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# Characterization of lipids in complex samples using comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry

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**Abstract**

Most lipids are a complex mixture of classes of compounds such as fatty acids, fatty alcohols, diols, sterols and hydroxy acids. In this study, the suitability of comprehensive two-dimensional gas chromatography coupled to a time-of-light mass spectrometer is studied for lipid characterization in complex samples. With lanolin, a refined wool wax, as test sample, it is demonstrated that combined methylation plus silylation is the preferred derivatization procedure to achieve (i) high-quality GC × GC separation and (ii) easily recognizable ordered structures in lipid analysis. Optimization of the GC × GC column combination, the influence of the temperature programme on the quality of the separation, and the potential and limitations of automated TOF-MS-based identification are discussed. The combined power of a 2D separation, ordered structures and MS detection is illustrated by the identification of several minor sample constituents.


*Keywords:* Gas chromatography; Comprehensive two-dimensional; Time-of-flight mass spectrometry; Lipids

## 1. Introduction

Lipids are key components of living organisms due to their metabolic importance, energy storage capacity and structural properties. Including a large range of chemical families, lipids have been operationally defined as being soluble only in non-polar solvents. Lipid characterization is a very important issue in different disciplines such as medicine, food science, biology, biochemistry, environmental sciences and pharmaceutical applications. Analysis has therefore to be carried out in a variety of samples: food, biological tissues, cosmetic preparations and environmental matrices. One of the most important lipid classes is the fatty acids (FA) which are essential parts of most living cells and cellular fluids. FA represent a complex chemical class, with different chain lengths and number of double bonds. Minor lipid compounds can be of major interest, as for example some allergens or molecular markers [1]. Lipids, and more specifically FA, are usually analysed by GC [2] and, to a lesser extent, by LC [3] and SFC [4]. In order to analyze FA and fatty alcohols (FAL) by GC, a derivatization step is needed [5]. As a consequence of sample complexity and of the interest in minor components, a multi-step procedure is usually required in order to fractionate samples prior to lipid determination [6]. That is, extensive sample preparation is needed in order to avoid co-elutions and interferences which can differ depending on the sample type analyzed.

Over the past decade, comprehensive two-dimensional gas chromatography (GC × GC) has emerged as a powerful separation technique which is especially suited for the characterization of complex samples [7,8]. This technique is an improvement over multidimensional GC–GC [9,10] because the peak capacity of the system is equal to the product of the peak capacities of the two dimensions. This permits a reduction of sample preparation and the type of sample

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analyzed becomes less important. Two other advantages of GC × GC are the increased analyte detectability due to the cryofocusing operated by the modulator and the presence of chemically ordered structures in the chromatograms. The modulator, which is the heart of a GC × GC system, has three functions. First, it is able to trap the compounds eluting from the first-dimension by means of a cold spot which is created by spraying the capillary column with cold fluids such as ambient-temperature air, low-temperature carbon dioxide or liquid nitrogen, depending on the application (range of the boiling points of the target analytes) [11]. Second, it refocuses the analytes obtaining sharper peaks in the second-dimension and, finally, it reinjects them in the second-dimension column.

Until recently GC × GC had to be coupled with detection methods such as a flame ionization (FID) or a micro-electron capture (μECD) detection which were the only ones providing the high acquisition rates which are needed to monitor the sharp chromatographic peaks eluting from the second-dimension column (50–600 ms peak width). Recently, time-of-flight mass spectrometry (TOF-MS), with its high scan speed capabilities, permits to add to GC × GC the advantages of MS techniques, primarily the possibility of analyte identification and/or confirmation. GC × GC–TOF-MS has already been used successfully for the characterization of oil volatiles [12–14], mixtures of volatiles [15], cigarette smoke [16–18], flavor compounds in food [19], aerosol particulates [20,21], oil samples [22], toxaphene [23], pesticides [24–26] and polychlorinated biphenyls in seals [27]. On the other hand, GC × GC has been scarcely used for the characterization of lipids and, then, always coupled to FID. FA were analyzed in oil samples with different orthogonal and non-orthogonal column sets [28–31] and in milk [32]. The technique was also applied for the characterization of fecal sterols in biological samples [33].

