auto-sampler arm thanks to its magnetic “base tip”. Samples were deposited into specially designed liner-inserts (~40 μl), which have been placed inside the sep-liner. After an analysis, the liner-insert is removed and a clean fresh one placed inside the sep-liner.

This way, “dirtying” of the sep-liner by difficult matrix samples after one or two runs is avoided and the sep-liner can be re-used. With this set-up, the liner of the GC injection interface can be used as a sample container and/or reaction chamber. The GC was operated at a constant column pressure of 70 kPa using He as carrier gas (see section 2.3 for Curie-point and 2.5 for GC-IRMS operational details).

Flow cytometric analyses and cell sorting of phytoplankton groups were conducted with an Epics Elite (Coulter, Hialeah, FL, USA) cell sorter equipped with an ion argon laser (excitation 488 nm, 30 mW power). The instrument optics were set to collect the red fluorescence of chlorophyll a (bandpass 675 ± 20 nm), and the orange fluorescence of phycoerythrin (bandpass 637±10 nm). For an effective entrapment of filamentous cyanobacteria, the instrument was adjusted one to two droplets beyond the default delay setting as described by Pel et al. [39]. Trichomes of lengths up to 350 μm, which greatly exceed the diameter of the droplets generated in flow sorting mechanism, were successfully collected.

A EuroEA (Eurovector) elemental analyser (EA) coupled on-line (via a conflo 2 interface) to Finnigan Delta-S IRMS was used for carbon isotopic analysis of authentic fatty acid samples, and the TMSH reagent. Tin cups (5 x 8 mm, Elemental Microanalysis Ltd, Okehampton, UK) were prepared by washing them in a 50/50
(vol/vol) methanol/chloroform solution to remove all possible (carbon-containing) contamination.

2.2 Materials

For THM, TMSH (0.05 M in MeOH, Fluka, Buchs, Switzerland) was used as reagent. As THM samples, sub-samples were created from flow-cytometric (FCM) sorted samples of the green algae Scenedesmus acutus, the diatom Asterionella formosa, and the filamentous cyanobacterium Limnothrix sp. strain MR1; all were obtained from the Centre for Limnology, Nieuwersluis (Netherlands Institute of Ecology, The Netherlands). Zooplankton specimens (cyclopoid copepods) were obtained from Lake Loosdrecht (The Netherlands). Cell suspensions delivered by FCM, containing approximately 1.5x10^5 microalgal cells or 3.5x10^5 cyanobacterial filaments, were concentrated by centrifugation to a final volume of 50 μL using oven-cleaned (at 500 °C) glass vials. 2 μL of a homogenous composition of this concentrate was then transferred to a Curie-point filament or DTD microvial/glass insert for analysis. For KIE investigation, selected authentic fatty acids (1 μg/μl) (such as myristic acid (C_{14:0}), palmitic acid (C_{16:0}) and stearic acid (C_{18:0}); Sigma-Aldrich, St. Louis, MO, USA) were methylated with 0.25M TMSH (2:3; vol:vol) and analysed. Pyrolysis and GC parameters were as described in sections 2.3 & 2.4.

2.3 On-line pyrolytic methylation of FCM sorted/monoalgal cultures and zooplankton specimens

Pyrolysis was conducted using a microvolume Curie-point reactor (FOM-4LX type series) developed by FOM Amolf (Amsterdam, The Netherlands), a device similar to that described by Dworzanski et al. [33]. Ferromagnetic wires with a Curie-point temperature of 480 °C were used in the experiments. A high-frequency power supply (0.5 MHz, 200 W maximum) was used to achieve a temperature rise time of approximately 0.5s, and a total pyrolysis time at 480 °C of 3 s.

For phytoplankton specimens, 2 μL of FCM-sorted samples were deposited on the tips of ferromagnetic wires (diameter, 0.4 mm). Loaded wires were allowed to dry at room temperature under reduced pressure and continuous rotation. After drying, 3 μL of 0.05 M TMSH reagent was added, dried and, finally, the wires were drawn into Pyrex glass tubes and inserted into the Curie-point pyrolyzer head. Zooplankton specimens, filtrated from Lake Loosdrecht (The Netherlands), were first sedated with
carbonated water to immobilise them, and then handpicked using syringe under a stereo microscope. Next, they were placed on pyrolysis wires, reagent was added and then dried before being subsequently drawn into Pyrex glass tubes. Finally, the tubes were placed into the Curie-point pyrolyzer head for analysis. Release of the cellular fatty acid fraction from the sample by \textit{in situ} methylation was achieved by a 3-s flash pyrolysis.

### 2.4 THM of FCM sorted/monoalgal cultures and zooplankton specimens using DTD interface

The use of the direct thermal desorption interface for the analysis of whole/intact phytoplankton in aqueous suspensions has been described earlier [35]. A short description of the procedure is as follows: The autosampler (also called \textit{FOCUS}) was programmed to inject 2 µL of an aqueous cell suspension (containing approx. 0.90 mg cell carbon/mL) into a 40 µL microvial/glass insert placed inside a capped liner. The liner and its content was then placed in a built-in vacuum system in the tray for sepliners with the aid of the magnetic tip of the autosampler and allowed to dry. A drying time of 3 min was found to be sufficient for complete drying of 2 µL of aqueous sample by the in-built at-line vacuum system. The at-line vacuum system was achieved by connecting the right hand upper corner hole of the sepliner holder to a vacuum source, which can be turned on/off by a valve fitted to it. Zooplankton specimens were treated as described in section 2.3 and then manually placed into the sepliner insert. The sepliner insert with its content was placed into the liner and then, finally, capped. Subsequently, the \textit{FOCUS} was programmed to perform the rest of the task (except sample injection) as described above for the phytoplankton specimens in this section.

A 1.7 m$^3$/h vacuum pump (Dijkstra Vereenigde BV, Lelystad, The Netherlands) served as the vacuum source. After complete drying, 3 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 by applying vacuum for 45 s.

It was transferred to the DTD interface and finally, THM was performed at 350 ºC by increasing the injector temperature from 40 ºC to 350 ºC at 16 ºC/s under splitless conditions. The liner is heated to effect thermally assisted methylation and simultaneously the fatty acid methyl esters (FAMEs) are introduced from the liner.
into the capillary GC column. At the start of the first sample analysis, the GC was programmed to perform a dummy run while the above-mentioned steps took place. Thus, while a sample analysis is running, the next sample is being prepared simultaneously and hence reducing sample throughput time. The automated liner exchange in DTD-GC has been well described by de Koning et al. [41].

2.5 GC/C-IRMS analysis of THM-created volatiles

Volatilized methylated fatty acid constituents were swept splitless into a capillary gas chromatograph coupled to a Finnigan Delta-S isotope ratio monitoring mass spectrometer via a Finnigan Type II combustion interface (Py-GC/C-IRMS). FAME mixtures were separated on a fused silica apolar analytical column (Hewlett-Packard Ultra-2, 25 m x 0.25 mm i.d., x 0.33 μm film) with helium as carrier gas at a flow of 1.8 ml min⁻¹. The column temperature was programmed from 35 °C to 130 °C at a rate of 30 °C min⁻¹ and to 300 °C at 6 °C min⁻¹. In practice, sufficiently accurate isotope ratio measurements at natural ¹³C abundance level (SD in δ¹³C at least ±0.4‰) were obtained with samples in which the individual major fatty acid(s) present delivered minimally 4 ng carbon on column (equivalent with a ±0.5 volt peak amplitude of the Mz 44 trace [42].

Carbon isotopic composition of FAME is reported in δ notation:

\[ \delta^{13}C = \left( \frac{^{13}C_{\text{sample}}}{^{12}C_{\text{sample}}} \right) \times 1000 - \left( \frac{^{13}C_{\text{reference}}}{^{12}C_{\text{reference}}} \right) \times 1000 \quad (1) \]

and it is expressed relative to the Vienna Pee Dee Belemnite standard. Fatty acid δ¹³C values are reported after correction for the methyl group originating from the derivatizing reagent, and the superimposed kinetic isotopic effect (KIE) associated with TMSH methylation of fatty acids in flash pyrolysis, according to Evans et al. [43]. In these corrections we applied the mean KIE value for fatty acids (C₁₄ - C₁₉) reported by Evans et al of 1.057 (SD = 0.002) [43]. The magnitude of the KIE in the methylation of fatty acids is given by the following equation:

\[ \text{KIE} = 1 + \frac{\Delta(n+1)}{1000} \quad (2) \]
where $\Delta$ is the difference between the measured $\delta^{13}C_{\text{FAME}}$ and the predicted $\delta^{13}C_{\text{FAME}}$ in the absence of any KIE, and $n$ is the number of carbons of the fatty acid under consideration.

So, by substituting KIE and the measured $\delta^{13}C_{\text{FAME}}$ in equation (2) the “predicted” $\delta^{13}C_{\text{FAME}}$ can be obtained, and subsequently the true (corrected) $\delta^{13}C_{\text{FA}}$ value derived by using a mass balance calculation according to:

$$\delta^{13}C_{\text{FA}} = \frac{(n + 1)\delta^{13}C_{\text{protein}} - \delta^{13}C_{\text{TMSH}}}{n} \quad (3)$$

where $\delta^{13}C_{\text{TMSH}}$ is the carbon isotopic composition of the TMSH reagent as determined by EA/IRMS.

3 Results and discussion

The final analytical parameters as described earlier [35,38] were again evaluated, optimized and applied to this procedure. A PTV temperature of 350 °C was found for maximum FAME yield. A distinct optimum (90% yield window) of reagent/analyte ratio in the range 0.02-0.05 (mol reagent/g sample) was also found for samples investigated. For maximum yield of FAMEs without causing and/or promoting isomerization/degradation of PUFAs, 3 µL of 0.05 M TMSH were added to 2 µL of sample (0.90 mg cell carbon/mL or 1-2 animals).

The advantage of a wet chemical procedure with a fractionation into neutral and polar lipids is the additional selectivity it provides in the differentiation of membrane-derived (i.e., “living biomass-related”) and other (i.e., free and/or neutral lipid-derived) fatty acid fractions. However, the use of a combination of flow-sorting and fatty acid profiling in our novel “two dimensional approach” (see also [42]) provides a good enough selectivity in identifying aquatic mesofauna species, which is very important in ecological-oriented (phytoplankton, food-web) studies. In cases where a substantial pool of free fatty acids may be present in the organisms/samples, (e.g. the cyanobacterium Prochlorothrix hollandica [42]), sample analysis with tetramethylammonium acetate (TMAAc) which has been shown to selectively derivatize only free fatty acids in the presence of esterified fatty acids and their salts, can be carried out prior to the main analysis in order to quantify the amount of free fatty acid present in the sample.
Since the pyrolysis device used by Evans et al. [43] and the Curie-point flash pyrolyzer used in this work are essentially the same in providing an almost instantaneous heating of sample and derivitization reagent to the desired reaction temperature, isotopic fractionation effects during the in situ pyrolytic methylation are not expected to differ, and so a repetition of the investigations on KIEs as performed by Evans et al. [43] was considered not to be necessary. An investigation of KIEs when DTD is used as an inlet was, however, performed due to its slower ramping rate (16 °C/s) to the reaction temperature, using some selected authentic fatty acids (C₁₄, C₁₆, and C₁₈) of known carbon isotopic composition.

The manual sample application in py-GC/C-IRMS procedure, as stated earlier (section 1.0), makes analysis of large time series very difficult. Results obtained using the DTD set-up as an inlet system (combined with at-line sample preparation) in a fully automated manner compares favourable to those obtained using the Curie-point pyrolysis inlet system.
Figs. 1A and 1B show the FAME profiles of the diatom *A. formosa*, and a freshwater cyclops species (predaceous copepod) obtained using both the conventional pyrolysis and the DTD as inlet systems for GC/C-IRMS measurements. The FAME profiles obtained are, except for peak shape, identical for both inlet systems for all the samples analysed. One striking similarity is the preservation of PUFAs without any significant degradation and/or isomerisation. For instance C\textsubscript{20:5} and C\textsubscript{22:6} PUFAs, which serve as biomarkers for the diatom *A. formosa* and the freshwater copepod, are nicely elucidated without any degradation and/or isomerisation. If this should happen, then the additional isomers formed will make it impossible to determine the natural occurrence of these PUFAs. This is especially true for ecological and food web studies, where the distinction between isomers originally present in an organism and those that are due to reagent degradation is a necessity.

The peak shape in the chromatograms obtained using the DTD (i.e. thermodesorption) as the inlet system was slightly sharper, (due may be to differences in the heating regimes of the two systems), than those obtained with the flash pyrolysis (i.e. thermochemolysis/pyrolysis) as an inlet system as can be seen in figures 1A and B.

As a result of this, some peaks (for instance the peaks appearing in front the C\textsubscript{20:5} in fig. 1B) could be detected which were not present or hardly detected in the pyrolysis chromatograms. Some typical examples are marked with asterix. At this stage of the research, their identity could not be verified due to the limited possibilities of the one dimensional GC/C-IRMS system which has a poor selective detection. This difficulty may be overcome by the application of multi-dimensional chromatography [44] or additional ion trap/quadrupole mass spectrometry (ITMS/qMS).

The repeatability of the fatty acid profiles with respect to peak area and carbon isotopic composition is shown in table 1 for *A. formosa*, two other microalgae and the copepod. The FAME yields obtained by the DTD and pyrolysis method were found to be similar for all samples tested. So apparently, there are no major differences in the effectiveness to release fatty acids from their cellular matrix, and the transfer efficiency of the analytes in both the pyrolysis and the DTD procedures. The RSD values with respect to peak area were less than 1% for algal species, and less than 3% for the freshwater copepod in both methods.
Table 1: Analytical data for the major FAMEs released using the DTD and pyrolysis methods obtained by comparison of the fatty acid profiles of cyclopoid copepod (1-2 individuals), S. acutus, A. formosa and Limnothrix sp. strain MRI (2 µl).

