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AppNote 5/2013

Thermochemolysis – A Simple and Rapid Methylation Method Based on TMAH for Gas Chromatographic Analysis of Linseed Oil and Amber

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KEYWORDS

Pyrolysis-GC-MS, Methylation, TMAH, Fatty Acid Methyl Esters (FAME), Linseed Oil, Amber

ABSTRACT

In order to improve pyrolysis chromatographic analysis of materials that release polar functional groups e.g. carboxylic acids, a simple and rapid methylation method based on TDU-pyrolysis/GC-MS in the presence of tetramethylammonium hydroxide (TMAH) was developed. Linseed oil was selected as test material because of its high triglyceride content comprising both saturated and unsaturated fatty acids. Pyrolysis was performed at 500, 600 and 700°C using a GERSTEL pyrolysis module (PYRO) with a heated platinum filament. The optimum pyrolysis temperature for linseed oil was found to be 500°C. The fatty acids in the linseed oil were found to have been quantitatively methylated when using a methanolic TMAH solution (~ 10 % in methanol). The use of an aqueous TMAH solution (25 wt. % in H₂O) for the methylation of fatty acids was found to result in lower fatty acid methyl ester (FAME) yields, indicating that

the formation of a homogeneous mixture of sample and reagent is essential. Additionally, it was evident that the reagent plays an active role in cleaving the triglycerides. Automated direct injection of the reagent into the TDU-PYRO is possible, but this function is a special adaptation. Chromatograms obtained from direct injection of 1 μ L TMAH solution into the linseed oil sample show no difference compared with those obtained after manually mixing linseed oil and the TMAH solution prior to pyrolysis.

The pyrolysis- and thermochemolysis-GC-MS methods were successfully used to determine the molecular composition of Eocene amber from the Ameki formation, Nigeria. The amber was pyrolyzed at 480°C for 20 s with and without adding TMAH. Free carboxylic acids were quantitatively methylated to their corresponding methyl ester products in the presence of TMAH. Both Pyrolysis-GC-MS and thermochemolysis-GC-MS chromatograms were used to determine the structural class and botanical source of the fossilized resin. The pyrolysis products were dominated by labdane type diterpenoids and some sesquiterpenoids, which point to a conifer (gymnosperm) botanical source of the resin.

INTRODUCTION

Pyrolysis-GC-MS is used to obtain structural information of macromolecules through analysis of their thermal degradation products. The heart of the GERSTEL TDU pyrolysis module (TDU-PYRO) is a platinum filament. The pyrolysis temperature can be set to a value between 350°C and 1000°C. TDU-PYRO fits into the heating tube of the GERSTEL Thermal Desorption Unit (TDU), enabling easy switching between normal TDU operation and pyrolysis operation. All process steps, including transporting the pyrolysis sample holders to and from the TDU and optionally performing liquid injections into the pyrolysis module are automated using a GERSTEL MultiPurpose Sampler (MPS).

Pyrolysis with in-situ derivatization is sometimes referred to as thermochemolysis. Organic compounds containing acidic protons, like carboxylic acids, alcohols and phenolic compounds can be methylated in a hot injector by a strong basic quaternary N-methylammonium hydroxide [1].

Tetramethylammonium hydroxide (TMAH) is a commonly used methylation reagent (fig. 1). It was first introduced by Robb and Westbrook as a methylation reagent for the GC analysis of carboxylic acids [2]. Acidic functional groups in sample molecules, originally existing or formed during pyrolysis, are deprotonated by the alkaline TMAH and the resulting tetramethylammonium salts are thermally converted to the corresponding methyl esters in the hot injection port of a gas chromatograph (fig. 2).

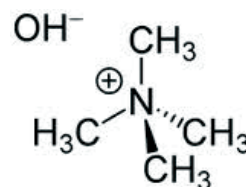


Figure 1. Tetramethylammonium hydroxide.

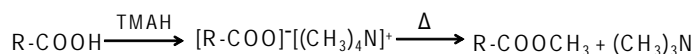


Figure 2. Methylation of acids using TMAH.

Thermochemolysis using TMAH is well established for studying the composition of fatty acids in oils, waxes, biopolyesters, and complex biological matrices, such as bacteria and sediments [3]. The advantages of methylation are (i) to prevent secondary degradation of fatty acids during pyrolysis and (ii) to obtain a better chromatogram using non-polar standard silica columns for polar and insufficiently volatile pyrolysis products. Thermochemolysis GC/MS provides more accurate information on sample composition. This paper describes fundamentals of the thermochemolysis technique using linseed oil as a test sample. The application of this technique is also demonstrated by the determination of the botanical origin of amber from Nigeria.

