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# Sampling techniques for gas chromatographic–mass spectrometric analysis of long-chain free fatty acids from insect exocrine glands

Roland Maile[a,*], Francesca R. Dani[a], Graeme R. Jones[a], E. David Morgan[a], Diethe Ortius[b]

[a]*Department of Chemistry, Keele University, Staffordshire ST5 5BG, UK*
[b]*Department of Ecology and Genetics, University of Aarhus, Ny Munkegade, Building 540, 8000 Aarhus C, Denmark*

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**Abstract**

Many features of gas chromatographic equipment can hinder the detection of free carboxylic acids, so that these polar and reactive substances do not lend themselves well to gas chromatography through adsorption and peak tailing. Therefore the normal practice is the derivatization to esters. The analysis of small quantities of insect exocrine secretions using this method is not quantitative. Factors affecting the quantitative analysis of free fatty acids have been explored. Two sampling methods, either by solid-phase micro-extraction (SPME) or the solid injector technique we developed and traditionally used, were found to be reliable if particular precautions are observed. These are the use of deactivated injection liners and glass wool in the injector. Deactivated pre- and post-columns prevent the detection of long-chain fatty acids even if the latter are present in μg amounts. By avoiding preheating of the samples in the injector and removing the glass powder of the sample capillaries after each injection, enhanced results have been observed with the Keele injector. For the SPME, absorption from headspace and moderate sample heating temperatures showed better results compared with high-temperature sample heating. 

*Keywords:* Sample heating; Injection methods; Carboxylic acids

## 1. Introduction

Carboxylic acids are highly polar and reactive substances and do not lend themselves well to gas chromatography. Therefore they are usually converted to methyl esters (for long-chain acids) or diphenylmethyl or other esters (for low-molecular-mass acids). The resulting esters are excellent subjects for gas chromatography (GC). This method works very well for the purposes of identification, finding the proportion of various acids in a mixture, or for quantitative determinations where large amounts of the samples are available to derivatize. However, quantitative determination of small samples, for example a few μl of insect secretion, presents considerable problems because the small sample cannot be quantitatively derivatized.

In this special case greater problems arise. By using a common GC–mass spectrometry (MS) analysis system with low polarity stationary phase any carboxylic acids present may either be absorbed in the injector system or appear as low, broad peaks in the chromatogram.

Since solvent-free sampling and avoidance of any

*Corresponding author.

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possible contamination are the ideal conditions for the analyses of tiny biological samples [1], in which small amounts are expected, carboxylic acids should be analyzed without additional treatment. Furthermore, since methyl or ethyl esters of long chain fatty acids appear to be quite common in insect samples, methylation of these acids may lead to misinterpretation. More difficulties may arise from unexpected transesterification of tissue constituents such as phospholipid fatty acids [2].

Presence of carboxylic acids has been reported in exocrine secretions of arthropods [3–5]. Saturated and unsaturated long chain fatty acids, such as palmitic, palmitoleic, stearic, oleic and linoleic acid, seem to be widespread in exocrine secretions and cuticular extracts of many different taxa. Since these compounds are important metabolites and intermediates in biological processes, the analytical techniques regarding long-chain fatty acids are of wide interest.

Two different techniques of solid sampling have been tested. Using the technique developed and successfully used for many years in the Keele laboratory [1,6,7] exocrine glands, exocrine secretions collected from insects, or small pieces of cuticle are first sealed in a soft soda glass capillary tube (20 mm×2 mm). Next the capillary is inserted in the gas chromatograph injector port, heated for 3 min and then crushed as the chromatography programme is started.

Solid-phase micro-extraction (SPME) is a recently developed technique which allows solvent-free sampling for GC or high-performance liquid chromatography (HPLC), either from water solutions or from the headspace above solutions or solids, through absorption of volatiles on fibres coated with different chromatographic stationary phases [8–10]. Polydimethylsiloxane coated fibres have been used for sampling pheromones from live insects maintained in small size containers [11,12] and long-chain cuticular hydrocarbons [13] by heating pieces of cuticle in small vials while sampling from the headspace. An application for the analysis of acids in insect secretions has not been reported so far. However different authors describe the analysis of carboxylic acids using polyacrylate and polydimethylsiloxane fibres [14,15].