<table>
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<th>Peak area</th>
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<tr>
<td></td>
<td>Raw Corr. SD</td>
<td>Raw Corr. SD</td>
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<tr>
<td></td>
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<td>DTD Pyrolysis</td>
</tr>
<tr>
<td>Cyclopoid Copepod</td>
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<tr>
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<td>6.1 (1.3)</td>
<td>6.9 (0.7)</td>
</tr>
<tr>
<td>C16:0</td>
<td>8.1 (0.9)</td>
<td>8.4 (1.4)</td>
</tr>
<tr>
<td>C18:0</td>
<td>1.9 (2.9)</td>
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<td>4.7 (1.5)</td>
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<tr>
<td>C22:6</td>
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Scenedesmus acutus

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<td>18.0 (0.3)</td>
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<td>5.7 (0.7)</td>
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<td>C18:0</td>
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Asterionella Formosa

<table>
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<th>FAME</th>
<th>Peak area</th>
<th>( ^{13}C (%a) )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Raw Corr. SD</td>
<td>Raw Corr. SD</td>
</tr>
<tr>
<td></td>
<td>DTD Pyrolysis</td>
<td>DTD Pyrolysis</td>
</tr>
<tr>
<td>C14:0</td>
<td>11.7 (0.8)</td>
<td>9.8 (0.4)</td>
</tr>
<tr>
<td>C16:0</td>
<td>8.1 (0.2)</td>
<td>9.5 (0.6)</td>
</tr>
<tr>
<td>C16:1</td>
<td>5.9 (0.4)</td>
<td>3.2 (0.1)</td>
</tr>
<tr>
<td>C16:0</td>
<td>6.6 (0.2)</td>
<td>5.8 (0.3)</td>
</tr>
<tr>
<td>C18:0</td>
<td>5.4 (0.3)</td>
<td>3.7 (0.1)</td>
</tr>
<tr>
<td>C20:5</td>
<td>26.1 (0.2)</td>
<td>21.8 (0.5)</td>
</tr>
</tbody>
</table>

Limnothrix sp. strain MRI

<table>
<thead>
<tr>
<th>FAME</th>
<th>Peak area</th>
<th>( ^{13}C (%a) )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Raw Corr. SD</td>
<td>Raw Corr. SD</td>
</tr>
<tr>
<td></td>
<td>DTD Pyrolysis</td>
<td>DTD Pyrolysis</td>
</tr>
<tr>
<td>C14:0</td>
<td>16.5 (0.1)</td>
<td>12.8 (0.3)</td>
</tr>
<tr>
<td>C16:0</td>
<td>8.7 (0.7)</td>
<td>10.1 (0.5)</td>
</tr>
<tr>
<td>C16:0</td>
<td>6.9 (0.6)</td>
<td>5.7 (0.3)</td>
</tr>
<tr>
<td>C18:0</td>
<td>22.8 (0.7)</td>
<td>21.1 (0.1)</td>
</tr>
<tr>
<td>C18:0</td>
<td>5.1 (0.5)</td>
<td>5.9 (0.3)</td>
</tr>
</tbody>
</table>

The table contains peak area plus RSD values of peak areas (%; n=6), and raw and corrected \( ^{13}C \)-signature plus SD (n=6). "Corrected values" refer to \( ^{13}C_{\text{FA}} \) values obtained according to equation (3). “Mainly made up of C18:1 with contributions from C18:2 and C18:3 fatty acids. Overall \( ^{13}C \) value for entire fatty acid complex given due to insufficient chromatographic separation in G/C-IRMS analysis.

Repeatability values expressed as relative standard deviation of the peak areas are very good for all measurements (≤ 3%). Noticeably, the values for the copepods are higher than the microalgal samples (up to 1.2 units) in both procedures. For
example, values for C_{20:5} for *A. formosa* are far below 1%, while for the same compound it is significantly above 1% in the case of copepods. Similar results are seen when the RSD values of C_{18:0} and C_{18:n} FAMEs of the other algal species are compared to those of the cyclopoid copepod as can be seen in table 1. The lower variation in repeatability values obtained for the algal species may be attributed to the homogeneity of the starting cell suspension and hence the sub-samples used after FCM-sorting. On the other hand sample variability, in terms of fatty acid content, is more pronounced in collected/selected zooplankton species due to the mode of sample selection/sorting, which is mainly based on physical appearance. And so, while certain animals/zooplankters may look physically similar in size, their fatty acid content may vary significantly depending on their age and growth stage, and this may result in higher (or lower) than expected variation in repeatability values compared to algae.

### Table 2: Effect of KIE on the δ^{13}C FAME values of some selected authentic fatty acids in THM by DTD

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>δ^{13}C (%)</th>
<th>Meas. FAME</th>
<th>Pred. FAME (Δ)</th>
<th>KIE⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>C\textsubscript{14}</td>
<td>−26.7</td>
<td>−30.4 (0.2)</td>
<td>−26.7 3.7</td>
<td>1.056</td>
</tr>
<tr>
<td>C\textsubscript{16}</td>
<td>−29.0</td>
<td>−32.6 (0.2)</td>
<td>−28.9 3.7</td>
<td>1.063</td>
</tr>
<tr>
<td>C\textsubscript{18}</td>
<td>−28.5</td>
<td>−31.7 (0.2)</td>
<td>−28.4 3.3</td>
<td>1.063</td>
</tr>
</tbody>
</table>

⁴Values represent average of 10 EA/IRMS measurements (±0.1); ⁵Values represent average of 10 GC/C-IRMS measurements (with corresponding std. dev.) after derivatization; ⁶obtained by mass balance calculation assuming no KIE: predicted δ^{13}C_{\text{FAME}} = (δ^{13}C_{\text{FA}} + δ^{13}C_{\text{TMSH}})/(n+1), where n is number of carbon atoms in the respective FA, and δ^{13}C of TMSH = -27.4; ⁷KIE = 1 + (Δ (n+1))/1000; KIE\textsubscript{mean}=1.061; std. dev. 0.004.

A mean KIE value of 1.061 (SD = 0.004) was obtained for this work (see table 2) which compares favourably to the 1.057 found by Evans et al [43]. This may be due to the fact that once the critical reaction temperature is reached (at a good enough ramping rate), the THM reactions involved remain essentially the same. Computed true (corrected) fatty acid δ^{13}C values are shown in table 1. For the computation of
true (corrected) fatty acid $\delta^{13}C$ values in this work, mean KIE values of 1.057 and 1.061 were used for the flash pyrolysis and DTD data, respectively. A difference of 2.3–4.1 (‰) was observed when the measured (uncorrected) $\delta^{13}C_{FAME}$ and the true (corrected) $\delta^{13}C_{FA}$ values of the samples investigated were compared as summarised in table 1, underlying the importance of KIE in the computation of “true” $\delta^{13}C_{FA}$ values in GC/combustion/IRMS analysis. Table 1 shows that measured (mean uncorrected) algal and copepod $\delta^{13}C_{FAME}$ values obtained by DTD and Curie-point pyrolysis did not significantly differ between the two techniques. Indeed, the similarities in analytical performance with respect to FAME yields and $\delta^{13}C$-values are strong evidence that DTD and flash pyrolysis provide very similar conditions for the THM reaction. The standard deviations of $\delta^{13}C_{FAME}$ in both procedures were quite similar (SD’s ≈ 0.4 ‰ in most instances) for both algal and zooplankton samples (see also table 1). This similarity may be due to the fact that unlike fatty acid content of an organism which may vary significantly depending on age and/or growth stage, and thus may result in significant differences with respect to fatty acid area response as noted above for the copepods, the carbon $^{13}C$/$^{12}C$ ratio for the same organisms living in the same environment under the same conditions is not expected to show significant differences.

Table 3 shows the preservation/degradation of FAME yield of A. Formosa which was stored in vial trays at room temperature and repeatedly analyzed for four

<table>
<thead>
<tr>
<th>FAME</th>
<th>Day (s)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0</td>
<td>100</td>
<td>99</td>
<td>98</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>C16:1</td>
<td>100</td>
<td>99</td>
<td>96</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td>C16:2</td>
<td>100</td>
<td>99</td>
<td>89</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>C16:0</td>
<td>100</td>
<td>99</td>
<td>96</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td>C18:0</td>
<td>100</td>
<td>98</td>
<td>89</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>C20:5</td>
<td>100</td>
<td>99</td>
<td>96</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td>C20:5</td>
<td>100</td>
<td>98</td>
<td>88</td>
<td>65</td>
<td></td>
</tr>
</tbody>
</table>

Table 3 shows the preservation/degradation of FAME yield of A. Formosa which was stored in vial trays at room temperature and repeatedly analyzed for four
days. It was observed that the loss of FAME yield after two days of storage at room temperature were not very significant for both saturated and (poly)unsaturated fatty acid (−1% and −1.5% respectively) table 3. However, after three days of storage a high loss in yield had become apparent (−4% and −12% respectively) for both saturated and (poly)unsaturated FAMEs, which substantially increased after four days of storage (−9% and −35%, respectively). Similar trend was observed for the fatty acid contents of Limnothrix sp. strain MRI and S. acutus samples. This loss was found to be higher in (poly)unsaturated fatty acids (14-35%) than in saturated fatty acid (4-9%). For instance while the loss in yields for saturated fatty acids in A. formosa after four days of storage at room temperature were 4%, 9% and 9% for C14, C16 and C18 respectively, the loss in yield for (poly)unsaturated fatty acids were 14%, 31%, 34% and 35% for C16:1, C16:2, C18:1 and C20:5 respectively, (see table 3). The most remarkable observation was that the higher the degree of unsaturation of the fatty acid, the more susceptible it is for FAME degradation to occur during storage. The higher loss in yield after four days of storage may be due to (rapid) cell deterioration as a result of the new environmental constraint imposed on it.

4 Conclusions

A fully automated inlet system has been developed as an alternative to the traditional pyrolysis GC/C-IRMS for the measurement of $^{13}\text{C}/^{12}\text{C}$ ratio of aquatic microorganisms at natural abundance. Thanks to the DTD interface, analysis of carbon isotopic composition of whole/intact FCM-sorted biological specimens were carried out using the liner of the GC injection interface as a sample container and/or reaction chamber. The profiles of the fatty acid methyl esters (FAMEs) produced are comparable to those obtained using the THM effected by flash pyrolysis. Very good analytical data were obtained; RSD values of FAME peak areas < 3% and standard deviation $\delta^{13}\text{C}$ (%) ≤ 0.4.

A major advantage of this novel procedure is the possibility of analysing samples in large time series. For optimum FAME yield results, especially for PUFAs, samples should be analysed within 48 hours of sampling/sorting.
4.1 References

15. MID-Sherlock Microbial Identification System, Technical note #101, July 2006, Microbial ID. Inc., Newark, DE.

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Chapter 3

DTD-GC-MS analysis of Ecological and Clinical samples
3.1 A new approach to sporopollenin research using direct thermal desorption–gas chromatography–mass spectrometry: A short communication

Abstract

A novel DTD-GC-ITD-MS method was developed to analyse p-coumaric and ferulic acids which form the monomers or building blocks of the UV-absorbing sporopollenin. Using thermally assisted hydrolysis and methylation, with tetramethyl ammonium hydroxide as methylation reagent, low amounts of pollen were analysed with this novel technique. This novel technique opens the door for the analysis of small amounts of pollen or spores from cultured plants, soil, sediment, and herbarium or peat cores (and residues of them).

DTD injector temperature, gas chromatography as well as mass spectrometric conditions were optimized for the analysis of minimal pollen amounts. A limit of detection value of ~6 fresh pollens of Alnus glutinosa and a relative standard deviation of ~10% were found for this novel procedure.

1. Introduction

Plants have been shown to have developed an active defence mechanism to protect vulnerable tissues against damages caused by UV radiation [1]. To this end, spores and pollen have been shown to have been protected from UV radiation thanks to the biomacromolecule sporopollenin which is present in their outer exine layer [1]. As a result of its ability to resist chemical as well as microbial degradation, sporopollen is well preserved in sediments and soils and thus can serve as a rich source of information on past/historical UV-B irradiation levels. Rozema et al. have demonstrated that chemical information recorded in pollen and spores may be used to reconstruct past UV-B radiation [2]. Alternative sources for information on historic UV-B radiation include plants from herbarium collections. Historic record on a wide variety of plant species over a long period of time may be unravelled with the analysis of small amounts of pollen, and thus providing valuable data for the reconstruction of
UV-B concentrations of the recent past. One of the main difficulties associated with the unravelling of the chemistry of fossil spores and pollen is the limited amount of organic material available for analysis. Fossil pollen and spores are part of a heterogeneous sediment/peat matrix and therefore require laborious manual work to isolate them under a microscope. Since spores and pollen of taxa of interest are usually present in low amounts in sediment/peat matrix, research studies in method development are on-going to develop sensitive analytical methods for their analysis.

For many years, the chemical nature of sporopollenin has been the subject of debate. Even though its chemical nature is not completely known, it is thought to be made up of an aliphatic chain with aromatic groups \([3–5]\), with p-coumaric acid and ferrulic acid monomers/building blocks forming an important part of this polymeric group \([6–8]\). These monomers have formed an intricate non-hydrolysable and insoluble bimolecular moiety which largely limits the choice of techniques that can be used for their analysis as it involves tedious chemical steps, (which are made even more complicated by their very small sizes (e.g. *Alnus glutinosa* pollen diameter~20 \(\mu m\)), prior to analysis. Techniques such as nuclear magnetic resonance, UV/visible and infrared spectroscopy have largely become unfeasible options due to the large amount of materials required for such analysis. Recently however, Blokker et al. [9] reported the use of a flash pyrolysis-GC/MS method for the analysis of low amounts of pollen.

In this paper, the development of a DTD-GC/MS method in which as low as 6 pollen could be analysed is reported.

2. **Experimental**

2.1 **Instrumentation**

A 3800 GC (Varian, Walnut Creek, CA, USA) equipped with an Optic-3 temperature and pressure programmable injection system (ATAS, Veldhoven, the Netherlands) and an ion trap mass spectrometer detector (ITD-MS) (Saturn 2000, Varian) was used. A DTD interface (ATAS) was mounted on top of an Optic-3 injector. With this set-up, the liner of the GC injection interface was used as a sample container and/or reaction chamber. The Optic can be heated up to 650 °C at heating...
rates between 1–30 °C/s, which creates the possibility to perform pyrolysis/chemolysis experiments. The GC was operated at a constant column pressure of 70 kPa using He as carrier gas and an HP-5MS (Agilent, little Falls, USA) fused silica capillary column (15 m x 0.32 mm i.d., 0.25 μm film thickness). The column was connected to the ion source of the ITD via a 1-m fused silica restriction (0.5 m x 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 target set at 10,000; scan time, 1.7 s and scan range, m/z 40–650.

The following technique was adopted for the analysis: Pollen grains were collected into glass vials whose lower halves have been deliberately narrowed/tapered (to approximately 2-2.5 mm). TMAH methylation reagent was added and the content allowed to incubate for 30 min and dried under vacuum. Thereafter, the tapered lower half was broken free with the help of a glass cutter and finally, placed inside the PTV liner. Thus, the broken half of the glass vial also doubles as a liner insert. The liner with its content was then placed in the DTD interface at 40 °C and after 5s of venting it was heated to 550 °C at a rate of 30 °C/s. Transfer onto the column of all products of the chemolysis/pyrolysis reactions was carried out in the splitless mode. The GC was programmed from 35 °C (3 min hold time) to 120 °C at 30 °C/min, followed by an 8 °C/min gradient to 300 °C (5 min hold time).

2.2 Materials and Methods

For thermally assisted hydrolysis and methylation (THM), tetramethylammonium hydroxide (TMAH) (0.25 M in MeOH, Sigma Aldrich) was used. *A. glutinosa* pollen was obtained from the faculty of Earth and Life Sciences, department of Systems Ecology (Vrije Universiteit, Amsterdam). PTV liners and crimp caps were purchased from Atas (Veldhoven, the Netherlands).