EXPERIMENTAL

Standards and samples. A tetramethylammonium hydroxide solution (~10 % in methanol) and a tetramethylammonium hydroxide solution (25 wt. % in H₂O) were purchased from Sigma-Aldrich (Steinheim, Germany). Methanol of analytical grade was obtained from Merck (Darmstadt, Germany). Linseed oil was purchased from a local supermarket. Eocene amber from the Ameki Formation, Nigeria was obtained from the Hunterian Museum (register number M 7432) of the University of Glasgow, United Kingdom.

Instrumentation. Pyrolysis-GC-MS was performed using a Thermal Desorption Unit (TDU) with pyrolysis module (TDU-PYRO) (all from GERSTEL) coupled directly to an Agilent 6890N gas chromatograph with a 5795B inert XL (triple axis) mass selective detector (MSD). A MultiPurpose Sampler (MPS) equipped with a 10 μ L syringe and a Cooled Injection System (CIS 4) programmed temperature vaporization (PTV) type inlet with liquid nitrogen cooling (LN₂) (all from GERSTEL) were used. The entire analysis system was operated under MAESTRO software control integrated in Agilent ChemStation software using one integrated method and one integrated sequence table.

RESULTS AND DISCUSSION

Linseed oil. In order to demonstrate the technique of thermochemolysis, linseed oil was selected as test material because of its unique triglyceride composition, comprising unsaturated fatty acids, such as palmitoleic acid (C16:1), oleic acid (C18:1), linoleic acid (C18:2), alpha-linolenic acid (C18:3) as well as lower percentages of triglycerides of saturated fatty acids such as palmitic acid (C16:0), stearic acid (C18:0) and arachidic acid (C20:0). During method development, the pyrolysis temperature and the entire methylation process for linseed oil had to be optimized. The methylation yields resulting from different commercially available reagents were also compared. The fatty acid methyl ester (FAME) profiles obtained using thermochemolysis with TMAH are presented in the following.

Analysis conditions. A series of 0.2 μ L aliquots of a diluted linseed oil (1:5 v/v in dichloromethane) were pyrolyzed at 500°C, 600°C and 700°C respectively for 20 s in order to determine the optimum pyrolysis temperature. Each diluted linseed oil sample was manually injected onto a plug of quartz wool placed in a tube type

pyrolysis sample holder. For the thermochemolysis experiments, 100 μ L diluted linseed oil was mixed with 100 μ L TMAH reagent (10 % in methanol or 25 % in water) and 0.2 μ L aliquots of this mixture were pyrolyzed at 500°C. In order to remove the methanol prior to pyrolysis, the TDU was operated in solvent vent mode (0.5 min, 40°C, 50 mL/min. The Cooled Injection System (CIS 4) PTV type inlet was set to a temperature of 300°C and used as a hot split interface. The GC was operated in split mode (1:100) and was equipped with a ZB-5MS fused silica column (30 m x 0.25 μ m x 0.25 mm, Phenomenex). The GC oven operating conditions were: initial temperature held at 40°C for 2 min, then increased from 40°C at a rate of 10°C/min to 220°C (held for 5 min) and finally increased to 320°C at a rate of 15°C/min (held for 5 min). Helium was used as carrier gas at a constant flow of 1 mL/min. The mass spectrometer was operated in electron impact (EI) ionization mode with the ionization energy set to 70 eV and the source and quadropole temperatures set to 230°C and 150°C respectively. Full scan mass spectra were recorded over a mass range from 50 to 550 Da. ChemStation software was used to acquire and process data. Individual compounds were identified by comparing mass spectra with MS library data and information from literature.

Method optimization - Sequential pyrolysis-GC-MS at different temperatures. Sequential pyrolysis was performed, i.e. a set of identical samples were analyzed under different pyrolysis conditions, in this case using increased pyrolysis temperature. Such a sequential procedure is a very useful analytical tool for optimizing a pyrolysis method, for example by varying the pyrolysis temperature, pyrolysis hold time or TDU vent time. An optimized pyrolysis temperature may very well lead to more detailed information on the sample. For example, overheating of the sample may hinder data interpretation due to the formation of secondary pyrolysis products. A 0.2 μ L sample of diluted linseed oil was prepared and pyrolyzed at 500°C, 600°C and 700°C in order to find the most suitable decomposition temperature for linseed oil (fig. 3).