## 2. Experimental

### *2.1. Standard solution*

Solutions of long-chain fatty acids with chain length between $C_{12}$ and $C_{18}$ were used in the analyses. Subsequently a standard solution containing dodecanoic (lauric), tetradecanoic (myristic), (*Z*)-9-hexadecenoic (palmitoleic), hexadecanoic (palmitic), (*Z,Z*)-9,12-octadecadienoic (linoleic), (*Z*)-9-octadecenoic (oleic) and octadecanoic (stearic) acids in hexane at 100 ng per μl was used. This solution will be referred throughout the text as solution 1.

### *2.2. Solid injection*

The Keele solid injection technique [1] has been tested and compared with the results obtained with liquid injections. Different quantities (2, 5 and 10 μl) of solution 1 were inserted in soda glass capillaries (20 mm×2 mm) closed at one end, the solvent was evaporated under a nitrogen stream and the capillaries were then sealed. This mimics a situation in which a secretion or an exocrine gland is sealed inside a capillary without any organic solvent. The capillary was then inserted inside the solid injector mounted in the injector port, the capillary was heated for 3 min and then crushed and the GC–MS analysis started.

### *2.3. Solid-phase micro extraction*

#### *2.3.1. Sampling from aqueous solutions*

The SPME sampling of fatty acids from aqueous solutions has been examined with fibres (Supelco, Bellefonte, PA, USA) coated with polydimethylsiloxane (PDMS, 7-μm film thickness), polyacrylate (PA, 85 μm) and Carbowax–divinylbenzene (CW–DVB, 65 μm). Solution 1 (1 μl) was dissolved in water (1 ml) in a 2-ml vial and stirred during the extraction (15 to 90 min).

#### *2.3.2. Headspace absorption*

In order to simulate the sampling of a small amount of an exocrine secretion containing long-chain fatty acids, different quantities of solution 1 were inserted in 2-ml glass vials and evaporated

under a nitrogen stream. Vials were then sealed, warmed in a block heater to 140, 180 or 225°C, while the fibre was lowered into the headspace for 5, 15 or 30 min.

### 2.4. GC–MS equipment

For setting up the methods and proving their reliability, two different GC–MS systems have been used. Helium was used as carrier gas on both systems using a 1 ml/min flow. All the analyses were performed in splitless mode.

#### 2.4.1. System 1

A Hewlett-Packard 5890 gas chromatograph coupled with a 5970B quadrupole mass spectrometer (using 70 eV electron impact ionization) was used. The system was controlled by a Hewlett-Packard series 300 computer equipped with a HP Chemstation.

Method 1a: 12 m×0.32 mm I.D. column covered with a 5% phenyl–95% methylsiloxane phase (0.25 μm thickness, BPX5, SGE, Milton Keynes, UK), SGE glass liner with Restek glass wool (Bellefonte, PA, USA), injection temperature 250°C, initial temperature 150°C, increment 5°C/min to 270°C, transfer line 270°C.

Method 1b: 15 m×0.32 mm I.D. column coated with a bonded polyethylene glycol stationary phase (0.25 μm thickness, Stabilwax, Restek). Quartz glass liner (homemade) with Restek glass wool, injection temperature 250°C, initial temperature 60°C, increment 11°C/min to 225°C, transfer line 225°C.

#### 2.4.2. System 2

A Varian 3400 gas chromatograph equipped with an Optic injector (Cambridge, UK) and connected to a Finnigan Incos 50 quadrupole mass spectrometer was used in the electron impact mode (70 eV electron impact ionization). Both the gas chromatograph and the mass spectrometer were controlled by a Data General DG 10 computer. The acquired data were transferred to a Finnigan Mat Datamaster II software for the analyses of the data.

Method 2: 15 m×0.32 mm I.D. column coated with a bonded polyethylene glycol stationary phase (0.25 μm thickness, Stabilwax, Restek). Optic glass liner with Restek glass wool, injection temperature 250°C, initial temperature 60°C, increment 11°C/min to 225°C, transfer line 225°C.