3. RESULTS AND DISCUSSION

Accuracy in the counting of pollen grains is a key factor in obtaining reliable results in analysis such as this and thus accurate counting of pollen grains was the greatest challenge in this study. To minimize this challenge, pollen grains of *A.
*glutinosa* which are easily recognizable under the microscope, due to their intermediate size compared to those of other taxa, were selected for this investigation. With the aid of micromanipulator (Leica Microsystems), pollen grains were placed into glass vials carefully avoiding spreading of the pollen in the vial. Excess reagent was also applied so as to minimize or avoid unequal/incomplete “wetting” of pollen with reagent, since in practice homogeneous distribution of reagent over the pollen is practically an almost impossible task.

The result of the optimization of the THM temperature of the at-line DTD procedure is shown in fig. 1. As can be seen, at temperatures between 220-400 °C an almost doubling of the analyte signal is observed. Additional increases of the PTV temperature above 420 °C show marginal increases as can be seen by the analyte signal strongly levelling off towards 600 °C. This observation may be due to the fact that analyte signal is still preserved (i.e. reproducibility) during chemolysis above 400 °C as indicated by flattening/levelling off of the curve above 420 °C. Even though ramping rates (slow or fast rates) of the pyrolysis temperature have been shown not to significantly affect the analyte yield [10], fast ramping rates are often preferable as slow ramping rates tend to, qualitatively, slightly increase chemical background noise as a result of the generation of various by-products. Furthermore, slow ramping rates create the condition(s) that allow compounds to react prior to thermal cracking and vaporization, and hence, the preference of fast heating regimes [9].

![Fig. 1: Response vs PTV temperature curve of p-coumaric acid methyl ester. Similar result was found for ferulic acid methyl ester](image-url)
The profile of the analysis of 50 pollen A. glutinosa is shown in fig. 2. and as can be seen both pCA and FA are clearly displayed. However, elution of peaks in very close vicinity of other analytes may present some difficulty in peak identification in certain samples. This difficulty may be resolved by analysing samples in selected ion monitoring (SIM) mode. For optimal selectivity and sensitivity, the three most intense mass fragments (p-coumaric acid methyl ester = 192, 161 and 133; ferulic acid methyl ester = 222, 191 and 163) were tested. Molecular ions m/z 192 and 222 were found to produce the highest signal and lowest background noise for p-coumaric acid methyl ester and ferulic acid methyl ester. To guide against memory effect which may occur due to long residence of analyte in the PTV injector, especially during analysis of high number of pollen, blanks were run (after 5-10 runs) at high interface temperature of 300 °C. This temperature suffices as it is above the elution temperature of the methylated ferulic acid (200 °C) and p-coumaric acid (278 °C) on the HP-5 MS column. In order to test for the detection limit of the novel procedure, a series of A. glutinosa pollen grains ranging from 10-200 were analysed. The largest source of error in this test, as reported earlier was the counting of pollen, especially for higher pollen counts.

A peak-to-peak signal-to-noise for FA is shown in fig. 3. As can be seen no appreciable/significant increase in S/N ratio is observed when 200 or more pollen is
analysed. This may be due to the significant increase in chemical noise which arises when large amounts of pollen are analysed.

The effect of UV-B irridation on p-coumaric and ferulic acids was investigated using fresh pollen of *Calam epigejos* species which was grown under low and high UV-B settings. As can be seen in figure 4, both p-coumaric and ferrulic acids response positively to UV-B radiation resulting in increase in relative peak abundance. As can be observed, however, this response differs between them with p-coumaric acid showing a higher response than ferrulic acid. This result shows that UV-B irradiation does induces some form of changes in the macro-molecular composition/structure of pollen which can be a very useful source of information/data in attempts to reconstruct past/historic UV-B levels/concentrations.

![Graph showing peak-to-peak signal-to-noise ratio vs pollen number curve](image)

**Fig. 3:** peak-to-peak signal-to-noise ratio vs pollen number curve
The detection limit for this novel procedure for pCA and FA, based on S/N ratio = 4, were found to be 6 and 30 pollen (of Alnus glutinosa) for these analytes respectively. The lower LOD achieved by this study may be partly attributed to the robustness of the Optic 3 system which allows for temperature reduction during runs and hence reduces/minimizes unwanted background noise that comes out of the injector. In addition, the Optic 3 system has a better temperature profile that results in better transfer of high boilers than the Optic 2. Finally, the Optic system allows for quantitative transfer of analytes onto the column and may be considered as a fully closed system. Repeatability expressed as RSD (%) values were within 10%.

4. CONCLUSIONS

A novel procedure has been developed for the analysis of pollen in low amounts. This procedure opens the way for the analysis of pollens, or spores from cultured plants, soil, sediment as well as peat cores etc., especially in studies such as analysis of fossil spores and pollen to develop a proxy to reconstruct past UV-B irradiation. In such cases/situations, not only are fossil spores or pollen present in low amount but lengthy and laborious work-up is often required to obtain just a few amount of sample.
4.1 References


3.2 Fatty acid profiling of crude soil lipid extracts using direct thermal desorption in combination with gas chromatography — mass spectrometry

Abstract

A novel method using direct thermal desorption interface combined with gas chromatography is used to profile fatty acids of crude soil lipid extracts. The FAME profiles obtained using the novel at-line procedure are similar to those produced when samples obtained from the traditional Bligh-Dyer off-line extraction procedure were analyzed. FAME yield for both procedures did not show significant differences. The major advantage of this method is the substantial reduction in the processing time of soil samples. The lengthy traditional manual fractionation of lipid classes and off-line methylation is greatly reduced from the sample processing scheme, saving several hours of laboratory work on a batch of 10 samples. Next to this, large amounts of solvents needed to elute lipid classes during fractionation are not necessary any more.

1. Introduction

Fatty acid analyses of micro-organisms are extensively used in studies of microbial ecology. Classification and identification of micro-organisms based on rapid and specific methods for determining the chemical composition have proven to be a valuable approach in microbiology [1]. Application of instrumentation-based techniques in microbiology to perform traditional/conventional procedures for the identification and differentiation of micro-organisms has increased over the past decade; for instance, Fast atom bombardment mass spectrometry (FAB-MS), Fourier transform infrared spectroscopy, UV resonance Raman spectroscopy, Flow cytometry, Gel electrophoresis, Laser-based enzyme profiling, Gas chromatography (GC) and Curie-point pyrolysis (Py-MS; Py-GC-MS) have all been used for identification and differentiation of micro-organisms [2–6]. Using established analytical methods, these techniques have been used to measure distinct biochemical properties and/or taxonomic relevant chemical distributions [7,8]. The combination of chromatographic methods such as gas chromatography (GC) and liquid chromatography (LC) combined with mass spectrometry (MS) techniques has led to the identification of a large number of chemical
constituents showing a degree of specificity suitable for chemotaxonomic and/or diagnostic purposes [9].

In fatty acid/lipids analysis, two main methodological approaches that convert non-volatile components to volatile products have been used widely. The first approach is based on wet chemical methods involving lipid extraction, saponification, and initial separation and/or derivatization of the lipid constituents prior to GC analysis [10,11], whiles the second procedure is based on thermal degradation (pyrolysis) of whole micro-organisms or cellular constituents [12].

Lipid extraction in the wet chemical procedure is typically achieved by the Bligh and Dyer method or its modification as introduced in environmental research by White and co-workers [13,14]. The extraction mixture is composed of chloroform, methanol and an aqueous phase in proportions to give a single phase, and this probably improves the extractant-cells contact in complex matrices such as soil/sediment compared to procedures with separate aqueous and organic phases. A modification of the Bligh and Dyer procedure for the dissolution of polar lipids in cell membranes has also been reported by Kates [15]. Brinch-Iversen et al. proposed the use of dichloromethane as an alternative to the more hazardous chloroform [16]. In combination with strong sodium bromide solution during phase separation, this modification results in phase inversion with physical separation of the organic solvent phase from sample residue following centrifugation [17]. Chemical methods of sample preparation enable determination of highly specific substances, e.g., fatty acids derived from bacterial cell walls.

A major disadvantage of the traditional wet chemical procedure is the rather laborious and long procedures it entails, thus affecting the analytical throughput. To overcome this drawback a lot of research efforts have been made to enable fast and accurate profiling of fatty acids [18–21] using such techniques as the pyrolytic methylation. Pyrolytic methods allow for a comparatively fast analysis of microorganisms since placing of a suspension of cells together with a derivatizing reagent on the pyrolytic probe, is the main step involved. However, compared to the wet chemical methods, the pyrolytic techniques are less accurate in terms of overall specificity as they are comparatively more prone to produce elevated levels of molecular ion fragmentation(s).
For identification of microorganisms by phenotype and genotype, a number of commercial systems are available. For instance, the Microbial Identification Systems (MIS) produced by MIDI (Newark, DE, USA) has been widely used for the identification of microorganisms by fatty acid analysis [22]. Nonetheless, for certain so-called difficult sample matrices such as soil and sediment, some form of sample preparation is still required prior to GC analysis. The current study explores the possibility of adapting a DTD inlet system developed earlier [23] to profile the fatty acid composition of soil/sediment samples in a fast, simple and accurate manner.

2.0 Experimental

2.1 Instrumentation

A 3800 GC (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 mass spectrometer (ITD-MS) (Saturn 2000, Varian) were used. A direct thermal desorption (DTD) interface (ATAS), which actually replaces the septum nut of the Optic 2/200, was mounted on top of an Optic 2/200 injector. The GC was operated at a constant column pressure of 70 kPa using He as carrier gas. A HP-5MS (Agilent, Little Falls, MN, USA) fused silica capillary column (30 m x 0.25 mm i.d., 0.1 µm film thickness) was connected to the ion source of the ITD via a 1-m fused silica restriction (0.5mm i.d., 75 µm). For detection and identification, the ITD settings were: trap temperature, 220 ºC; interface temperature, 270 ºC; manifold temperature, 50 ºC; A/M amplitude, 4.0 V; emission current, 10 µA; AGC target set at 10,000; scan time, 1.7 s and scan range, m/z 40–650. A special pneumatic opening/closing device facilitates the opening/closing of the DTD injector head.

In order to operate in a fully automated manner, the upper part of the PTV liner and the “base tip” of the “programme unit” of the PTV, had to be adapted. The liner should be sealed with a stainless-less steel cap and the “base tip” of the auto-sampler arm should be magnetic, such that the auto-sampler arm can lift and transport a capped liner. With this set-up, the liner of the GC injection interface can be used as a sample container and/or reaction chamber.

2.2 Materials

For thermally assisted hydrolysis and methylation (THM), trimethylsulfonium hydroxide (TMSH) (0.25 M in MeOH, Fluka; Buchs, Switzerland) was used, while tetramethylammonium acetate (TMAAc) (Sigma-Aldrich, St. Louis, MO, USA) was used for
selective methylation of free fatty acids. For re-evaluation of the selective methylation action of TMAAc, heptadecanoic acid (margarinic acid, 17:0) and glyceryl tripalmitate (Sigma-Aldrich, St. Louis, MO, USA) were used. As internal standard (IS), C22 n-alkane (Polyscience Corporation, Niles, IL, USA) was used. Soil samples were obtained from the Centre for Limnology, Nieuwersluis (Netherlands Institute of Ecology, The Netherlands). Soils were taken from two floodplain locations (“Ewijkse Waard” (N 51° 52’ E 05° 53’) and “Oude Waal” (N 51° 51’ E 05° 53’) at the River Waal (The Netherlands), the main tributary of the River Rhein as has been described by Kemnitz et al 2004. Ewijkse Waard is a river bank that gradually rises from the water level of the river. The gradient in elevation results in gradient of flooding events. Two sites within the Ewijkse Waard area were selected according to different duration and frequency of flooding. They were termed according to their elevation: HL (high level) and LL (low level) sites. The area Oude Waal is an oxbow lake that was a former arm of the river. Samples of the site PM (permanent marshland) were taken in the emergent macrophyte (Glyceria maxima) zone. Physico-chemical parameters of the sampling sites have been described earlier (Kemnitz et al. 2004). Soil cores from the 3 sites were sampled (length 20 cm, diameter 3.8 cm). The soil cores were sectioned into three layers; 0-5 cm (depth 1), 5-10 cm (depth 2) & 10-20 cm (depth 3) and homogenized.

2.3 Off-line soil sample extraction

Off-line lipid extraction was carried out from 4g of freeze-dried soil using a modified version of the Bligh-Dyer extraction procedure as previously described [24,25]. After the first step of this procedure, one-half of the sample, referred to as the crude sample in this study, was used for the at-line transesterification procedure and the other half for the traditional off-line procedure. By means of sequential elution with chloroform, acetone and methanol, lipid extracts were fractionated on silicic acid into different polarity classes. Using mild-alkaline methanolysis, the methanol fraction containing polar lipids was derivatized and 2μl of the solution was injected for GC analysis.

2.4 THM of crude soil samples using TMSH (at-line transesterification)

Analysis of crude soil sample was carried out by re-optimisation of a generic analysis similar to the analysis of whole/intact phytoplankton in aqueous suspensions [26]. For transesterification of samples, trimethylsulfonium hydroxide again turned out to perform best compared to tetramethylammonium hydroxide (TMAH) and trimethylphenyl hydroxide (TMPAH). Compared to the latter two, TMSH has been found to produce firstly higher yield
of PUFAs and, secondly, cause no isomerisation/degradation of PUFAs [27]. In addition, TMSH is reported to be a better reagent for on-column methylation because methylation with TMSH is achieved at lower temperatures and thus causes less pyrolytic side-reaction(s) to occur, although it has similar alkalinity as TMAH and TMPAH (pkb=12) [28].

For thermal hydrolysis methylation of the crude sample, the interface which is the robotic arm of the autosampler, was programmed to inject 2ul of crude sample into 40-μl vial placed inside a capped liner. Next, 3μl of 5mM TMSH solution in methanol was applied and the reaction mixture allowed to incubate for 15 min. Finally, after applying vacuum for 15 s to evaporate the content of the insert, the liner and its content was transferred to the DTD interface where THM was performed at 350 °C by increasing the injector temperature from 40 °C to 350 °C at 16 °C/s under splitless conditions. The liner is heated to effect thermally assisted methylation, and simultaneously the fatty acid methyl esters (FAMEs) are introduced from the liner into the capillary GC column. The GC was programmed from 35 °C (5min hold time) to 120 °C at 30 °C/min, followed by a 6 °C/min gradient to 300 °C (hold time 5min).

While the temperature-programmed analysis was performed, the steps for preparation of the next crude sample took place. Thus, after cooling down of the GC oven, the prepared liner containing the prepared sample was ready to be placed into the DTD interface for analysis. In this way sample throughput was increased to a sample per 45 min.

The at-line vacuum system has been described earlier [41] and a short description is as follows: The right hand upper corner hole of the sep-liner holder was modified such that a 1.7 m³/h vacuum pump (Dijkstra Vereenigde BV, Lelystad, The Netherlands) could serve as the vacuum source. To improve evaporation as well as desorption of analytes, a glass vial/insert of the same length as the sep-liner and with a ‘pinched’ hole just above its midpoint (to provide an additional outlet for analytes) has been developed into which samples can be placed for analysis.