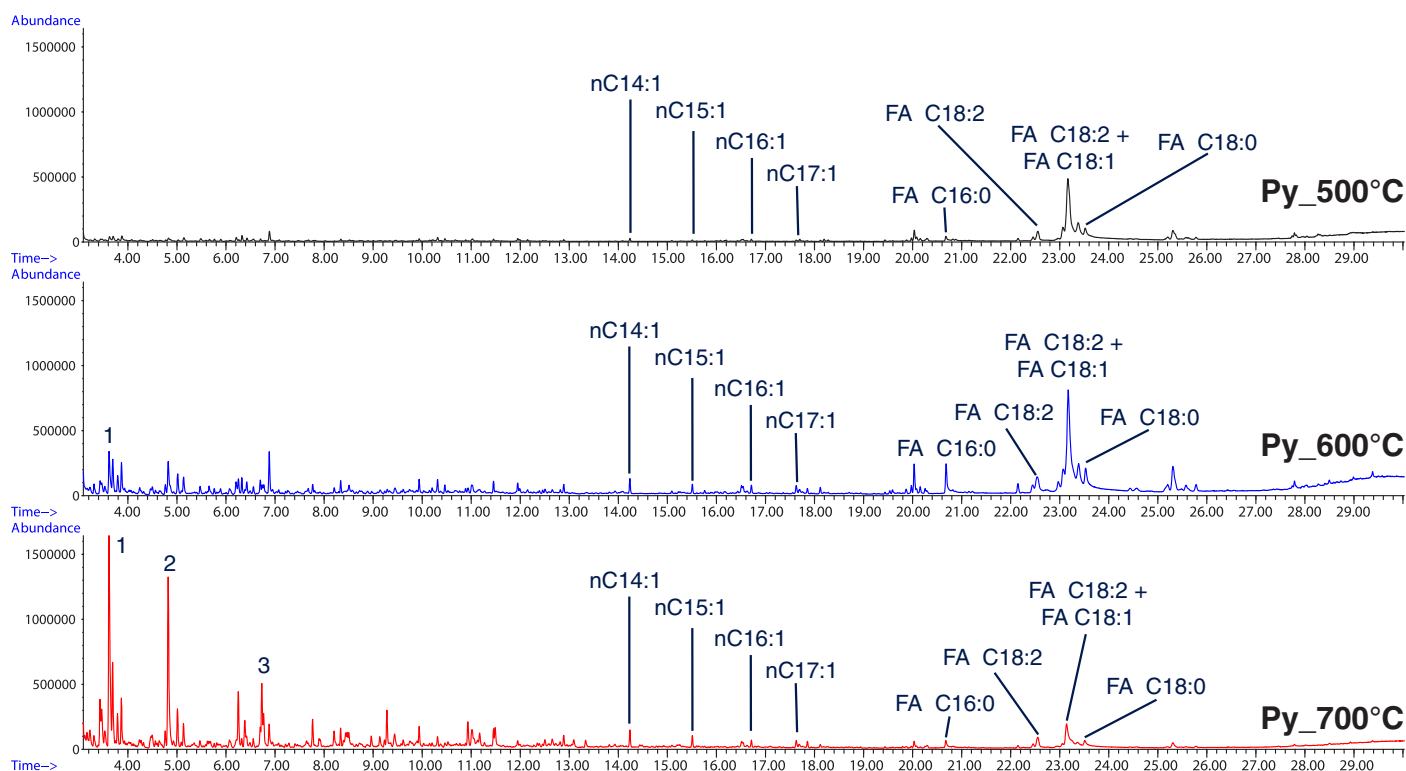


Figure 3. Pyrolysis-GC-MS total ion chromatograms of 0.2 μL diluted linseed oil pyrolyzed at 500°C, 600°C and 700°C respectively. (1. benzene; 2. toluene; 3. styrene; n (n-alkenes); FA (fatty acids) C X (carbon chain length) : Y (number of double bonds)), e.g.: FA C15:1.

The pyrolysis-GC-MS total ion chromatograms of linseed oil show typical fatty acids like palmitic acid (C16:0), linoleic acid (C18:2), oleic acid (C18:1) and stearic acid (C18:0). Additionally all the chromatograms are characterized by a homologous series of n-alk-1-enes up to 17 carbon atoms derived from the radical scission of alkyl chains of fatty acids, which were released during decomposition of their corresponding esters [3]. Aromatic compounds like benzene, toluene and styrene appear when pyrolysis temperatures are set to 600°C or 700°C. This indicates that pyrolysis at temperatures over 500°C would lead to secondary degradation reactions. It was found that 500°C is an adequate pyrolysis temperature for linseed oil.

Thermochemolysis-GC-MS with TMAH. In order to obtain reproducible methylation results, it is important to keep the ratio of diluted linseed oil to TMAH constant. To achieve this, 100 μL of diluted linseed oil (1:5 v/v with dichloromethane) and 100 μL TMAH (10 % in methanol) were transferred into a 1.5 mL vial and shaken manually to homogenize the mixture. A 0.2 μL aliquot of this mixture was taken and injected into a tube type sample holder filled with quartz wool using a 1 μL syringe. Samples prepared based on this procedure were pyrolyzed at 500°C, 600°C, 700°C and 800°C respectively for 20 s to determine the optimum

thermochemolysis temperature. 500°C was chosen as the most suitable temperature because no secondary pyrolysis products like benzene and toluene could be detected and the yields of the FAMES were similar compared to results obtained using temperatures between 600°C and 800°C.