In this paper, a new analytical approach based on GC × GC–TOF-MS was developed for the characterization, without pre-fractionation, of known and unknown compounds from various classes of lipids. Lanolin, the wool wax secreted by the sebaceous glands of sheep, was chosen as a complex sample model [34,35]. In order to achieve our goal, analytical parameters such as type of modulation, column selection and derivatization technique were optimized. Furthermore, preliminary identification techniques using the GC × GC–TOF-MS system capabilities are discussed.

## 2. Experimental

### 2.1. Samples and reagents

Fatty acids are the most important class of lipids; therefore, special care has been given to their separation. For this reason, a standard solution of 37 fatty acid methyl esters (FAME), with different chain lengths and number of double bonds, was purchased from Supelco (Supelco Park, PA, USA). FA will be named using the formula **C***a*:*b***n***c*, where *a* is the number of carbon atoms, *b* the number of double bonds and *c* is the position of the first double bond beginning at the methyl terminal group. Commercial purified lanolin (Corona lanolin) was from Croda (Snaith Goole, UK).

### 2.2. Derivatization procedures

#### 2.2.1. Methylation

Methylation was done using a 0.005 M solution of trimethylsulphonium hydroxide (TMSH) from Fluka (Buchs, Switzerland) in methanol. An equal volume of reagent and sample (10 μL, 3000 μg/g) were mixed and the mixture was held at room temperature for 30 min. Under these mild conditions, trans-esterification of esters is minimal.

#### 2.2.2. Silylation

Bis-silyltrifluoroacetamide (BSTFA) from Merck (Darmstadt, Germany) was used as silylating reagent. Twenty microliters of reagent were added to the sample (10 μL, 3000 μg/g) and the derivatization was carried out at 70 °C during 1 h. Next, the sample was evaporated to dryness under a gentle nitrogen flow. Then the sample was redissolved in the injection solvent, ethyl acetate (20 μL).

#### 2.2.3. Dual derivatization

In dual derivatization, first the methylation is carried out and then the silylation. That is, the individual procedures presented above were now applied sequentially. The only difference was that after the methylation of the FFA, the sample was evaporated to dryness under a gentle flow of nitrogen to avoid the presence of methanol during silylation. Next, the sample was directly redissolved in BSTFA and silylation took place as described.

### 2.3. GC × GC–TOF-MS

The GC × GC–TOF-MS system consisted of a HP 6890 (Agilent Technologies, Palo Alto, CA, USA) gas chromatograph equipped with an Optic 2 programmable injector (ATAS, Veldhoven, The Netherlands) in the splitless mode at 300 °C (2 min). The MS system was a Pegasus II TOF system (LECO, St. Joseph, MI, USA) working at −70 eV, transfer line 280 °C, ion source 250 °C and scanning from 70 to 800 *m*/*z* at 50 Hz with a detector voltage of 1950 V. Details of the various column sets are shown in Table 1. Experimental conditions for column set 1, which was used in almost all experiments were: oven temperature, 70 °C (1 min), to 360 °C at 5 °C $min^{-1}$ with a final hold of 20 min. The carrier gas, helium, was used at a constant flow of 1.2 mL $min^{-1}$. The optimal ambient temperature air modulation time was 5 s.

For the modulation, an in-house developed modulator was used. This modulator uses two ambient-temperature air jets (Fig. 1a), but small variations in the oven temperature caused slight movements of the jets, which prevented

Table 1
Column sets used during GC × GC method optimisation

| Column set | Dimension | Phase[a] | Length (m) | Internal diameter (mm) | Phase thickness (μm) |
|---|---|---|---|---|---|
| 1 | First | XTI-5, 95% dimethyl–5% diphenyl polysiloxane, Restek | 10 | 0.25 | 0.25 |
| | Second | BPX-50, 50% phenyl polysilphenylene-siloxane, SGE | 1 | 0.10 | 0.10 |
| 2 | First | XTI-5, 95% dimethyl–5% diphenyl polysiloxane, Restek | 10 | 0.25 | 0.25 |
| | Second | BGB-WAX, poly(ethylene glycol), BGB Analitik AG | 0.5 | 0.05 | 0.10 |
| 3 | First | BP-1, 100% dimethyl plysiloxane, SGE | 15 | 0.25 | 0.25 |
| | Second | HT-8, 8% phenyl Polycarborane-siloxane, SGE | 1 | 0.10 | 0.10 |
| 4 | First | ZB-5, 95% dimethyl–5% diphenyl polysiloxane, Phenomenex | 30 | 0.25 | 0.25 |
| | Second | BPX-50, 50% phenyl polysilphenylene-siloxane, SGE | 1 | 0.10 | 0.10 |
| 5 | First | DB-Wax, poly(ethylene glycol), J&W Scientific | 25 | 0.32 | 0.25 |
| | Second | BPX35, 35% phenyl polysilphenylene-siloxane, SGE | 1 | 0.10 | 0.10 |