## 3. Results

The following paragraphs explain step-wise the successful set-up of the GC–MS method for the analysis of free fatty acids.

### 3.1. Effect of different liners

The deactivation of glass liners (through a 3-h immersion in a solution of Silyl-8 (Pierce Europe, Oud-Beijerland, The Netherlands), in toluene) was found to be quite inefficient. The less time-consuming use of commercially available straight glass liners (SGE, Optic and Restek) and homemade untreated quartz liners of the same shape led to much better results.

### 3.2. Effect of glass wool plugs

The use of glass wool inside the injection liners is reported to hinder the detection of fatty acids [14]. Phosphoric acid-treated glass wool (Supelco), which according to the catalogue description is suitable for the analysis of free fatty acids, gave extremely poor results. However, no significant differences were found between liners containing deactivated fused-silica wool purchased from Restek and empty liners when 100 ng of the standard was injected.

### 3.3. Effect of deactivated silica pre- and post-columns

The presence of pre- and post-columns of deactivated silica tubing were found to hinder the detection of free fatty acids in a dramatic way. The separation, peak shape and peak area were all affected and therefore the achievement of quantitative results was strongly hindered (system 1, method 1b). After removing the pre- and post-columns, the results improved considerably. The effect of the deactivated pre- and post-columns were even stronger if non-polar columns, commonly used for the analysis of

insect exocrine secretions, were used (system 1, method 1a)

### 3.4. Effect of the injector temperature

Injections of solution 1 on the Stabilwax column (system 2, method 2), at different injector temperatures varying from 200 to 350°C showed the best results with the highest temperatures. As little differences were found in the peak areas and because of the sensitivity of the Stabilwax column to high temperatures, 250°C was considered to be a good compromise for the analyses. By injecting solution 1 using 300°C and 250°C for the injector port a slight negative discrimination was observed against the highest molecular mass acids (stearic, oleic and linoleic acids).

### 3.5. Solid injection

The Keele injector [1] in early experiments was quite inefficient for the analyses of the acidic compounds (system 2, method 2). Soon it was found that the presence of glass fragments from crushed capillaries in the injector hindered the detection of the fatty acids. Probably because of absorption of the free fatty acids on the glass fragments at high temperature. This problem could be partially overcome as the glass fragments were regularly removed from the liner and the glass wool was changed after each analysis. However, even by taking these precautions the peak areas obtained were lower than those obtained by liquid injections (Fig. 1). Higher peak areas and more consistent results were obtained when the capillaries were crushed immediately after the insertion in the solid injector (Fig. 1).

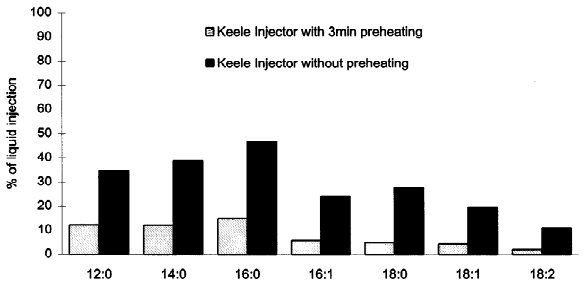


Fig. 1. Peak area obtained by the injection of 200 ng (2 μl of solution 1) of dodecanoic, tetradecanoic, hexadecanoic, (*Z*)-9-hexadecenoic, octadecanoic, (*Z*)-9-octadecenoic and (*Z,Z*)-9,12-octadecadienoic acids with the Keele injector with and without heating the capillaries before the beginning of the chromatography. The areas are given as percentages of the peak areas obtained by liquid injections of the same amount.

### 3.6. Solid-phase micro extraction

#### 3.6.1. Sampling from aqueous solutions

The SPME sampling of fatty acids from aqueous solutions and the direct GC–MS analysis has been examined with different fibres. The PDMS-coated fibre gave the best results for the saturated fatty acids. Improved results for palmitoleic, oleic and linoleic acids were obtained with the PA fibre. The experiments also indicated that equilibrium between the fibre coating and the aqueous phase was reached only with a 60 to 90 min absorption. As this absorption time was rather long and the Stabilwax column, used for the direct GC–MS analyses, is very sensitive to inorganic acids, which are present in the aqueous solution at lower pH to increase the extraction of the acids [14,15], no additional examinations have been carried out.