Figure 4 shows a comparison of pyrolysis-GC-MS and thermochemolysis-GC-MS chromatograms of linseed oil. Fatty acid methyl esters (FAMES) are formed at significant levels when pyrolysis of linseed oil is performed in the presence of TMAH. It can be seen clearly from figure 4 that (i) the FAME peaks in the thermochemolysis chromatogram are much bigger and sharper, resulting in better separation compared to the fatty acid (FA) peaks obtained through pyrolysis-GC-MS; (ii) the FAs are quantitatively derivatized to FAMES during thermochemolysis-GC-MS; (iii) the alkenes (C14 to C17) are not seen in the thermochemolysis-chromatogram. These findings point to a successful and complete methylation using the thermochemolysis method described above. Furthermore, thanks to the improved separation, the chromatograms obtained were easier to interpret. Palmitic acid (C16:0), linoleic acid (C18:2), oleic acid (C18:1) and stearic acid (C18:0) were all methylated to their corresponding FAMES. Alpha-linolenic acid methyl ester (FAME C18:3), the methylation product

from alpha-linolenic acid (FA C18:3), was detected in the thermochemolysis chromatogram and identified according to its mass spectrum (Figure 5). This acid is a key nutrient found in linseed oil and it could not be detected in the pyrolysis chromatogram, perhaps due to co-elution with other broad peaks. This compound was easily detected as FAME using thermochemolysis, which allows more accurate profiling of fatty acids in linseed oil. It was previously reported that the methyl ester of alpha-linolenic acid (FAME C18:3) could not be determined using TMAH [1, 2]. Based on the experimental set-up described above, the methyl ester of alpha-linolenic acid produces a big peak that can be identified with high confidence even when it is not completely separated from the methyl ester of oleic acid.

Compounds with the same mass spectra as linoleic acid methyl ester (FAME C18:2) and alpha-linolenic acid methyl ester (FAME C18:3) are assigned to their E or Z conjugated isomers respectively (FAME iso C18:2 and FAME iso C18:3). Methylation probably occurs as a result of the high basicity of the TMAH solution, which leads to base catalyzed isomerization of polyunsaturated fatty acids. From literature it is known that this problem can be solved by diluting the TMAH solution [4] or by removing excess TMAH with acetic acid [5]. These artifacts are reportedly not produced when TMAH is replaced by the commercially available reagents MethPrep 1 and MethPrep 2, which are trifluoromethylphenyl trimethylammonium hydroxide solution in water and methanol [6].