2.5 GCxGC-ToF-MS

An HP 6890 (Agilent Technologies, Palo Alto, CA, USA) gas chromatograph equipped with an Optic 2 (ATAS) operating in the splitless mode was used. For MS detection a Pegasus II ToF MS (LECO, St. Joseph, MI, USA) operating at -70 eV, 280 °C transfer line, 280 °C ion source and scanning between 70 and 800 m/z at 50 Hz and a detector voltage of 1950 V was applied. The conventional 1st dimension column was an HP-5MS (5% Phenyl + 95% Dimethyl Polysiloxane) 30 m x 0.25 i.d., 0.25 μm film thickness (Agilent, Little Falls, MN, USA). The 2nd dimension column (fast) was a BPX50 (50% Phenyl Polysilphenylene-
siloxane) 1m x 0.10 mm I.D., 0.10 µm film thickness (SGE International, Rinhwood, Australia). A temperature programme of: 70 °C (1min) slowly heated to 300 °C at 3 °C/min with a final hold time of 15 min was used. Helium was used as carrier gas at a constant flow of 1.2 ml/min. A 5 s modulation time was applied in all analysis and initiated by the chemstation (HP) programmed external events, which through an electronic controller, also activates the modulator valves. Carbon dioxide (CO₂) was used for cryogenic trapping of analytes. For data collection, total ion current (TIC) MS signal was used. For data transformation as well as visualization, a program to convert the raw data into a two-dimensional array (software provided by Ph.J. Marriot) and a program for the generation of contour plots from this array (“Transform”, part of Noesys software package; Research Systems Int., Crowthorne, UK) were used.

2.6 Identification and quantification

The identities of sample methyl esters were determined by comparing their relative retention times with those of well-known FAME standards. Quantification of FAMEs (area response) was accomplished with the help of the C₂₂ n-alkane internal standard.

3.0 Results and discussion

GC conditions as reported earlier [23] were again re-evaluated and optimized for this work. A THM temperature of 350 °C was found to produce maximum fame yield as shown in figure 1.

![Figure 1](image_url)

**Fig. 1:** Optimisation of injection temperature for DTD-GC-MS analysis
Incubation time (for the crude soil extract) of 15 min. was used. TMSH (0.005M) was chosen as methylation reagent as it produced no significant amount of degradation/transesterification isomers compared to other methylation reagents such as tetramethylammonium hydroxide (TMAH) and trimethylphenyl hydroxide (TMPAH).

For a more accurate comparison of analysis of the crude soil lipid extracts and derived PLFA fractions, a control on the possible presence of substantial amounts of free fatty acids pools in the soil/sediment samples was included in our evaluation. The ability of TMAAc to discriminate between free and esterified fatty acids has been nicely demonstrated by Hardell et al [29] and this was re-evaluated using heptadecanoic acid and glyceryl tripalmitate as shown in fig. 2A and 2B for this study. A mixture of heptadecanoic acid and glyceryl tripalmitate was analyzed with TMSH as methylation reagent. As can be seen, both heptadecanoic acid and glyceryl tripalmitate were methylated into their respective fatty acid methyl esters (fig. 2A). However, when TMAAc was applied to the same mixture, only heptadecanoic acid methyl ester was formed as shown in fig.2B, demonstrating the selective methylation of only free fatty acids in the presence of esterified fatty acids by TMAAc.

![Glyceryl tripalmitate + 17:0 + TMSH](image1)

![Glyceryl tripalmitate + 17:0 + TMAAc](image2)

**Fig. 2:** TMAAc selective methylation of free fatty acids in the presence of esterified fatty acids.
THM analyses of the crude soil sample with TMSH as methylation reagent was preceded by one with TMAAc as methylation reagent as shown in fig. 3A. The presence of \( C_{16:1} \)\(^{7c} \), \( C_{18:1}^{9c} \), and \( C_{18:0} \) free fatty acids are clearly observed as they are selectively methylated into their corresponding methyl esters by the TMAAc methylation reagent and these are corrected for in the final computation of the FAME MS-response of the crude soil extract. The FAME profile of the crude soil extract is similar to that obtained when PLFA methanol-fraction of the traditional procedure was analysed. In both profiles, \( C_{16} \) and \( C_{18} \) saturated FAMEs, the mono- and di- unsaturated FAMEs of \( C_{16} \) and \( C_{18} \) remain the dominant FAMEs.

**Fig. 3:** FAME profile of DTD-GC-MS analysis of crude soil extract and off-line soil lipid extract from permanent marshland (0-5cm) after at-line TMSH (C), off-line mild alkaline (B) and TMAAc methylations (A); \( aC_{14}=1 \), \( C_{14:0}=2 \), \( iC_{15}=3 \), \( aC_{15}=4 \), \( C_{17}=5 \), \( iC_{16}=6 \), \( C_{16:1}^{8c}=7 \), \( C_{16:1}^{9c}=8 \), \( C_{16:1}^{7c}=9 \), \( C_{16}=10 \), \( brC_{17}^{7c}=11 \), \( aC_{17}=12 \), \( C_{17:1}^{7c}=13 \), \( C_{17:1}^{6c}=14 \), \( cyC_{17}^{7c}=15 \), \( C_{17}^{7c}=16 \), \( aC_{18}=17 \) \( C_{18:2}^{6c}=18 \), \( C_{18:1}^{7c}=19 \), \( C_{18:1}^{9c}=20 \), \( C_{18:1}^{7c}=21 \), \( C_{18}^{0}=22 \), \( brC_{19}^{9c}=23 \), \( C_{19}^{0}=24 \), \( C_{20:5}^{3c}=25 \), \( C_{20:4}^{6c}=26 \), \( C_{20:1}^{7c}=27 \), \( C_{20}^{0}=28 \)
Comparison of FAME yield for both procedures is shown in figure 4. As can be seen, even though FAME yield using the DTD at-line analysis of crude soil extract compares favourably to the FAME yield after analysis of soil samples that has undergone the traditional extraction procedure, the differences in yield is not as high as one would have expected given that the crude soil extract would contain neutral triacylglycerol (i.e. neutral lipid fatty acids; NLFA) in addition to polar lipids (i.e. phospholipid fatty acids; PLFA).

For improved Fame detection, soil samples were analysed with comprehensive DTD-GCxGC (2D) technique. As shown in figure 5, Fames that co-elute in one dimension GC (1D) analysis are nicely separated, thanks to the high resolving power of the 2D technique. For instance, C_{18:3w3} which co-elutes with C_{18:1w9} in 1D analysis, is nicely displayed in the GCxGC chromatogram (fig. 5). In addition, the high sensitivity of the 2D technique makes it possible for analytes that are present in low amounts in the sample matrix to be detected (e.g. C_{16:1w7} and C_{18:1w9}).
The distribution of FAMEs at various layers for the permanently flooded, intermittently flooded and the lowly flooded areas are shown in table 1. Even though saturated FAMEs such as C_{16}, C_{18} and C_{20} do not show any significant differences in terms of FAME yield, the same cannot be said of the unsaturated FAMEs. For instance, while differences of 0.15 and 0.05 units are found for C_{16} and C_{18} respectively, differences of 0.34, 0.28 and 0.25 units are observed for C_{16:1\text{9t}}, C_{18:2\text{6c}} and C_{18:3\text{3c}} mono-, di- and tri-unsaturated FAMEs respectively as can be seen in table 1. Percentage composition of FAMEs (w%) for PM, HL and LL layers are shown in table 1. It can be seen that the percentage FAME composition are similar for the PM HL and LL samples. A high degree of repeatability was realised by this novel method as shown by RSD values of less than 3% as shown in table 1.

Fig. 5: C_{16} and C_{18} FAME region after DTD-GCxGC analysis of crude soil lipid extract from permanent marshland (0-5cm). Similar results were observed for off-line extracted soil lipids.
<table>
<thead>
<tr>
<th>FA</th>
<th>PM</th>
<th>HL</th>
<th>LL</th>
<th>PM</th>
<th>HL</th>
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<th>PM</th>
<th>HL</th>
<th>LL</th>
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<td>0.7</td>
<td>0.41</td>
<td>0.34</td>
<td>0.33</td>
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<tr>
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<td>2.48</td>
<td>2.61</td>
<td>1.2</td>
<td>0.9</td>
<td>1.3</td>
<td>0.42</td>
<td>0.40</td>
<td>0.39</td>
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<tr>
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<td>9.62</td>
<td>8.15</td>
<td>0.8</td>
<td>0.9</td>
<td>1.2</td>
<td>1.56</td>
<td>1.55</td>
<td>1.22</td>
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<td>0.92</td>
<td>0.83</td>
<td>0.8</td>
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<td>0.7</td>
<td>0.18</td>
<td>0.15</td>
<td>0.12</td>
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<tr>
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<td>4.95</td>
<td>5.24</td>
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<td>0.86</td>
<td>0.80</td>
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<td>-</td>
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<td>9.63</td>
<td>10.3</td>
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<tr>
<td>C18:1o9t</td>
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<td>9.71</td>
<td>10.2</td>
<td>0.3</td>
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<td>1.58</td>
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<td>0.59</td>
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<td>1.3</td>
<td>0.39</td>
<td>0.31</td>
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</table>

**Table 1:** Percentage FAME compositions, FAME yield and RSD values of selected FAMEs after DTD–GCxGC-ToF-MS analysis of crude soil lipid extract. Similar results were obtained for the methanol-PLFA fraction.

The PLFA C16:1o5t, C16:1o8c and C18:1o8c are highly specific biomarkers for methane oxidizing bacteria (Bodelier et al. 2000, Mohanty et al. 2006), an environmentally highly relevant group of bacteria involved in the degradation of the greenhouse gas methane. C16:1o5t and C16:1o8c are indicative for Type I methane oxidizing bacteria (MOB) such as Methylomonas, Methylobacter and Methylocibacter whereas C18:1o8c is highly specific for Type II MOB such as Methylocystis and Methylosinus. The distribution of these PLFA in the “flooding gradient” is presented in Figure 6. As can be seen, C18:1o8c fatty acid is present in PM but undetectable in both the HL and LL sample areas, indicating that type II methane oxidizing bacteria are clearly much more abundant in the permanently flooded soils whereas type I methane oxidizing bacteria are equally abundant throughout the flooding gradient. It can also be seen that whereas no substantial changes in terms of MS FAME response for C16:1o5t occur for HL and LL flooded soil (figure 6b and 6c), substantial changes are seen for...
PM flooded areas with the highest amount found at 0-5cm depth and the lowest at 20cm depth as can be seen in figure 6a. On the other hand, changes in MS response for C₁₆:₁₆:₀ do not show any significant differences for PM, HL and LL flooded soil regions for the three soil depth of 0-5cm, 5-10cm and 10-20cm investigated. Hence, the new method is clearly capable of detecting microbial population dynamics in complex environmental matrices like soils.

Fig. 6: Distribution of important PLFA (polar lipid derived fatty acid) within a flooding gradient with depth in the soil profile. 1= 0-5 cm soil layer depth; 2=5-10 cm soil layer depth; 3=10-20 cm soil layer depth;
4. Conclusions

Cellular fatty acids present in crude soil lipid extracts have been successfully profiled using at-line DTD-GC-MS. The FAME profile obtained using the novel at-line procedure is similar to those obtained when samples obtained from the traditional Bligh-Dyer off-line extraction procedure were analyzed. FAME yield for both procedures did not show significant differences. The method could clearly monitor and quantify population dynamics of environmentally relevant bacteria in complex environmental matrices.

The major advantage of this method is the substantial reduction in the processing time of soil samples. The traditional manual fractionation of lipid classes and off-line methylation is greatly reduced from the sample processing scheme, saving 2 days of laboratory work on a batch of 10 samples. Next to this, large amounts of solvents needed to elute lipid classes during fractionation are not necessary any more.

4.1 References


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3.3 Fatty acid profiling of raw human plasma and whole blood using direct thermal desorption combined with gas chromatography — mass spectrometry

Abstract

Gas chromatography (GC) has in recent times become an important tool for the fatty acid profiling of human blood and plasma. An at-line procedure used in the fatty acid profiling of whole/intact aquatic micro-organisms without any sample preparation was adapted for this work. A direct thermal desorption (DTD) interface was used to profile the fatty acid composition of human plasma and whole human blood of eight volunteers in a procedure omitting the usual lipid extraction steps that precede sample methylation in the traditional (off-line) protocols. Trimethylsulfonium hydroxide (TMSH) was used as reagent for thermally assisted methylation. In a fully automated manner, the liner of the GC injector is used as a sample-and-reaction container with the aid of the DTD interface.

The fatty acid methyl ester (FAME) profiles obtained using this novel approach, were very identical to those obtained when the traditional off-line protocol was applied. FAME yields obtained in the at-line DTD method were found to be very similar for saturated fatty acids, but significantly higher for polyunsaturated fatty acids compared to off-line yields. As a result of the contribution of circulating cell membranes in blood, substantial differences were observed when the amount of FAMEs obtained in whole human blood and human plasma samples were compared after their analysis. Thanks to the fully automated operation of this novel procedure, large series of analyses can easily be performed.

Keywords: Direct thermal desorption; Methylation; Blood; Plasma; Fatty acids

1.0 Introduction

Over the years, fatty acid profiling of biological (i.e. nutritional and biomedical) samples has gained tremendous importance as far as the understanding of
the relation between dietary lipids and the development of ailments such as diabetes and cardiovascular diseases are concerned [1–3]. In recent decades, the relationship between fat consumption and health has been investigated in several epidemiological as well as clinical studies. The determination of fatty acids in human plasma by GC has become a useful and routine tool for the understanding of the significance of dietary fat for human health [4]. Particularly, the consumption of an n-3 polyunsaturated fatty acid (PUFA)-rich diet has been positively linked with optimal infant development [5,6], cardiovascular protection [7], prevention of neurodegenerative diseases [8] and behavioral disorders [9] as well as improvement of immune defenses [10].

Since the publication at the end of the 1950s when the first GC application on human plasma was made [11], a lot of studies (both nutritional and biomedical) have been carried out not only to determine the effects of fat biomarkers on nutritional status but also to establish their relationship with some major pathologies such as cardiovascular diseases [12–16]. In addition, with the aid of isotope labelling, studies have been carried out to investigate the digestion of dietary triglycerides by pancreatic lipase and the absorption of fatty acids. For this purpose single tracer or dual tracer studies have been applied in 13C breath tests [17,18] or isotope enrichment measurements in blood [19–21] with the aid of gas chromatography/combustion-isotope ratio-mass spectrometry (GC/C-IRMS). Another important research question is the determination of the endogenous capacity to synthesize fatty acids from acetyl co-enzyme A (acetyl-CoA). For this purpose [1,13C]-acetate is infused intravenously and the 13C incorporation in serum palmitic acid is measured with GC/MS. Data are deconvoluted using the mass isotopomer distribution analysis (MIDA) procedure originally described by Hellerstein et al. [22–25]

Conventional methods for the profiling of fatty acids consist of several steps; viz, lipid extraction with organic solvents, hydrolysis, methylation/derivatization, and finally, analysis and quantification with GC [26,27]. The derivatization step involves the conversion of all fatty acids that may be present in the sample into their corresponding volatile fatty acid methyl esters (FAMEs). This step can be carried out by acid catalysis using hydrochloric acid [28,29], sulfuric acid [30] or boron trifluoride [31,32], all in methanol, by alkaline catalysis using sodium methoxide in anhydrous methanol [33] or by a combination of the two procedures [34]. Diazomethane has also been used as a methylation reagent [35].
The application of the conventional procedure to biological samples is, however, bedevilled with disadvantages such as high risk of contamination and recovery losses in multi-step procedures. Furthermore, these methods become cumbersome and impractical for analysis of large series of samples especially for limited sample amounts. To overcome these disadvantages, methods that combine extraction and derivatization in a single step have been developed. Direct (i.e. without previous lipid extraction) methylation of fatty acids has been carried out by several investigators using acetyl chloride [36], hydrochloric acid in methanol [26] or boron trifluoride in methanol [37,38]. Ohta et al [39] have described a rapid method for the analysis of the fatty acids (FAs) of plasma phospholipids, based on the direct application of plasma aliquots to the silica layer of a TLC plate without previous lipid extraction, followed by a chromatographic run.