#### 3.6.2. Headspace absorption

As the aim of this work was to set up a solvent-free method, headspace analyses was attempted, although the experiments were indicating less absorption for the fatty acids compared with the absorption from the liquid (Fig. 2). In contrast with

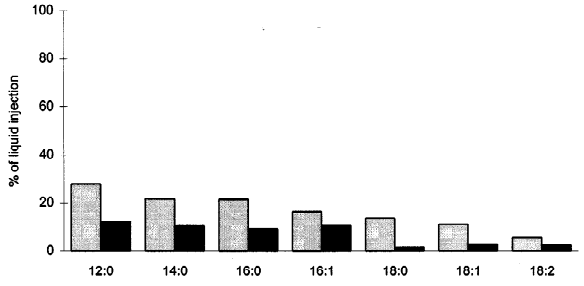


Fig. 2. Peak area obtained by the injection of 100 ng (1 μl of solution 1) of dodecanoic (12:0), tetradecanoic (14:0), hexadecanoic (16:0), (*Z*)-9-hexadecenoic (16:1), octadecanoic (18:0), (*Z*)-9-octadecenoic (18:1) and (*Z,Z*)-9,12-octadecadienoic (18:2) acids with a carbowax–divinylbenzene SPME fibre from liquid solution (grey bars) and from headspace (black bars). The areas are given as percentages of the peak areas obtained by liquid injections of the same amount.

the results obtained in the extraction from water, the best results were given by the CW–DVB fibre, which was therefore used in subsequent experiments. The first set of experiments with different block heater temperatures (140, 180 or 225°C) indicated, that the absorption was worse at the high temperatures, probably because they tend to desorb more easily from the SPME phase. Because of these results, in the later experiments vials were kept at 140°C.

The effect of the absorption time was checked by keeping the fibre in the vials for 5, 15 or 30 min. The results indicated that a 15-min absorption gave better results than a 5-min absorption. Increasing the absorption time to 30 min gave larger peak areas for palmitic, palmitoleic, stearic, oleic and linoleic acids, but worse for lauric and myristic acids; however a statistically significant difference was only found for linoleic acid. In another set of experiments the best recovery of all the compounds was obtained when the fibre was inserted in the sample vials at the same time when the heating of the vials started.

### *3.7. Quantitative aspects of the methods*

The linearity of the methods was checked at six points, with a total number of 18 analyses per method, corresponding to 50 ng, 100 ng, 200 ng, 400 ng, 600 ng and 1000 ng of each of the acids.

Linear regression analysis data are presented in Table 1 for three acids, lauric, an acid least affected by adsorption, palmitoleic, moderately adsorbed and linoleic acid, the most difficult to analyze. The lower slope of the regression line for the Keele injector and SPME method indicates that there is discrimination against long chain fatty acids through adsorption in the system, but the Keele injector gave better linearity as judged by the regression coefficient (Table 1).

### *3.8. Analysis of insect secretions through solid-phase micro extraction and solid sampling*

To demonstrate the practical application of these methods, CW–DVB fibres were used for the analyses of *Polistes dominulus* (Paper wasp) sternal glands. It was known from previous work that they contain long chain fatty acids [16], when diazomethane esterification was used. In these analyses the sternal glands were inserted in 2-ml vials of the same kind as described above and kept at 140°C while the absorption took place. The results showed the presence of palmitic, palmitoleic, oleic, linoleic and stearic acids.

The Keele injector was used for the analysis of the acidic secretion from the metapleural glands of workers of the Leaf-cutter ant *Acromyrmex octospinosus* (Fig. 3). Although the studies with pure acids showed the SPME method to be more sensitive for the analysis of acids, the SPME method could not detect a single acid in the secretion of the ants. A possible explanation may be, that during the 15 min headspace sampling at 140°C with SPME, the acids are absorbed at the glass surface or react with other compounds present in the secretion.