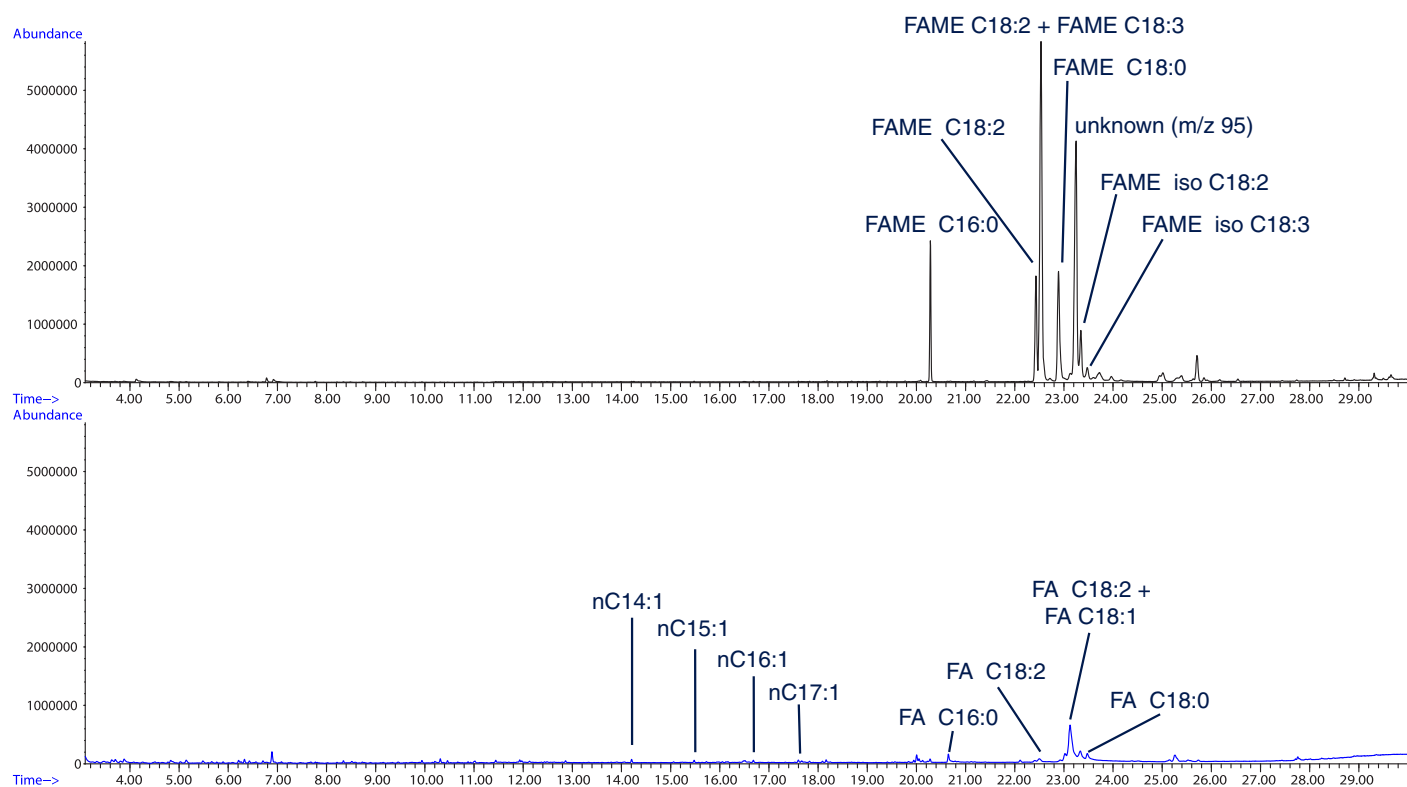


Figure 4. Thermochemolysis-GC-MS total ion chromatogram of diluted linseed oil with TMAH pyrolyzed at 500°C (Black); Pyrolysis-GC-MS total ion chromatogram of diluted linseed oil pyrolyzed at 500°C (Blue). (n (n-alkene); FAME (fatty acid methyl ester) C X (carbon chain length):Y (number of double bonds)).

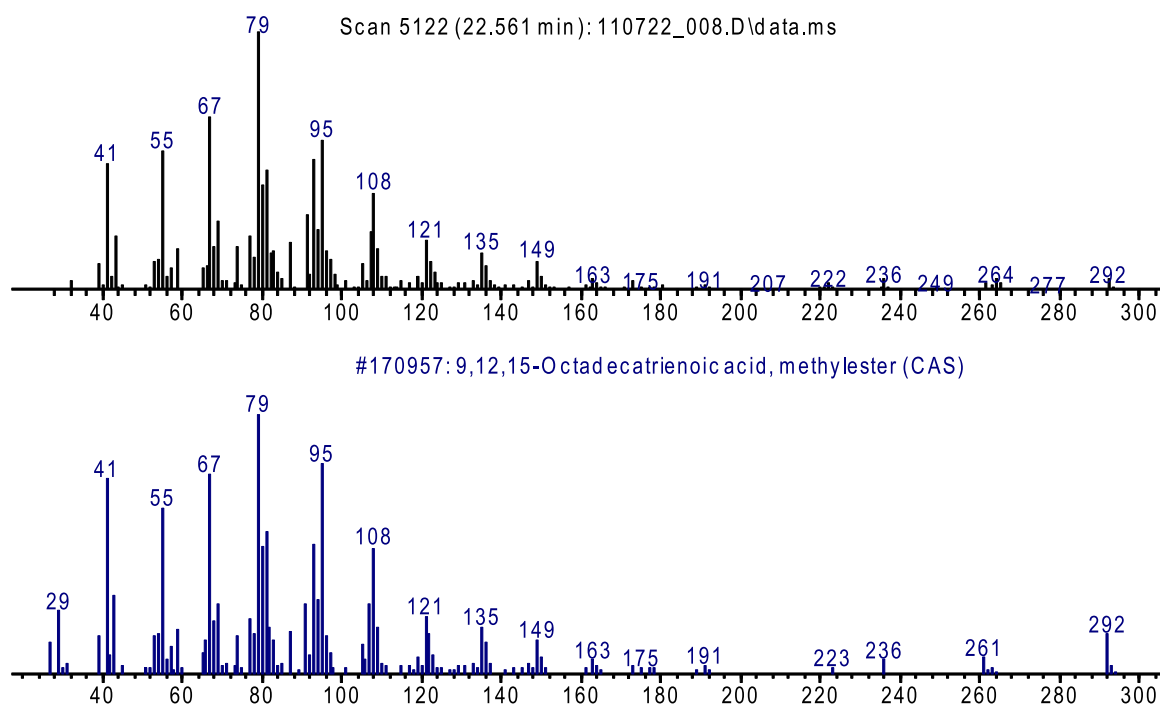


Figure 5. Obtained mass spectrum of alpha-linoleic acid methyl ester (9, 12, 15-octadecatrienoic acid methyl ester (FAME C18:3)) compared with a spectrum from the Wiley6n library.