In all the so-called direct analyses such as mentioned above, some form(s) of minimal sample preparations were required. In this study, the results of analysis of raw human plasma and whole blood samples carried without any sample preparation are compared with the conventional procedure.

2.0 Experimental

2.1 Instrumentation

A 3800 GC (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 mass spectrometer (ITD-MS) (Saturn 2000, Varian) were used. A direct thermal desorption (DTD) interface (ATAS) was mounted on top of an Optic 2/200 injector as described earlier in section 2.2.

The GC was operated at a constant column pressure of 70 kPa using He as carrier gas. A HP-5MS (Agilent, Little Falls, MN, USA) fused silica capillary column (30 m x 0.25 mm i.d., 0.1 μm film thickness) was connected to the ion source of the ITD via a 1-m fused silica restriction (0.5mm i.d., 75 μm). 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 target set at 10 000; scan time, 1.7 s and scan range, m/z 40–650.

A special pneumatic opening/closing device facilitates the opening/closing of the DTD injector head. In order to operate in a fully automated manner, the upper part
of the standard fritted optic interface/PTV liner was adapted so as to make it possible to be tightened in the same way as an autosampler vial.

These new liners, otherwise referred to as sep-liners, were sealed with a stainless-steel cap. This allowed capped liners to be lifted and transported, courtesy the magnetic “base tip” of the auto-sampler arm. The design of the sep-liner as well as automated liner exchange in DTD-GC has been well described by de Koning et al. [40]. Specially designed liner-inserts (~40μl) were put inside the sep-liner into which samples were placed. The “dirty” liner-insert could be removed after an analysis and a clean fresh one placed inside the sep-liner for the next analysis. This way, the sep-liner can be re-used several times as “dirtying” of the sep-liner by difficult matrix samples after one or two runs is avoided. With this set-up, the liner of the GC injection interface can be used as a sample container and/or reaction chamber.

2.2 Materials

For thermally assisted hydrolysis and methylation (THM), trimethylsulfonium hydroxide (TMSH) (0.25 M in MeOH, Fluka; Buchs, Switzerland) was used. For reference purposes, transesterification with BF₃ (12%) in MeOH (Merck, Darmstadt, Germany) was used. Human plasma and whole blood samples, collected from eight individuals, were obtained from University Medical Center Groningen (venous blood collection), The Netherlands. As internal standard (I.S.), C₂₂ n-alkane (Polyscience, Niles, IL, USA) was used.

2.3 Off-line transesterification of plasma and whole blood samples

The off-line sample preparation technique/method as described by Bondia-Pons et al. [41] was adapted for the analysis of human plasma and whole blood samples. Human plasma and whole blood samples were either immediately processed or stored (airtight) at 4 °C, prior to analysis. For storage periods exceeding two weeks, addition of butylated hydroxytoluene (BHT, in ethanol) is necessary to prevent the loss of polyunsaturated fatty acid. 100 μg of either sample (i.e. human blood or human plasma) were saponified in PTFE screw-capped Pyrex tubes containing 0.25 μg of the I.S. by adding 1 ml of sodium methylate (0.5%/w/v) and heating to 100 °C for 15min. After cooling to room temperature (25 °C), samples were esterified with
1mL of BF₃ in methanol (also at 100 °C) for 15 min. Again after cooling of the tubes, 500 µL of n-hexane was added to extract the fatty acid methylesters (FAMEs). They were then shaken for 1 min. and 1 mL of saturated sodium chloride solution added. Finally, the tubes were centrifuged for 8 min at 2200 X g. After drying with anhydrous sodium sulfate, the clear n-hexane top layer was transferred into an injection vial, evaporated to dryness under a gentle stream of nitrogen and then redissolved in 100 µL n-hexane. 2 µL of the final solution was injected in the splitless mode at an injector temperature of 40 °C; that is, the same amount as in the in the at-line approach should reach the column. GC–MS conditions were the same as those reported above.

2.4 **THM of plasma and blood samples using TMSH**

*(at-line transesterification)*

A procedure for the use of direct thermal desorption (DTD) interface for the analysis of whole/intact phytoplankton in aqueous suspensions which has been described earlier was adapted for this work [42]. Trimethylsulfonium hydroxide instead of other alkaline based methylating reagents such as tetramethylammonium hydroxide (TMAH) and trimethylphenyl hydroxide (TMPAH) was again chosen as methylating reagent as it has been found to cause no significant amount of isomerization/degradation of (poly)unsaturated fatty acids (PUFAs) compared to the others [43]. In addition, TMSH is reported to be a better reagent for on-column methylation because methylation with TMSH is achieved at lower temperatures and thus causes less pyrolytic side-reaction(s) to occur, although it has similar alkalinity as TMAH and TMPAH (pKb=12) [44].

To optimise the FAME profile of the plasma/blood samples, the robotic autosampler arm (also known as the FOCUS) was programmed to inject 2 µL of plasma/blood sample into 40-µL vial/insert placed inside a capped liner. The liner was then placed in a built-in vacuum system in the tray for sep-liners, using the magnetic tip of the autosampler in combination with a stainless-steel cap for the sep-liner. Next, it was allowed to dry for 3 min. Then, 3µL of 5 mM TMSH solution in methanol was injected and the reaction mixture allowed to incubate for 15 min. Finally, after applying vacuum for 45 s to dry the contents of the insert and the addition of 2 µL C₂₂ n-alkane internal standard, the liner and its content was transferred to the DTD
interface where THM was performed at 280 °C by increasing the injector temperature from 40 °C to 280 °C at 16 °C/s under splitless conditions.

The liner is heated to effect thermally assisted methylation, and simultaneously the fatty acid methyl esters (FAMEs) are introduced from the liner into the capillary GC column. The GC was programmed from 35 °C (4 min hold time) to 120 °C at 30 °C/min, followed by a 7 °C/min gradient to 280 °C (hold time 5 min). The system was programmed to perform a dummy run at the start of the first sample preparation procedure, while the steps mentioned above took place. Thus at the start of the next procedure, the prepared liner is placed into the injector for analysis and, so, while the GC is running, the next sample is being prepared at the same time.

The at-line vacuum system has been described earlier [42] and a short description is as follows: The right hand upper corner hole of the sep-liner holder was connected to a vacuum source, which can be turned on/off by a valve fitted to it. A 1.7 m³/h vacuum pump (Dijkstra Vereenigde BV, Lelystad, The Netherlands) served as the vacuum source. To improve evaporation as well as desorption of analytes, a glass vial/insert of the same length as the sep-liner and with a ‘pinched’ hole just above its midpoint (to provide an additional outlet for analytes) has been developed into which samples can be placed for analysis.

2.5 Identification and quantification

The identities of sample methyl esters were determined by comparing their relative retention times with those of well-known FAME standards. Quantification of FAMEs (area response) were accomplished with the help of the C22 n-alkane internal standard.

3.0 Results and discussion

Analytical parameters of the final procedure described earlier [42] were evaluated, optimised and adapted for this work. For 2 μl of sample, 3 min. of drying time and 3 μl of 5 mM TMSH methylation reagent were applied. For sample volumes of more than 3 μl, “cooking/boiling” as a result of vacuum drying, (leading to the spread of samples along the inner walls of the liner insert), was observed.
This may lead to low FAME yields during analysis as the methylation reagent may not react with the entire sample delivered into the liner insert. A step-by-step sample drying of 2 μl or 3 μl (depending on the sample volume) per step was found to overcome this problem.

Maximum response for FAMEs was reached at a PTV temperature of 280 °C instead of 300 °C as in the earlier study. An incubation time of 15 min instead of 30 min as in the earlier study and a TMSH concentration of 5 mM, the same as in the earlier study, were applied. The result of temperature optimization for the at-line DTD procedure for the whole blood sample is shown in fig. 1. The highest FAME yield was found around a temperature of 280 °C. Similar result was found for human plasma sample.
The fatty acid profile of the whole blood sample after GC analysis using the conventional extraction procedure and the at-line DTD method is shown in fig. 2A and 2B respectively. The FAME profiles were found to be essentially identical, with the at-line procedure giving a better baseline than the off-line method. No degradation/isomerization of (poly)unsaturated fatty acids was observed using the DTD method. This may be explained by the use of TMSH as reagent which causes no significant effect of degradation/isomerization (side)-reactions compared to other at/on-line methylation reagents such as TMAH and TMPAH as reported by Ishida et al. [45]. Similar results were found when the FAME profiles of the at-line and off-line analysis of human plasma sample were compared.
The percentage compositions of the main fatty acids present in both samples after conventional (i.e. lipid extraction followed by methylation into FAMEs) and the at-line (i.e. direct methylation or so-called transesterification) procedures are shown in table 1. The results show that, for both methods of analysis, the amount of each individual FAME relative to the total FAME amount (i.e. percentage FAME composition), are very similar for both the whole human blood as well as the plasma analysis. For instance in both procedures, the total FAME yield in the analysis of the whole blood sample was dominated by saturated FAMEs (40%), followed by mono- and (poly)unsaturated (21% each) and finally di-unsaturated (18%) FAMEs. Similar results were obtained for plasma samples (table A).

A comparison of FAME yields obtained when whole blood samples were analysed using the traditional extraction procedure and the DTD method is shown in fig. 3. FAME yields were computed according to the area amount/response of the individual FAME under consideration with respect to the area amount/response of n-

### Table 1: Fatty acid (FA) percentage composition and RSD (%) of whole human blood and plasma total lipids of a volunteer after direct transmethylation (B and D) and conventional extraction procedures (A and C) (n=6). Similar results were obtained for seven other volunteers.

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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| FA | Whole blood | Plasma |  |
| B (DTD) | A (Conventional) | D (DTD) | C |
| FA% RSD | FA% RSD | FA% RSD | FA% RSD |
| C14:0 | 1.22 0.55 1.37 0.47 | 2.10 1.39 2.32 0.95 |
| C16:0 | 19.7 0.72 19.9 0.94 | 26.3 0.78 28.9 0.58 |
| C16:1 | 1.60 2.37 1.75 3.14 | 2.10 1.53 2.94 1.34 |
| C18:0 | 18.0 0.53 19.6 0.63 | 19.3 0.79 24.8 2.14 |
| C18:1 | 18.1 1.08 19.2 0.98 | 17.4 0.90 16.5 1.49 |
| C18:2 | 18.3 0.57 19.7 1.65 | 16.5 0.75 15.2 0.43 |
| C20:0 | 1.13 0.68 1.53 0.97 | 1.52 2.93 0.85 1.89 |
| C20:1 | 1.07 0.54 1.57 1.50 | 1.50 2.26 0.79 1.78 |
| C20:2 | 1.85 1.67 1.99 1.05 | 1.55 0.49 0.91 0.16 |
| C20:4 | 9.02 0.57 5.96 0.15 | 6.22 1.03 3.03 1.12 |
| C20:5 | 0.98 1.07 1.23 0.47 | 1.52 0.80 0.79 1.16 |
| C22:6 | 4.49 0.78 2.97 1.68 | 2.04 1.42 1.05 0.97 |
| C22:5 | 2.27 0.96 1.63 0.25 | 1.02 0.49 0.93 0.81 |
| C22:4 | 2.28 1.31 1.61 1.29 | 0.98 1.11 0.91 1.28 |

The percentage compositions of the main fatty acids present in both samples after conventional (i.e. lipid extraction followed by methylation into FAMEs) and the at-line (i.e. direct methylation or so-called transesterification) procedures are shown in table 1. The results show that, for both methods of analysis, the amount of each individual FAME relative to the total FAME amount (i.e. percentage FAME composition), are very similar for both the whole human blood as well as the plasma analysis. For instance in both procedures, the total FAME yield in the analysis of the whole blood sample was dominated by saturated FAMEs (40%), followed by mono- and (poly)unsaturated (21% each) and finally di-unsaturated (18%) FAMEs. Similar results were obtained for plasma samples (table A).
alkane C₂₂ internal standard. Both the traditional extraction procedure and the at-line DTD method were carried out six-fold.

The result shows that the FAME yields using the at-line DTD (with trimethyl sulfonium hydroxide as a reagent) method in the analysis of whole human blood were very similar (saturated FAMEs) or even increased for the mono- and polyunsaturated FAMEs (except C₂₀:₁ and C₂₀:₅) compared to the traditional procedure. Indeed, substantially higher FAME yields were observed for the more important PUFAs of whole human blood such as C₂₀:₄, C₂₂:₆, C₂₂:₅ and C₂₂:₄ using the at-line DTD method (fig. 3) compared to the traditional off-line extraction method (fig. 3). Even though the traditional method is more susceptible to sample losses/contamination, this observation may be attributed to the ability of TMSH to produce higher yields of PUFAs at low concentrations compared to other transesterification reagents [42] rather than methodical differences (i.e. DTD vs classical methods). Similar results were obtained in the analysis of human plasma.
Compared to their saturated counterparts, differences in the FAME yields for PUFAs were found to be even more pronounced when the versatility of the novel DTD method using TMSH was evaluated against whole blood and plasma samples as shown in fig. 4. This observation was consistent in the entire eight volunteers investigated (fig. 5). While small differences were observed for mono and di-unsaturated FAME yields, differences in FAME yields were relatively substantial for poly-unsaturated FAMEs (except for the minor C20:5). For example, while a difference of 1.3, 1.0 and 1.3 (relative area units) were obtained for C16:1, C18:2 and C20:1 respectively, substantially higher differences of 2.6, 3.0, and 3.3 (relative area units) were observed for C20:4, C22:6 and C22:5 PUFAs, respectively. The higher PUFA yields in the whole blood sample may be due to the contribution of circulating cells with membranes which are mainly composed of phospholipids. These phospholipids are reported to be rich in such poly-unsaturated FAMEs as arachidonic acid (AA, 20:4 n-6) and docosahexaenoic acid (DHA, 22:6 n-3) [46].