[a] Restek (PA, USA); SGE (Ringwood, Australia); BGB Analitik (Anwill, Switzerland); Phenomenex (Torrance, CA, USA); J&W Scientific (Folsom, CA, USA).

proper spraying of the capillary column during the whole chromatographic run. In order to avoid this problem, the jet shape was modified as shown in Fig. 1b. With the new configuration temperature variations had no effect anymore. A minor drawback is that the new configuration is more noisy than the original one.

For data transformation and visualization two additional programs were used, a program to convert the raw data into a two-dimensional array (software provided by P.J. Marriott) and a program to generate contour plots from this array ("Transform", part of Noesys software package; Research Systems International, Crowthorne, UK).

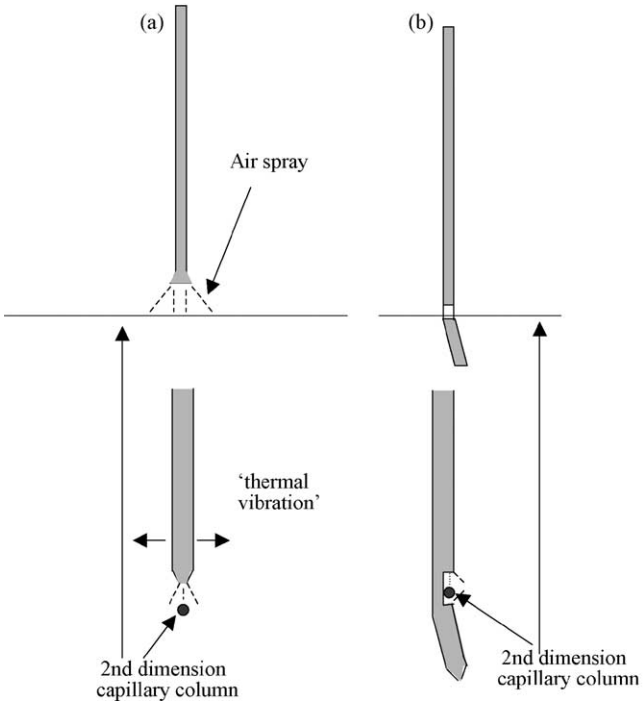


Fig. 1. (a) Original air jet modulator and (b) modification of original air jet modulator (a) to enable proper functioning (no 'thermal vibration') in GC × GC–TOF-MS.

## 3. Results and discussion

### 3.1. Modulation

As already stated, there are various options available to carry out modulation. These include the use of liquid nitrogen, carbon dioxide and air (cooled or ambient temperature). Modulation selection is a compromise between efficiency, boiling-point range of the analytes, cost effectiveness and robustness. In this study, ambient air was tested since it is the most inexpensive and robust technique. With that modulation system, the first *n*-alkane that is correctly modulated was $C_{17}$; this agrees with the previous results [11]. Furthermore, if we consider the FAME, the first compound to be efficiently modulated is $C_{14}$ and in, for example, environmental samples the first FAME that is important as molecular marker is $C_{15}$ and its isomers. This justifies the use of ambient air as cooling agent for modulation.

### 3.2. Column selection

Several column sets were tested in order to optimize the resolution of target analytes. There are two main types of sets, orthogonal and non-orthogonal ones. The orthogonal systems, like column sets 1–4 of Table 1 consist of a non-polar first column which separates on the basis of the boiling points of the analytes, and a polar second-dimension column with a separation based on the interaction between the analyte(s) and the polymeric stationary phase. For a non-orthogonal system, such as column set 5, the separation in the first-dimension is based on both compound/stationary phase interaction and boiling point. In order to compare these different chromatographic systems, several co-elutions to be expected for the FAME mixture were studied in terms of their chromatographic resolution ($R_s = \Delta R_t/\omega_b$) in the first ($R_{s_1}$) and second ($R_{s_2}$) dimension, with $\Delta R_t$ being the retention time difference between the two peak maxima and $\omega_b$ the peak width at the baseline. For $R_{s_1}$, reconstructed peak widths in the first-dimension were used for the calculation.