Influence of solvent type on TMAH reagent efficiency. TMAH solutions are commercially available at a concentration of 10 % in methanol and 25 % in water. These two reagents are commonly used for thermochemolysis. In order to evaluate if they are equally useful for methylation of fatty acids during thermochemolysis, both TMAH solutions were applied in thermochemolysis under identical pyrolysis conditions. It was found that using the 10 % TMAH solution in methanol resulted in significantly higher FAME yields compared with the 25 % TMAH solution in water. Presumably, the water based TMAH does not mix very well with the linseed oil. The result indicates that good contact between the methylation reagent and sample matrix is crucial in order to obtain good reaction efficiency during thermochemolysis.

Eocene Amber. The application of pyrolysis-GC-MS and thermochemolysis-GC-MS for a detailed analysis of the molecular composition of different fossilized plant resins was part of the project carried out by O. O. Sonibare, T. Hoffmann and S. F. Foley at the Institute of Inorganic and Analytical Chemistry and the Department of Geosciences and Earth System Research Centre, University of Mainz, Germany. This project was aimed at carrying out geochemical characterization of ambers using different analytical techniques such as infrared spectrometry, gas chromatography-mass spectrometry and pyrolysis gas chromatography. The project was

sponsored by Alexander von Humboldt Foundation. GERSTEL is honoured to have participated with the development of a method for analysing amber using GERSTEL TDU-PYRO. The first result was published recently in the journal of Organic Geochemistry under the title “Molecular composition and chemotaxonomic aspects of Eocene amber from the Ameki Formation, Nigeria” [7]. In their work, Sonibare et al. describe ambers as “fossilized resins produced from plant exudates, which perform a range of function including sealing and protecting wounds, repelling insects and discouraging herbivores”. Fossilization of plant resin is described as a complex maturation process involving loss of volatile components, polymerization and crosslinking of terpenoid components which occurs over a period of up to 100 million years. Ambers are complex mixtures of mono-, sesqui-, di-, and triterpenoids. The knowledge about the chemical composition of amber is useful to differentiate between natural (genuine) and fake (imitations) amber and is important for chemotaxonomy, palaeovegetation and palaeoclimatic studies.

Major analytical techniques used for characterization of amber are infrared spectroscopy, Raman spectroscopy, GC-MS analysis of amber extracts and Pyrolysis-GC-MS. Among them, infrared spectroscopy (IR) can be used to differentiate Baltic amber from others by the appearance of “Baltic shoulder” at 1250-1175 cm^{-1} . The “Baltic shoulder” is associated with succinic acid

present in Baltic amber. The IR technique generally has limitations for this use since most amber types exhibit rather similar patterns and do not allow recognition of individual components in amber. Raman spectra are only useful for the determination of differences in maturation rather than geographical origin. GC-MS allows only the molecular structural elucidation of soluble components in amber, which rarely account for more than 20 % of the whole resin. Pyrolysis-GC-MS is arguably the most useful technique for a detailed analysis of amber. It has the advantage of breaking polymeric constituents, insoluble and non-volatile macromolecules in ambers into individual components which can be separated and their molecular structures elucidated from mass spectrometric data.

Analysis conditions. Ground amber samples (~200 µg) were pyrolyzed at 480°C for 20 s. For thermochemolysis, the samples were pyrolyzed in the presence of 10 µL TMAH (10 % in methanol) to ensure methylation of the carboxylic, alcoholic and phenolic products formed. The method parameters used were the same as those used for the thermochemolysis of

linseed oil (described above), except for the split ratio (1:20); the GC oven program and the MS mass range (50 to 650 Da). The GC oven program used here: Initial temperature 60°C (2 min), 6°C/min to 300°C (10 min).

Pyrolysis-GC-MS and Thermochemolysis-GC-MS for Eocene Amber. In fig. 6 the pyrolysis-GC-MS chromatogram and thermochemolysis-GC-MS chromatogram of the Eocene amber are shown. Detailed information about the peak identification can be found in literature [7], in which important components like sesquiterpenoids, diterpenoids and others are listed and described. Labelled peaks in fig. 6 were identified fatty acid methyl esters (FAMES) in the thermochemolysis chromatogram and their corresponding fatty acids (FAs) in the pyrolysis chromatogram, which are not listed in the literature. Compound identification and molecular masses are listed in table 1, the molecular weights of the FAMES are 14 Da higher than their corresponding FAs, since hydrogen (H+) was replaced with a methyl group (-CH₃+) during methylation.