Fig 4: Comparison of relative FAME area response of whole human blood (black) and human plasma (grey) of a volunteer using the at-line method. Similar results were obtained for seven other volunteers (see figure 5). Yield = area response vs C22 internal standard.
Again, this observation may be attributed to the ability of the TMSH reagent to perform transesterification reaction without causing significant isomerization and/or degradation of PUFAs and thus enabling the realization of “real” yields, and

**Fig 5**: Comparison of relative FAME area of some important whole human blood (■) and human plasma (▲) FAMEs of seven (7) volunteers using the at-line method.
hence the “real differences in yields”, of the PUFAs in the samples under investigation.

4. Conclusion

The fully automated at-line DTD method has successfully been adapted for the analysis of whole human blood and plasma. The FAME profiles obtained using this novel approach, are identical to those obtained when the traditional off-line procedure was applied. Again, TMSH was the reagent of choice, since it produces no significant isomerization/degradation products. FAME yields obtained using the at-line DTD method were found to be very similar (saturated fatty acids) or even higher (polyunsaturated fatty acids) than the results obtained when the traditional off-line procedure was applied. The repeatability (assessed through FAME analysis of the same whole blood/plasma sample within the same day) expressed as RSD (%) values, were 0.5 – 2.9. Similar results were found for the human plasma sample.

In addition to its advantage of being operated in a fully automated manner and thus allowing the analysis of large sample series, another major advantage of this procedure is that very low sample amounts (2-3μl) are required compared to the 50-100μl usually needed for the conventional off-line procedure.

4.1 References

Chapter 4

Comprehensive two-dimensional DTD-GCxGC-ToF-MS analysis of aquatic mesofauna species
Improved fatty acid detection in micro-algae and aquatic mesofauna species using direct thermal desorption interface combined with comprehensive gas chromatography — time of flight — mass spectrometry

Abstract

GCxGC with a time-of-flight mass spectrometer as a detector is used to profile the fatty acid composition of whole/intact aquatic micro-organisms such as the common fresh water green algae *Scenedesmus acutus* and the filamentous cyanobacterium *Limnothrix* sp. strain MRI without any sample preparation steps.

It is shown that the technique can be useful in the identification of lipid markers in food web as well as environmental studies. For instance, new mono and di-unsaturated fatty acids were found in the C_{16} and C_{18} regions of the green algae *Scenedesmus acutus* and the filamentous cyanobacterium *Limnothrix* sp. strain MRI samples. These fatty acids have not been detected in the conventional one dimension (1D) GC analysis due to either co-elution and/or their presence in low amounts in the sample matrix. In GC x GC, all congeners of the fatty acids in these micro-organisms could be detected and identified due to the increased analyte detectability and ordered structures in the two-dimensional separation space. The combination of DTD-GCxGC-ToF promises to be an excellent tool for a more accurate profiling of biological samples and can therefore be very useful in lipid biomarker research as well as food-web and ecological studies.

1. Introduction

Lipids are major cellular components which occur as diverse compounds both structurally and functionally. The principal components of most natural lipids are fatty acids (FAs) and like lipids, portray an immense diversity in terms of chain-length, degree of unsaturation, geometry and substitution pattern. Fatty acids can be present either as the
free fatty acid or the esterified form. As a result of their importance, the profiling of fatty acid of living organism has become a necessary tool for the classification and determination of biological interactions [1,2], bioremediation and medical microbiology [3–5] and biomarker research [6,7] etc.

Over the years, several procedures have been developed to profile the fatty acid composition of organisms. For instance, gas chromatography has been used in the profiling of the fatty acid composition of human plasma in order to understand the significance of dietary fat in human health [8]; GC/combustion/IRMS has been applied to investigate the digestion of dietary triglycerides by pancreatic lipase and the absorption of fatty acids using single/dual tracer studies in $^{13}$C breath tests or isotope enrichment measurements of blood [9 –13]; pyrolysis gas chromatography (Py-GC-MS) has been applied in various compositional studies such as the analysis of membrane-derived fatty acids, biopolymers, humic substances and even whole soils [14 –20]. Procedures for the characterization of fatty acids of various samples have gradually improved from the traditional manual extraction methods [21], through one-step pyrolysis GC-MS technique [22] to DTD-GC/MS technique of profiling of fatty acids of raw biological samples [23]. These new developments have reduced, or in some cases have eliminated, sample treatment prior to analysis and have led to remarkable reduction in analysis time as well as increase in sample throughput. Still, these novel procedures end up with complex chromatograms. Even in cases where very selective (manual) sample clean-up procedures are applied, the problem of co-eluting FAME compounds of different carbon chain lengths and degree of unsaturation still persist.

For characterization of such complex mixtures, GCxGC has in the past decade emerged as a powerful separation technique [24]. In order to solve the problem of peak co-elution in the study of lipids, GCxGC-MS has been applied in profiling the fatty acids of oils [25 –29], in lipid characterization of milk, faecal sterols in biological samples as well as complex lipidic samples [30–32].

GCxGC coupled to mass spectrometry has emerged as a powerful tool in the analysis and identification of analytes present in complex samples over the past decade [24]. The peak capacity in GCxGC system is equal to the product of the peak capacities in the two-dimensional system, thus making it a superior technique to the
multidimensional GC (i.e GC–GC) technique whose peak capacity is the sum total of the peaks of the one-dimensional systems involved. Improved chromatographic resolution, decrease in detection limits (due to cryofocusing) and the ability to present structures of chromatograms in a chemically ordered manner are the main advantages of GCxGC [24]. Furthermore, it provides such information as second dimension retention times, peak intensity etc. The cryofocusing is induced by the modulator which is the heart of the GCxGC system. The main functions of the modulator are to trap compounds eluting from the first dimension (by creating a cold spot with the aid of cold fluids such as liquid nitrogen, liquefied carbon-dioxide or air (ambient-temperature) depending on the application), refocus analytes (in order to obtain sharper second dimension peaks) and finally, re-inject the refocused analytes into the second dimension column. Coupling of GCxGC systems has been limited to such detectors as the flame ionisation detector (FID) and the micro-electron capture detector (μECD) due to their ability to provide the high acquisition rate required to monitor the sharp chromatographic peaks (usually of 50-600 ms peak width). For instance GCxGC has been coupled to FID in fatty acid characterization of oils and milk [25– 29].

While the use of such detectors as FID permits peak recognition, they do not provide structural information. For identification and/or confirmation of the numerous compounds that are separated in complex mixtures using the GCxGC procedure, the use of a spectrometric detector, specifically, mass spectrometer (MS) has become indispensable. Presently, the Time of Flight Mass Spectrometer (ToF-MS) has become the detector of choice for GCxGC systems thanks to its ability to acquire the fifty or more mass spectra per second (high acquisition rate) that are required for the proper reconstruction of GCxGC chromatograms and quantification. Unlike in one dimensional chromatography, GCxGC characterization of lipids has not received as much attention as the characterization/analysis of petroleum, cigarette smoke, pesticides, flavour compounds [33 –35] etc.

In an earlier paper [23] the possibility of profiling the fatty acids of intact/whole aquatic micro-organisms in a fully automated manner was demonstrated. However, due to the one-dimension nature of the system set-up, separation of co-eluting fatty acids (e.g. C₁₈ω in Limnothrix sp. strain MRI and S. acutus) were not realised. In ecological and
food-web oriented studies which involve isotope ratio measurement ($^{13}$C/$^{12}$C ratio) of signature fatty acids, a complete peak resolution is mandatory to improve substantially on the reliability and “exactness” of the $\delta^{13}$C values obtained for lipid biomarkers. Furthermore, the application of GCxGC technique in lipid research may enhance biomarker research, especially in the discovery of FAMEs which may be present in low amounts in complex sample mixtures.

In the present paper, the possibility of using a DTD interface coupled to GCxGC-ToF/MS to resolve co-eluting peaks, detect and identify FAMEs present in low amount in the sample matrix of one dimension (1D) analysis of certain aquatic micro-organisms [23] is explored.

2. Experimental

2.1 Instrumentation

**GC/C/IRMS**

A 3600CX GC system (Varian, Walnut Creek, CA, USA) equipped with an Optic 2 temperature and pressure programmable injection system (ATAS, Veldhoven, The Netherlands) was used. A direct thermal desorption (DTD) interface (ATAS), which actually replaces the septum nut of the Optic 2, was mounted on top of an Optic 2 injector.

To allow for a full automation of the system, the upper part of the PTV liner was adapted. The sep-liners, (as the adapted PTV liners are called), were sealed with a stainless-steel cap and thus makes it possible for capped liners to be lifted and transported by the auto-sampler arm with the help of its magnetic “base tip”. Samples were placed into specially designed liner-inserts (~40μl), which have been placed inside the sep-liner. After an analysis, the liner-insert is removed and a clean fresh one placed inside the sep-liner. This way, “dirtying” of the sep-liner by difficult matrix samples after one or two runs is avoided and the sep-liner can be re-used. With this set-up, the liner of the GC injection interface was used as a sample container and/or reaction chamber. The automated liner exchange in DTD-GC has been well described by de Koning et al. [36].
The GC was operated at a constant column pressure of 70 kPa using He as carrier gas. Volatilized methylated fatty acid constituents were swept splitless into a capillary gas chromatograph coupled to a Finnigan Delta-S isotope ratio monitoring mass spectrometer via a Finnigan Type II combustion interface (DTD-GC/C-IRMS). FAME mixtures were separated on a fused silica capillary column (HP-5MS, 30 m x 0.25 mm i.d., 0.25 μm film thickness, Agilent, Little Falls, MN, USA) with helium as carrier gas at a flow of 1.8 ml min\(^{-1}\). The column temperature was programmed from 35 °C to 130 °C at a rate of 30 °C min\(^{-1}\) and to 300 °C at 6 °C min\(^{-1}\).

### 2.2 GCxGC-ToF-MS

An HP 6890 (Agilent Technologies, Palo Alto, CA, USA) gas chromatograph equipped with an Optic 2 (ATAS) operating in the splitless mode was used. For MS detection a Pegasus II ToF MS (LECO, St. Joseph, MI, USA) operating at -70 eV, 280 °C transfer line, 280 °C ion source and scanning between 70 and 800 m/z at 50 Hz and a detector voltage of 1950 V was applied. The conventional 1\(^{st}\) dimension column was an HP-5MS (5% Phenyl + 95% Dimethyl Polysiloxane) 30 m x 0.25 i.d., 0.25 μm film thickness (Agilent, Little Falls, MN, USA). The 2\(^{nd}\) dimension column (fast) was a BPX50 (50% Phenyl Polysilphenylene-siloxane) 1m x 0.10 mm i.d., 0.10 μm film thickness (SGE International, Rinhwood, Australia). A temperature programme of; 70 °C (1 min) slowly heated to 300 °C at 3 °C/min with a final hold time of 15 min was used. Helium was used as carrier gas at a constant flow of 1.2 ml/min. A 5 s modulation time was applied in all analysis and initiated by the chemstation (HP) programmed external events, which through an electronic controller, also activates the modulator valves. Carbon dioxide (CO\(_2\)) was used for cryogenic trapping of analytes. For data collection, total ion current (TIC) MS signal was used. For data transformation as well as visualization, a program to convert the raw data into a two-dimensional array (software provided by Ph.J. Marriot) and a program for the generation of contour plots from this array (“Transform”, part of Noesys software package; Research Systems Int., Crawthorne, UK) were used.
3. **Materials and methods**

A fatty acid methyl ester standard solution containing 37 FAMEs of different chain lengths and degree of unsaturation was purchased from Supelco (Supelco Park, PA, USA). For thermally assisted hydrolysis and methylation (THM), TMSH (0.25 M in MeOH, Fluka) was used as reagent. As THM samples, sub-samples were created from flow-cytometric (FCM) sorted samples of the green algae *Scenedesmus acutus* and the filamentous cyanobacterium *Limnothrix sp.* strain MR1 which were all obtained from the Centre for Limnology, Nieuwersluis (Netherlands Institute of Ecology, The Netherlands). Larvae of the mosquito *Chironomus plumosus* and other zooplankton specimens such as *Daphnia galatea* and cyclopoid copepods (Lake Loosdrecht, The Netherlands) were also obtained from the Centre for Limnology. Cell suspensions delivered by FCM, containing approximately 1.5x10^5 microalgal cells or 3.5x10^5 cyanobacterial filaments, were concentrated by centrifugation to a final volume of 50 µL using oven-cleaned (at 500 °C) glass vials. 2 µL of a homogenous composition of this concentrate was then transferred into a 40 µL microvial/glass insert placed inside a capped liner for analysis.

A 1.7 m³/h vacuum pump (Dijkstra Vereenigde BV, Lelystad, The Netherlands) served as a vacuum source for drying aqueous sample suspensions.

3.1 **THM of FCM sorted/monoalgal cultures and zooplankton specimens using DTD interface**

The use of the direct thermal desorption (DTD) interface for the analysis of whole/intact phytoplankton in aqueous suspensions has been described earlier [30]. A short description of the procedure is as follows: The FOCUS, which is the programmable arm of the Optic system, was programmed to inject 2 µL of an aqueous cell suspension (containing approx. 0.90 mg cell carbon/mL) into a 40 µL microvial/glass insert placed inside a capped liner. The liner and its content was then placed in a built-in vacuum system in the tray for sepliners with the aid of the magnetic tip of the autosampler and allowed to dry. A drying time of 3 min was found to be sufficient for complete drying of 2 µL of aqueous sample by the built-in vacuum system. The vacuum system was achieved by connecting the right hand upper corner hole of the sepliner holder to a vacuum source, which can be turned on/off by a valve fitted to it. Zooplankton
specimens, filtrated from Lake Loosdrecht (The Netherlands), were first sedated with carbonated water to immobilise them and then handpicked using syringe under a stereo microscope. They were subsequently manually placed into the sepliner insert. The sepliner insert with its content was placed into the liner and, finally, capped. Subsequently, the FOCUS was programmed to perform the rest of the task (except sample injection) as described above for the phytoplankton specimens in this section.

After complete drying (in the case of aqueous sample suspensions), 3 µL of a 0.005 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 by applying vacuum. It was transferred to the DTD interface and finally, THM was performed at 350 °C by increasing the injector temperature from 40 °C to 350 °C at 16 °C/s under splitless conditions. The liner is heated to effect thermally assisted hydrolysis and methylation, and simultaneously the fatty acid methyl esters (FAMEs) are introduced from the liner into the capillary GC column (i.e. the 1st dimension column). At the start of the first sample analysis, the GC was programmed to perform a dummy run while the above-mentioned steps took place. Thus, while a GC run is taking place, the next sample is being prepared simultaneously and hence increasing sample throughput.

4. Results and discussion

Optimised GC conditions as reported earlier [23] were re-evaluated and applied to this work. In order to enable a single procedure to be applied to as many different microflora and aquatic mesofauna as possible, a PTV temperature of 350 °C was again chosen even though maximum response for FAMEs was already achieved at a PTV of 300 °C and no losses were observed at 350 °C. Again, TMSH was chosen as methylation reagent as it produced no significant amount of degradation/transesterification isomers compared to other methylation reagents such as tetramethylammonium hydroxide (TMAH) and trimethylphenyl hydroxide (TMPAH). As in an earlier study [23], an incubation time of 30 min. and a concentration of 0.005 M TMSH was applied.