The presence of acidic compounds like 3-hydroxydecanoic acid and indoleacetic acid were already known from previous investigations in which the secretion was treated with diazomethane [17]. A number of short chain acids ($C_2$, $C_5$, $C_7$), long chain fatty acids from $C_{10}$ to $C_{18}$ and different 4-ketocarboxylic acids, overlooked in the previous studies, could be detected by crushing the capillaries immediately in the Keele injector. However the analysis of

Table 1
Linear regression relationships obtained by the analysis of 50, 100, 200, 400, 600 and 1000 ng of lauric, palmitoleic and linoleic acid (system 1, method 1b)

| | Liquid injection | | Keele injector | | SPME | |
|---|---|---|---|---|---|---|
| | $y\ (\cdot 10^{-4})$ | $R^2$ | $y\ (\cdot 10^{-4})$ | $R^2$ | $y\ (\cdot 10^{-4})$ | $R^2$ |
| Myristic acid | 68 | 0.87 | 40 | 0.94 | 29 | 0.80 |
| Palmitoleic acid | 74 | 0.87 | 28 | 0.93 | 46 | 0.80 |
| Linoleic acid | 80 | 0.95 | 11 | 0.80 | 31 | 0.93 |

$y$=Slope of regression line, $R^2$=regression coefficient.

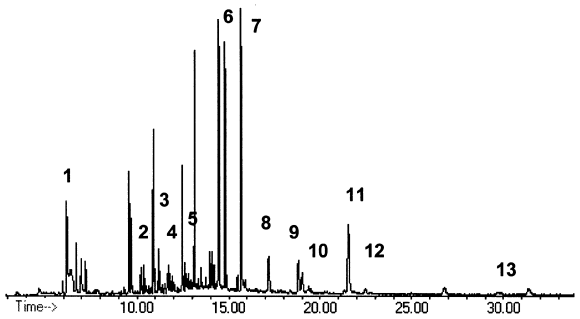


Fig. 3. Reconstructed total-ion chromatogram (TIC) of a GC–MS analysis from injection of the metapleural gland content of one *Acromyrmex octospinosus* (Leaf-cutter ant) major worker by solid-sampling technique (system 1, method 1b). 1=Acetic acid; 2=pentanoic acid; 3=heptanoic acid; 4=nonaonic acid; 5=decanoic acid; 6=indoleacetic acid; 7=4-oxo octanoic acid; 8=myristic acid; 9=4-oxo decanoic acid; 10=pentadecanoic acid; 11=palmitic acid; 12=palmitoleic acid, 13=stearic acid. Time in min.

more polar compounds like 3-hydroxydecanoic and 3-hydroxydodecanoic acid was only partially successful, probably because of the high boiling points and greater polarity of these compounds. Moreover the absorption from the headspace with the SPME method for this problem failed completely.

## 4. Discussion

In the course of this work many aspects of the gas chromatographic equipments used in routine analyses were found to hinder the detection of fatty acids. In particular, the use of user-deactivated liners and glass wool in the injector and the use of deactivated pre- and post-columns may prevent the detection of long chain fatty acids even if present in μg amounts.

Both absorption from sample headspace through CW–DVB-coated SPME fibres and the solid injection method using the Keele injector, are methods suitable for sampling carboxylic acids. Moreover both of them give quantitative results in analyses on columns coated with polar stationary phases. For the Keele method we observed that better results were obtained if the capillaries were crushed immediately after being inserted in the injector. Although the results obtained with the SPME method were below expectations, further modifications such as the use of smaller volume vials or the use of deactivated glass vials may improve the absorption from the headspace.

Even though the recovery of the target compounds was moderate, both methods are preferable to extraction with solvent, especially when the secretion of individual specimens has to be analysed. In fact, the extraction of insect secretion by solvents and the liquid injection of a fraction of the extract gave worse results than those obtained with either of the methods reported because only a fraction of the secretion can be injected. The gas chromatography of derivatized fatty acids presents fewer problems, but derivatizations may introduce contaminants or induce artefacts in the biological sample. Furthermore derivatization cannot give quantitative results when dealing with small insect samples. For these reasons the analysis of untreated samples may be preferable for confirming the genuine presence of free carboxylic acids in insect samples.

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