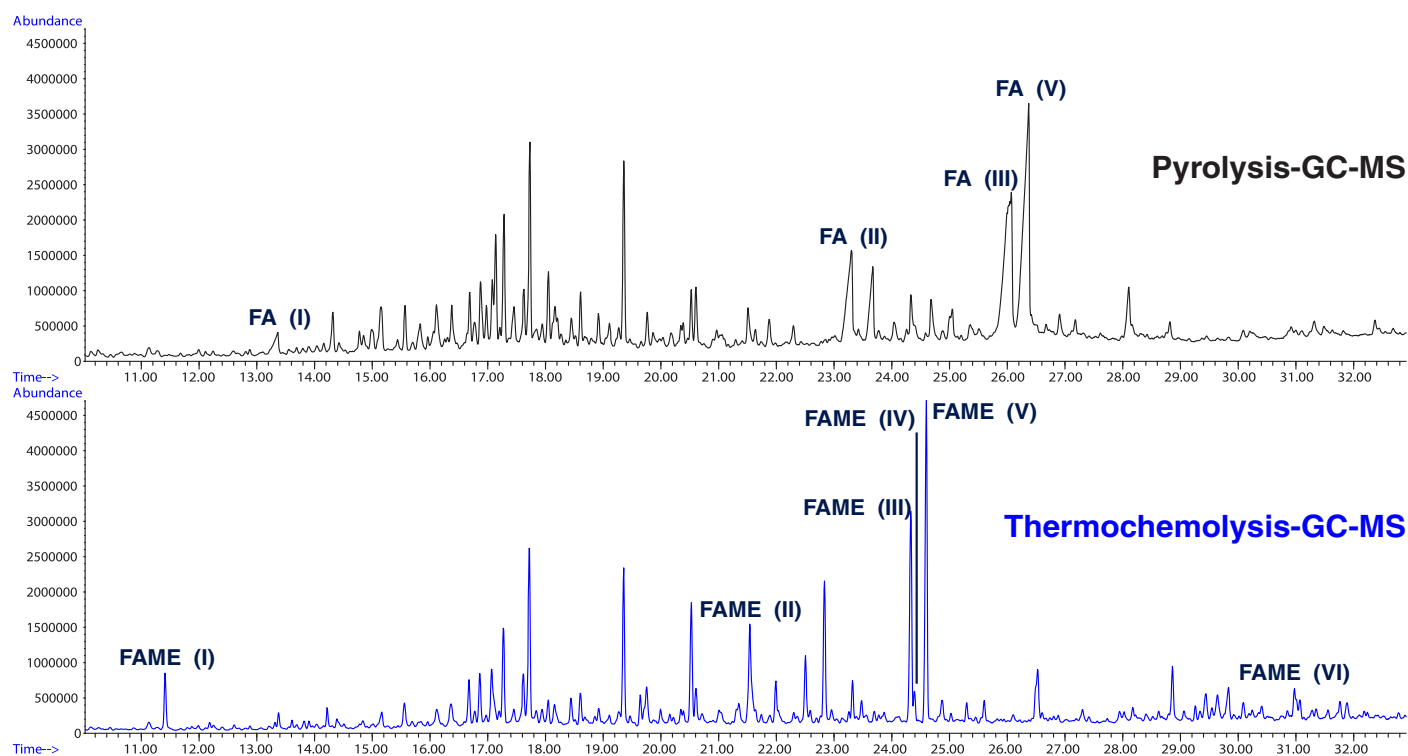


Figure 6. Pyrolysis-GC-MS total ion chromatogram (black) and thermochemolysis-GC-MS total ion chromatogram (blue) of Eocene amber from Ameiki Formation, Nigeria (FA = fatty acid; FAME = fatty acid methyl ester, For peak identification, please see table 1).

Table 1. Compounds identified in pyrolysis-GC-MS and thermochemolysis-GC-MS from the Eocene amber.