For the purposes of this manuscript, fatty acids are named according to the following designation: Cx:yoz where x represents the number of carbon atoms, y represents the degree of unsaturation and z represents the position of the first unsaturated...
carbon atom counting from the terminal methyl group of the alkyl chain. For instance, a fatty acid with a designation of \( C_{16:3,6} \) can simply be interpreted as an unsaturated fatty acid containing 16 carbon atoms, having three double bonds of unsaturation, and that the first unsaturated bond is located on the sixth carbon atom counting from the terminal methyl group of the alkyl chain. FAMEs were identified with the aid of FAME standards (ordered GCxGC structures) combined with the log \( K_{ow} \) (i.e. octanol-water partition coefficient) vs \( \log RT \) (i.e. second dimension retention time) correlation technique for FAME identification as suggested by Jover et al [31]. To answer, in future, the question as to how accurate isotopic numbers computed for coeluting FAMEs using the present GC-IRMS technique are, 1D GC-IRMS and 2D GCxGC profiles of micro-algae and aquatic mesofauna species are compared.

\[ \text{Fig. 1: 1D DTD-GC-IRMS analysis of } S. \text{ acutus (A) and a cyclopoid copepod (B) samples showing poor resolution (co-elution) of certain peaks} \]

\[ \text{acutus (1a) and cyclopoid copepod (1b). As can be seen, peak separation of certain} \]
(poly)unsaturated fatty acid methyl esters is quite poor. For instance, a number of PUFAs (especially in the C18:n and C20:n regions) are not well resolved to enable unambiguous identification.

Similar results were observed when the cyanobacteria Limnothrix sp. strain MRI was analysed. In addition, the low analyte detectability of the 1D system compared to the
2D systems, makes identification of PUFAs which may be present in low amounts in the complex sample matrix very difficult (e.g. asterix (*) labelled peaks of *S. acutus* and the cyclopoid copepod in fig. 1). For unambiguous peak identification in fatty acid biomarker studies as well as precise computation of isotopic numbers in IRMS measurements, complete peak resolution of FAMEs is very important.

In order to select the appropriate column-set for optimum FAME separation in this study, a typical orthogonal (HP-5MS – BPX50) and non-orthogonal (DB-Wax – BPX35) column-set systems were investigated. Figures 2a and 2b show the two dimension (2D) chromatograms of orthogonal and non-orthogonal column-set system analysis of FAME standards respectively. The chromatograms depict the two main different modes of PUFA separation in 2D systems; that is, separation based on the degree of unsaturation for orthogonal system and one based on the carbon chain length in the case of the non-orthogonal system respectively. The orthogonal column system shows a better two-dimension profile of FAMEs than the non-orthogonal column system. In this column combination, FAMEs are separated in the 2nd dimension (2tr) according to the degree of unsaturation with saturated FAMEs eluting first followed by FAMEs with increasing degree of unsaturation.

On the other hand, the non-orthogonal system does not show the use of two-dimensional space and thus FAMEs are grouped together according to their carbon chain lengths and not degree of unsaturation (fig. 2b). A close look at fig. 2b shows that in the non-orthogonal column-set system, FAMEs are separated more according to 1st dimension retention time (1tr) than the 2nd dimension retention (2tr). Even if some compounds are shown to have 2tr of two or more seconds, this is only caused by the temperature limitation of 260° C of the 1st dimension column. Compounds with higher carbon numbers than C18 have under these conditions higher retention times in the 2nd dimension. For instance, C24:0 and C24:1 elute at the same retention as can be seen in figure 2b. This is, in actual fact a one-dimension separation. Since one of the main goals of this study is to elucidate clusters of co-eluting peaks and in addition detect compounds that may be present in low amounts in the complex sample matrix, the orthogonal column-set system (HP-5MS–BPX50) was chosen to analyse various aquatic micro-organisms. For fast FAME identification, FAME standards were injected and their positions identified by
spectral comparison. Templates were made and then fitted onto real life samples in order to aid in the identification of FAMEs present in the real life samples.

As stated above, resolution of co-eluting peaks is one of the major problems in the conventional GC as can be seen in figure 1. Figures 2c and 2c’ show the GCxGC analysis of the cyanobacteria *Limnothrix* sp. strain MRI. This figure clearly shows that, unlike the 1D analysis of the same sample, the application of the GCxGC technique resulted in complete resolution of the C18:n (see fig. 2c insert) FAME cluster into C18:1, 7, C18:2, 6 and C18:3, 9 fatty acids. Two FAMEs, C18:1, 7 and C18:3, 9 are coeluting in the first at 3250 s are now fully separated. The second dimension retention times (2.4 s and 2.75 s for C18:1, 7 and C18:3, 9 respectively) are however distinct for these FAMEs and thus enables better peak resolution in GCxGC (fig. 2c). The capability of the GCxGC technique to detect analytes present in low amounts in the complex analyte matrix enables the detection of for instance, C18:3, 9 FAME which as far as we know has not yet been reported. This FAME is not detected in the 1D analysis due to its presence in low amounts in the analyte complex matrix and will thus be embedded in the noise level of the chromatogram as amply demonstrated by the GCxGC spectrum in figure 2c’.

Figure 3a shows the 2D chromatogram of the C16 FAME region of the green algae *S. acutus*. As can be seen, all detectable FAMEs present in this region are nicely separated. Surprisingly, a new FAME C16:2, which to our knowledge has also not been previously reported (may be due to its presence in low amounts in the complex matrix), was detected thanks to the high detectability of the 2D system. Similarly, FAMEs of the C18 region are nicely resolved (see figure 3b). Again, C18:1 and C18:3 FAMEs, which will otherwise co-elute in 1D analysis, are beautifully separated. Again, C18:2 FAME which is present in very low amount in the complex matrix is well detected.

Another previously unreported FAME, C18:4, was detected. Figure 3c shows the 2D chromatogram of *D. galatea* which was reared on *S. acutus*. All FAMEs detected reflect the FAME compositions as measured for *S. acutus* in this study, showing a complete qualitative FAME (energy) transfer from the microalga to this zooplankter, an
observation which can be of much interest in the interpretation of trophic interactions in food-web studies.

Fig. 3: Chromatographic 2D elucidation of the C_{16} and C_{18} regions of S. acutus and D. galeata samples
In figures 4a and 4b, the high detectability of the 2D system is once again utilized to detect C18:2, C18:4, C20:4 and C22:5 FAMEs which are also present in low amounts in the complex matrix of cyclopoid copepod. In figures 4c and 4d, the zoom function of the “transform” software program was utilized to enhance the detection of analytes that are present in very low amounts in the analyte matrix complex.

**Fig. 4:** Chromatographic 2D elucidation of the C16, C18, C20 and C22 regions of a cyclopoid copepod and the larval stage of *C. plumosus*
This property is applied to detect fatty acids in the C\textsubscript{20} and C\textsubscript{22} regions of \textit{C. plumosis} (mosquito larvae) as shown in figure 4d.

As can be seen, FAMEs detected in C\textsubscript{22} region for example are present in very low amounts and are therefore found in the noise region of the chromatogram. One way analytes that are present in low amounts in the analyte complex matrix may be detected is to increase the sample concentration/amount. This, however, will lead to the overload of FAMEs that are present in higher amounts in the sample matrix. A compromise may be to run two exploratory sample analyses; one with normal sample concentration of the matrix complex (\textasciitilde 0.90 mg cell carbon/ml) for the detection of analytes that may be present in adequate amount in the matrix complex and another with an increased sample concentration for analytes that may be present in low amounts in the matrix complex.

The combination of the high resolving power as well as the high sensitivity by the comprehensive GCxGC technique makes it possible to detect and identify certain PUFAs which were not detected and/or identified in the application of the 1D technique as a result of co-elution and/or the low amount of the analyte present in the sample matrix. For instance, C\textsubscript{16:2}:\textsubscript{4} \textit{(S. acutus)}, C\textsubscript{16:2}:\textsubscript{4} & C\textsubscript{18:3}:\textsubscript{6} \textit{(D. galeata)}, C\textsubscript{18:2}:\textsubscript{6}, C\textsubscript{20:4}:\textsubscript{3}, C\textsubscript{20:4}:\textsubscript{6} & C\textsubscript{22:5}:\textsubscript{3} (cyclopoid copepod) and C\textsubscript{20:1}:\textsubscript{7}, C\textsubscript{22:4}:\textsubscript{6}, C\textsubscript{22:5}:\textsubscript{3} & C\textsubscript{22:6}:\textsubscript{3} \textit{(C. plumosus)} are nicely detected using the GCxGC technique.

This novel procedure in which the high resolving power as well as the high sensitivity of the comprehensive GCxGC technique is utilised to profile the fatty acids of micro-algae and aquatic mesofauna, may find interesting application in studies employing compound-specific isotope-ratio measurements of lipid biomarkers where good peak resolution is essential in the computation of accurate isotopic numbers. For reliable \textsuperscript{13}C measurement at natural abundance level in fatty acid isotope ratio measurement, at least 4 ng C per fatty acid must be admitted on column which means high sample load and hence, the use of GC columns with thick films (at least 0.2 μm). Improving the slow acquisition rates of the present isotope ratio systems (i.e. detectors) will be the major challenge in realising a fast and reliable GCxGC-combustion-IRMS system.
5. Conclusion

DTD-GC\timesGC-ToF has been successfully used to profile the fatty acid composition of aquatic micro-organisms such as the green algae *S. acutus*, the cyanobacteria *Limnothrix* sp. strain MRI, the mosquito larvae, *C. plumosus, D. galatea* and the cyclopoid copepod. The capability of a direct thermal desorption (DTD) set-up allows solid and / difficult sample matrices to be analyzed without any solvent extraction and/or additional sample preparation step(s).

It is shown that the technique can be useful in the identification of lipid markers in food web and/or ecological studies. For instance, new mono, di and tri-unsaturated fatty acids were found in the C\textsubscript{16} (*S. acutus*), C\textsubscript{18} (*S. acutus, Limnothrix* sp. strain MRI, *D. galatea* and the cyclopoid copepod), C\textsubscript{20} (the cyclopoid copepod and *C. plumosus*) and C\textsubscript{22} (*C. plumosus*) regions of samples analyzed. These fatty acids have not been detected in the conventional one dimensional (1D) GC analysis due to either co-elution and/or their presence in low amounts in the sample matrix.

In the GC\timesGC technique, all congeners of the fatty acids in these (micro)organisms could be detected and identified due to the increased analyte detectability and ordered structures in the separation space. The novel DTD-GC\timesGC system can be very useful in lipid biomarker research as well as food-web, ecological and clinical studies. It may also serve as a promising inlet system for IRMS measurements.

5.1 References

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Summary

Profiling of cellular fatty acids plays an important role in clinical, microbiological and ecological/environmental studies. Fatty acids are the principal components of most natural lipids. They show a great degree of structural diversity in terms of chain-length, geometry, degree of unsaturation, position of double bonds as well as substitution pattern. The specificity and definiteness of the fatty acid composition, often referred to as the fatty acid profile or fingerprint/pattern, is particularly characteristic of the lipid and the provenient organism. This thesis discusses the development of a fully automated at-line analysis system for fatty acid profiling of whole/intact biological samples - that is samples/living specimens receiving little or no pre-treatment at all before the actual fatty acid analysis. The thesis is divided into three main parts. The first part deals with the development of the novel procedure (Chapter 2). The second part discusses the application of the novel technique to various biological samples (Chapter 3). In the third and final part of this thesis, coupling of the novel inlet system to comprehensive two-dimensional time-of-flight mass spectrometry (GCxGC-ToF-MS) is discussed (Chapter 4).

Traditionally in microbiology, the analysis of fatty acid methyl esters (FAMEs) is carried out by extraction of membrane-derived and/or storage lipids, followed by saponification (hydrolysis) and methylation, and finally, analysis with gas chromatography (GC). The resulting FAME profiles are diagnostic for the identification and differentiation of several microbial taxa, in some cases even down to the species level. Some of the disadvantages of fatty acid profiling using the traditional method are the rather lengthy steps required to extract and derivatize the microbial fatty acids, and, the potential risk of contamination due to the manual mode of operation. In addition, enough microbial biomass must be available to provide adequate amounts of fatty acids in the extraction and derivatization step.

The first chapter attempts to give an overview of the various analytical methods used in fatty acid/lipid analysis.
Chapter 2 describes the development of a novel procedure to circumvent the problems associated with the traditional method. Using direct thermal desorption (DTD) interface, fatty acids are released from the membranes of microbial cells, converted into their corresponding fatty acid methyl esters, and finally, transferred into the GC column via an Optic injector. In order to operate in a fully automated manner, the upper part of the standard fritted Optic interface/PTV liner was adapted so as to make it possible to be tightened in the same way as an autosampler vial, and was sealed with a stainless-steel cap. This allowed capped liners to be lifted and transported, courtesy the magnetic “base tip” of the auto-sampler arm. For the final analysis, each liner is transported to the injector for thermal desorption. This was realised with the help of a special pneumatic opening/closing device. A built-in at-line vacuum system enabled water and other solvents to be evaporated. The DTD interface allows the liner of the Optic injector to be used as a sample and reaction container. The new procedure yields similar FAME profile as the tedious conventional off-line method.

Optimisation of several parameters revealed the following procedure; a volume of 2 µl of cell culture (0.90 mg/ml) was injected into a 40 µl specially designed liner-insert/micro-vial placed inside a stainless-steel capped DTD liner. Next, the liner was transported by the auto-sampler, courtesy its magnetic “base-tip”, to a vacuum port to evaporate the solvent. After complete drying, 2 µl of a 50 mM trimethylsulfonium hydroxide was added. After 30 min incubation, the content of the insert was dried in a specially designed vacuum port in the liner tray-holder, and then transported to the DTD interface. Finally, thermally assisted hydrolysis and methylation with simultaneous transfer into the GC column was performed at 350 °C followed by GC–ion trap MS detection. Using the DTD-interface, at-line fatty acid analysis of flow-cytometric sorted green micro-algae and cyanobacteria and hand-picked freshwater mesofauna species were performed. It was shown that the at-line procedure was suitable for direct thermally assisted hydrolysis and methylation (THM) of the cellular fatty acids of whole/intact cells and animals. The FAME profiles of lipids produced using the novel technique were the same as those obtained using the conventional off-line lipid extraction procedure followed by hydrolysis and methylation with BF₃ in methanol. The good performance of this novel procedure with respect to fatty acid profiling, automation and the absence of
side-reactions/isomerization of polyunsaturated fatty acids (PUFAs) opened the way to use DTD-THM as an inlet technique in compound-specific stable isotope-ratio \(^{13}\text{C}/^{12}\text{C}\) measurements. The DTD in-let system was coupled to a gas chromatography linked online to an isotope-ratio mass spectrometer (GC-IRMS), and the results thus obtained compared to the performance of at-line flash pyrolysis-GC-IRMS in analysis of phyto- and zooplankton specimens. Fatty acid profiles and the associated \(^{13}\text{C}\)-isotopic signatures were shown to be very similar in both techniques. Analysis of fatty acids with the new procedure was performed in an automated manner, which allows a high sample throughput. To this end, preparation of the next sample is performed simultaneously with GC analysis of the \(n\)th sample.