Peak	Compound name	MW	Peak	Compound name	MW
FAME (I)	Norchrysanthemic acid methyl ester	168	FA (I)	Norchrysanthemic acid	154
FAME (II)	Naphthalene-1-carboxylic acid-1,2,3,4,4a,7,8,8a-octahydro-1,4a,6-trimethyl methyl ester	236	FA (II)	Naphthalene-1-carboxylic acid-1,2,3,4,4a,7,8,8a-octahydro-1,4a,6-trimethyl	222
FAME (III)	Naphthalene-1-carboxylic acid-1,2,3,4,4a,7,8,8a-octahydro-1,4a,5,6-tetramethyl methyl ester	250	FA (III)	Naphthalene-1-carboxylic acid-1,2,3,4,4a,7,8,8a-octahydro-1,4a,5,6-tetramethyl	236
FAME (IV)	Methyl-1,2,3,4-tetrahydro-1,5,6-trimethyl-1-naphthalene carboxylate	232			
FAME (V)	Naphthalene-1-carboxylic acid-1,2,3,4,4a,5,8,8a-octahydro-1,4a,6-trimethyl-5-methylene methyl ester	248	FA (V)	Naphthalene-1-carboxylic acid-1,2,3,4,4a,5,8,8a-octahydro-1,4a,6-trimethyl-5-methylene	234
FAME (VI)	Methyl-16,17-dinor callitrisate	286			

In the pyrolysis chromatogram, the FA (I), FA (II), FA (III) and FA (V) peaks are broad with some peak fronting, a typical phenomenon for polar compounds like fatty acids separated on a non-polar column. In contrast the corresponding methylated compounds, FAME (I), FAME (II), FAME (III) and FAME (V) in the thermochemolysis chromatogram elute as well resolved sharp peaks. FAME (IV) and FAME (VI) could only be identified in the thermochemolysis chromatogram. It is clearly seen that pyrolysis in the presence of TMAH provides more detailed information on the sample.

A previous study [7] has presented the first available information on the molecular composition of fossil resin from the Eocene Ameki Formation, Nigeria. Pyrolysis-GC-MS analyses clearly indicated that the amber belongs to Class Ib type derived from regular labdatriene structures that lacked succinic acid. The pyrolysis products of the amber were dominated by labdane type diterpenoids and some sesquiterpenoids. The exclusive presence of labdane type diterpenoids and the absence of plant triterpenoids in the amber points to a conifer (gymnosperm) source for this resin.

Complete methylation of acids with TMAH. To verify that the free carboxylic acids formed during pyrolysis of the amber sample were completely methylated with TMAH, further experiments using excess reagent were carried out. 10 μ L and 20 μ L aliquots of TMAH solution were added to separate amber samples, which were then pyrolyzed under identical conditions. It should be noted that the cavity of the vial type sample holder with slit can contain a maximum of 20 μ L of liquid. A comparison of the resulting thermochemolysis

chromatograms revealed that no additional peaks had been introduced when excess reagent was used, the peak areas remained unchanged and no previously detected peaks had disappeared. These results indicate that the addition of 10 μ L of TMAH solution was sufficient to ensure complete methylation of the free carboxylic acids formed from pyrolysis of a 200 μ g amber sample.

The experiment described above also confirmed that TMAH did not evaporate before reacting with the sample, for example, during the solvent vent step in the TDU when residual methanol is purged. One sample to which 10 μ L of TMAH had been added was placed in a fume hood for approx. 30 min to evaporate the methanol and subsequently pyrolyzed at 480°C. The resulting chromatogram showed no difference to the chromatograms obtained when using the TDU solvent vent to evaporate methanol. Further experiments were done adding the same amount (10 μ L) of TMAH solution, but increasing the solvent vent time to 0.5, 1.0 and 1.5 min while keeping the initial temperature of 30°C. No significant difference for the analytes of interest was noticed in the thermochemolysis chromatograms. Only the methanol peak area decreased with increased solvent vent time. A solvent vent step of 0.5 min at 30°C with a gas flow of 50 mL/min was found to be perfect for the derivatization step. Since the methanol peak elutes early, a 4 min solvent delay time was set for MS data acquisition. To keep methanol entirely from entering the column, a column back flush can be used during the solvent vent step.

CONCLUSION

Pyrolysis-GC-MS has proven to be a powerful tool for obtaining information on the molecular composition of highly polymerized, insoluble and non-volatile macromolecules, which are usually not amenable for gas chromatographic analysis. Using thermochemolysis-GC-MS with tetra methyl ammonium hydroxide (TMAH) as methylating reagent, carboxylic acids, alcohols and phenolic decomposition products were converted to their methylated derivatives and easily determined on standard apolar GC columns. Pyrolysis-GC-MS and thermochemolysis-GC-MS were applied successfully in determining the fatty acid profile in linseed oil as well as the chemical composition of fossilized plant resin. Prior to pyrolysis, the use of the GERSTEL Thermal Desorption Unit with solvent vent functionality as a basis for the Pyrolyzer module allows purging of the solvent used to add the derivatization reagents. Thus, additional manual sample preparation steps are avoided.

ACKNOWLEDGEMENTS

The authors would like to thank the Alexander von Humboldt Foundation for granting Dr. O. O. Sonibare the George Forster Fellowship. We thank Dr. John Faithful of The Hunterian Museum, University of Glasgow, United Kingdom for supplying the fossilized resin samples.

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