Chapter 3 discusses the application of the new procedure in the analysis of clinical, ecological and geochemical samples. To this end whole blood/plasma samples, crude soil lipid extracts and pollen grains were analysed. The profiles of the fatty acid methyl esters obtained were identical to those obtained when the conventional off-line method was applied. FAME yields were similar or in some cases higher than the yields obtained with the conventional method. A major advantage of this new method in the analysis of these samples is that the tedious and lengthy sample treatment steps associated with the traditional Bligh-Dyer off-line lipid extraction and fractionation method are either completely avoided (e.g. blood/plasma samples) or greatly reduced (e.g. soil/sediment samples). For instance, by applying this new procedure in the analysis of crude soil lipid extracts, up to 2 days of laboratory work on a batch of 10 samples can be saved. Next to this, very small amounts of solvents are required.

Chapter 4, which is the final part of this thesis, describes the coupling of the new inlet system to a comprehensive GCxGC-ToF-MS system for the analysis of whole/intact aquatic micro-organisms in which improved separation of co-eluting peaks as well as detection of FAMEs that are present in very low amounts in the sample matrix were realised. For instance, new mono- and di-unsaturated fatty acids were found in the \(C_{16}\) and \(C_{18}\) regions of the green algae Scenedesmus acutus and the filamentous cyanobacterium Limnothrix sp. strain MRI samples. These fatty acids have not been detected in the conventional one-dimensional (1D) GC analysis due to either co-elution and/or their presence in low amounts in the sample matrix.
Samenvatting

De analyse van cellulaire vetzuren heeft een belangrijke plaats in veel klinische, microbiologische en ecologische/milieu studies. In de meeste natuurlijke lipiden zijn vetzuren de karakteristieke samenstellende component. Zij vertonen een grote mate aan structurele diversiteit met betrekking tot ketenlengte, substitutiepatroon, graad van onverzadigdheid als wel de positie van dubbele bindingen met hun geometrie. De specificiteit en de (relatieve) onveranderlijkheid van de vetzuursamenstelling- vaak aangeduid als het “vetzuurprofiel” – is een opvallende karakteristiek van veel lipiden en daarmee van het organisme dat haar produceerde.

In dit proefschrift wordt de ontwikkeling van een volledig geautomatiseerd analyse systeem beschreven en bediscussieerd ten behoeve van een directe zgn. “at-line” bepaling van het vetzuurprofiel aan intacte/complete biologische monsters (i.e., ruwe monsters die nauwelijks of geen behandeling hebben ondergaan voorafgaande aan de eigenlijke vetzuuranalyse). Het proefschrift valt in drie delen uiteen. Het eerste deel behandelt de ontwikkeling van de nieuwe methodiek (Hoofdstuk 2). Het tweede beschrijft de toepassing van de methode in de vetzuuranalyse van verschillende typen biologische monsters (Hoofdstuk 3). In het derde en laatste deel (Hoofdstuk 4) worden vervolgens de uitkomsten behandeld van de koppeling van de nieuwe techniek met zgn. twee-dimensionale gaschromatografie/“time-of-flight” massa spectrometrie (GCxGC-ToF-MS).

In de microbiologie wordt de analyse van vetzuur-methylesters (zgn. FAMEs) traditioneel uitgevoerd d.m.v. een extractie van membraan- en opslaglipiden, gevolgd door hun verzeeping (hydrolyse) en methylering van de gevormde vrije vetzuren met tenslotte een FAME-bepaling met behulp van gaschromatografie. De resulterende FAME-profielen zijn diagnostisch te hanteren in de identificatie en differentiatie van verschillende microbiële taxa, soms zelfs tot op soortsniveau toe (sommige bacteriegroepen). Nadelen van deze traditionele manier van het bepalen van het vetzuurprofiel zijn de tamelijk langdurige/bewerkelijke opwerkingsstappen om de microbiële vetzuren te extraheren en te methyleren (met daarbij de kans op contaminatie omdat het gehanteerde protocol geheel handmatig is). Bovendien moet er genoeg microbiële biomassa als uitgangsmateriaal aanwezig zijn om een voldoende hoeveelheid vetzuren te leveren in de extractie- en derivatizeringsstap, terwijl dit in
veel studies vaak gelimiteerd voorhanden is. Een overzicht van de gangbare methoden
ten behoefte van lipide-en vetzuuranalyse wordt gegeven in hoofdstuk 1.

In hoofdstuk 2 wordt de ontwikkeling van de nieuwe methodiek beschreven
waarmee de problemen verbonden aan de traditionele procedures kunnen worden
ondervangen. Door middel van een zgn. “direct thermal desorption (DTD) interface”
worden cellulaire vetzuren kwantitatief vrijgemaakt uit micro-organismen, omgezet in
de corresponderende vetzuur-methylesters en vervolgens naar een GC-kolom geleid
via een Optic injector. Om volledig ge-automatiseerd te kunnen werken, werd het
bovenste deel van de standaard “Optic interface/PTV-liner” zo aangepast dat deze
overeenkomstig een autosampler flesje - afgesloten kon worden met een roestvrij
stalen kapje. Dit maakte het mogelijk dergelijke liners op te tillen en te verplaatsen
middels een magneet-bevestiging aan het uiteinde van de autosampler-arm. In de
laatste stap van de analyse wordt elke liner naar de injector getransporteerd voor
thermische desorptie. Dit werd gerealiseerd middels een speciale pneumatisch
deiende klep om de injector te openen en te sluiten. Om water en oplosmiddelen te
cunnen laten verdampen werd er gebruik gemaakt van een ingebouwd at-line vacuum
systeem. De DTD-interface maakte het mogelijk de liner van de Optic injector te
hanteren als een gecombineerde monster- en reactiecontainer. De nieuwe procedure
leverde FAME-profielen sterk vergelijkbaar met de bewerkelijke conventionele off-
line methoden.

Optimalisering van verschillende reactie-parameters leidde tot het volgende
standaard protocol: 2 μl cell suspensie/cultuur (0.90mg/ml) werd geïnjecteerd in een
speciaal ontworpen 40 μl “micro-liner-insert” die vooraf in de DTD-liner was
geplaatst. Vervolgens werd deze DTD-liner naar de vacuum-poort verplaatst om het
water volledig te laten verdampen. Na deze droogstap werd 2 μl van een 50 mM
trimethylsulfoniumhydroxide oplossing geïnjecteerd. Na 30 min incubatie met dit
reagens werd de inhoud van de insert gedroogd middels vacuum en de DTD-liner
vervolgens naar de Optic-injector getransporteerd. Tijdens een snelle opwarming naar
350 °C hydrolyseert het reagens de lipide-esterbindingen in het monster en vindt er
een gelijktijdige methylering van de gevormde vetzuren plaats in een zgn. THM-
reactie (thermally assisted hydrolysis and methylation). Na GC-scheiding van de
gevormde FAMEs vond detectie plaats middels “Ion-trap” massaspectrometrie (GC-
MS).
De DTD-interface werd gebruikt om directe at-line vetzuuranalyses uit te voeren aan flow-cytometrisch geselecteerde groenalgen en cyanobacteriën en met de hand gesorteerde kleine zoetwaterfauna soorten (o.a. waterflooien en copepoden). Er kon getoond worden dat de at-line procedure geschikt was om middels THM de cellulaire vetzuren van intacte cellen en organismen vrij te maken en te kwantificeren.

De aldus geproduceerde FAME-profielen kwamen goed overeen met de conventionele methode waarin lipiden off-line geëxtraheerd worden en na verzinging de methylering met BF₃ in methanol plaatsvindt. De goede prestaties middels de nieuwe methode met betrekking tot “FAME-profiling”, de afwezigheid daarin ook van isomerisatie/degradatie van de meervoudig-onverzadigde vetzuren (zgn. PUFAs) en de mogelijkheid tot automatisering, openden de mogelijkheid om DTD-THM als inlaat-techniek te hanteren in zgn. component-specifieke stabiele isotoop-ratio (¹³C/¹²C) massaspectrometrie (GC-IRMS).

De DTD-interface werd hiertoe gekoppeld aan een GC-IRMS en de analyses uitgevoerd aan phyto- en zooplankton vergeleken met die verkregen middels at-line “flash pyrolyse”-GC-IRMS. De vetzuurprofielen en hun ¹³C-isotoop signatuur bleken in beide technieken sterk overeen te komen. Omdat in de nieuwe (geautomatiseerde) methode een monster al geprepareerd/geïncubeerd kan worden terwijl het vorige de GC-MS analyse doorloopt, kan een grotere analysecapaciteit bereikt worden dan met de “flash pyrolyse” techniek.

In hoofdstuk 4 – het afsluitende hoofdstuk van dit proefschrift – wordt de koppeling van het nieuwe inlaatsysteem aan GCxGC-ToF-MS beschreven. In de analyse van intacte aquatische micro-algen en mesofauna soorten werd een verbeterde scheiding van co-eluerende pieken bereikt en een gevoeliger detectie van FAMEs die in zeer lage concentraties in de monstermatrix aanwezig waren. Nieuwe enkel- en tweevoUDig-onverzadigde vetzuren werden bijvoorbeeld aangetroffen in de C_{16} en C_{18} regio’s van de groenalg *Scenedesmus acutus* en de filament-vormende cyanobacterie *Limnothrix* sp. stam MRI. Deze vetzuren werden niet eerder gedetecteerd in de meer gebruikelijke éénimensionale GC analyse ten gevolge van co-elutie en/of hun aanwezigheid in kleine hoeveelheden.
Acknowledgements

I wish to thank the Lord Almighty for his abundant grace and mercies, indeed his words are true and he is the one and “only immortal and invincible only” God.

I wish to express my deepest gratitude to all those who helped me in diverse ways to get this thesis complete. Special mention must be made of my supervisors Dr. R.J.J Vreuls and Dr. R. Pel for their superb guidance. I could not have wished for a better supervision. I wish also to thank Prof. Dr. H. Irth, my promotor and also Prof. Dr. U. A. Th. Brinkman the co-initiator of this research as well as members of the project review committee; Prof. Dr. Peter Schoenmaker, Prof. Dr. H-G. Janssen and Dr. Frans Stellaaard. My gratitude also goes to all the staff members of the Analytical Chemistry and Applied Spectroscopy (ACAS) department, my colleagues in ACAS as well as those at the NIOO namely; Cees, Nel, Freek, Peter Blokker, Mohamed Adalchour, Maria Kristenson, Jeroen Carol, Natasja Visser, Reyer Dijkstra, Arjen Bader, Eva de Rijk, Junko Hirata, Thomas Litzel, Rico Derks, Hans Crabbe, Johannes Hoos, Arjen de Boer, Evtim Efremov, Alois Bonifacio, Jens Dalluge, Leo van Steel, Diego Millo, Mirka Smoluch, Lineke van der Spleen, Michele Antonnik Presta, Joost Bijs, Dik Kamminga, Pim Voogt, Dick van Eperen, Marcel, Virgilio, Marion, Dr. Paul Bodelier, Miranda, Barbette, Maiko and Hans Hoogvedl.

To the ever so helpful and ever so patient secretary of Acas, the affable Gisèle M. Cassée, I say thank you so very much. My special thanks also go to the pastors and the whole congregation of Love Christian Centre under the able leadership of pastor Baiden for their support; both spiritually and physically. I will like to thank the Netherlands Science Foundation and Atas GL (especially Geert Alkema) for their financial and technical support for this research respectively. I wish also to thank all former colleagues at IHS/Wageningen University Research for their enthusiastic support.

To the Ghanaian community in Amsterdam south-east, I say thank you for your support and encouragement, especially Mr. Ernest Owusu Sekyere (Emo oooooooooo!!!!!!!!! Emo?) Edward Edusei, Charles van der Puye, M. Ofori, Adu Acheampong, Adjekumhene, Dr. Nimako, Cpt. Rtd. Ampomah Nketa, Madam Pofam, Bro. Yoofi, Ms. Harriet Neckaps and family, Mr. and Mrs. Anane, Nana Akwasi Anyani (Anum Asamankeshehene), Mr. Samuel Tiase, the entire staff of the Recogin secretariat, Recogin Youth, Recogin media, colleagues at Genscape Int. as well as former colleagues at EPA, Ghana, not forgetting the Old students’ association of Obuasi Sec. Tech. Sch./Tema Sec. school.

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I wish to thank the leadership as well as members of Okuapemman Fekuw of Holland. My special thanks go to Nana Addo Kwaata, Sahoo, E.M. Manukure, Opanyin Kwame Addo, Siisie Linda and Kwame Kwam. Kwaapong.

To my lovely and ever supportive family I say thank you very, very much. You are simply the best! To my dearest mother I say, the task is done. To the adult members of the Akoto and allied families I say thank you for your unflinching support and to the young ones I say thanks for keeping me on my toes, but the ball is now in your court. This acknowledgement will not be complete without specially thanking Mr. Eugene Atiemo, Mr. David Osae Akoto, Mr. Philip Akoto, Mrs. Mercy A. N. Adjar, Mrs. S. Adjar, Mrs. Regina Awuku, Bro. Odum, Sister Mansua, Marian Arhin, Mr. Daniel Adjei, Dr. and Mrs. Edward Narh of Narh-Bita hospital, Tema, Eric Glover, Joana Ameyaw, Victoria Laast, Mr. and Mrs. Victor Owusu, Mr. Jonathan Odame, Pastor Kesse of Love Baptist Church, Tema and all SOTGGANs for their inspiration and encouragement during every step of the way.

Finally, I will like to thank my adorable better-half, Sylvia, for being there to lean on whenever the going got tough. My dear, thanks a lot. To my kids Maame Yaa and Owura Kwaku, I say congratulations as well for you deserve a pat on your shoulders for your endurance and patience at such young ages. As for Maame Yaa, my little daughter, I say thank you for keeping me awake at night!!!!!!!!!!!!!!! To all my friends and acquaintances whose name I may not have mentioned I say, you have not been forgotten, thank you all for everything and God bless you.

Lawrence Akoto
List of publications


On a hill far away stood an old rugged cross,  
The emblem of suffering and shame;  
And I love that old cross where the dearest and best  
For a world of lost sinners was slain.

Refrain

So I’ll cherish the old rugged cross,  
Till my trophies at last I lay down;  
I will cling to the old rugged cross,  
And exchange it some day for a crown.

O that old rugged cross, so despised by the world,  
Has a wondrous attraction for me;  
For the dear Lamb of God left His glory above  
To bear it to dark Calvary.

Refrain

In that old rugged cross, stained with blood so divine,  
A wondrous beauty I see,  
For ’twas on that old cross Jesus suffered and died,  
To pardon and sanctify me.

Refrain

To the old rugged cross I will ever be true;  
Its shame and reproach gladly bear;  
Then He’ll call me some day to my home far away,  
Where His glory forever I’ll share.

Words of George Bennard, 1913, in his famous soul inspiring song “Old Rugged Cross”.

“The cross of our Lord Jesus Christ” (Galatians 6:14)
Dedicated to my dear parents