Table 2
Resolution between target analytes using different column sets

| Analyte pairs | Set 3 | | Set 2 | | Set 4 | | Set 1 | | Set 5 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | First | Second | First | Second | First | Second | First | Second | First | Second |
| C18:0/C18:1n9cis | 1.0 | –[a] | 2.3 | 0.6 | 2.6 | 0.8 | 2.5 | 1.2 | 1.0 | –[a] |
| C18:1n9cis/C18:1n9trans | –[a] | –[a] | 0.5 | –[a] | 0.6 | –[a] | 0.8 | –[a] | –[a] | –[a] |
| C18:3n3/C18:3n6 | 1.0 | –[a] | 2.0 | 0.5 | 2.4 | –[a] | 2.0 | –[a] | 1.4 | 0.5 |
| C18:1n9cis/C18:3n6 | –[a] | –[a] | –[a] | 1.1 | –[a] | 1.7 | –[a] | 2.2 | 3.1 | –[a] |
| C20:3n6/C21 | 4.3 | –[a] | 11.8 | 1.2 | 11.8 | 2.2 | 10.6 | 6.8 | –[a] | 2.5 |

[a] $R_s < 0.5$.

In this way, the $R_s$ of the following compound pairs were evaluated: C18:0/C18:1n9cis; C18:1n9cis/C18:1n9trans; C18:3n3/C18:3n6; C18:1n9cis/C18:3n6; C20:3n6/C21. The experimental results are summarized in Table 2. For column set 3, its second-dimension separation is completely inefficient, with $R_{s_2} < 0.5$ for all compound pairs studied. This is due to the low polarity of the second-dimension column. That is, $R_{s_2}$ can be strongly improved by increasing the polarity of the second-dimension column. For example, if we consider the C18:0/C18:1n9cis pair, $R_{s_2}$ improves when going from sets 3 to 1 ($R_{s_2}$ 1.2) and set 2 ($R_{s_2}$ 0.6). Actually, the best result was expected for set 2 [poly(ethylene glycol)] as it is the most polar system. However, because the second column has a 50 μm I.D., there is a huge difference in the average carrier speed in the first-column and second-dimension columns: it is 25 times higher in the second-dimension, and one cannot achieve optimal carrier conditions, especially in the second-dimension [36]. This explains the relatively low $R_{s_2}$ obtained with this system.

The main difference between column sets 1 and 4 is the length of the first-dimension columns (set 1, 15 m; set 4, 30 m); the other parameters are the same, including the nature of the stationary phase. As expected, $R_{s_1}$ improves by increasing column length as plate numbers are increased. However, this increase adversely affects $R_{s_2}$, as can be read from Table 2. This is due to the fact that with a longer column, the elution temperatures from the first-dimension column increase. Therefore, isothermal separation in the second column is carried out at higher temperatures which reduces the analyte selectivity. This effect can be diminished by slowing down the temperature program of the GC oven; however, this will unfortunately drastically increase the analysis time. Therefore, depending on the resolution requirements of each application, set 1 or 4 will be preferred.

Completely different analytes distributions are obtained when working with a non-orthogonal system, as was earlier stated in [30,37]. Some compounds which coeluted in the first-dimension of the orthogonal system, are fully separated in the non-orthogonal system (C18:1n9cis/C18:3n6), but the opposite also occurs, for example, for the C20:3n6/C21 pair. In fact, what is most important is not to avoid coelution in the first-dimension, but to have a complementary system, where a coelution problem in one dimension can be solved in the other one. In our case better separation is obtained when using orthogonal systems; furthermore, a wax column such as is used as first-dimension in the non-orthogonal system, presents maximum-temperature limitations, with serious bleeding close to the maximum temperature. Therefore, for the present application an orthogonal system is preferred and more precisely column set 1 will be used in the further work.

### 3.3. Ordered structures

As has repeatedly been demonstrated [7] chemical ordering, i.e. the positioning of compounds in a GC × GC contour plot according to their chemical properties, occurs in orthogonal systems. That is, compounds with similar chemical properties will appear as ordered structures. Since FAME are a complex chemical class, their chemical ordering will be somewhat complex. That is, sub-structures will show up which reflect the number of double bonds (Fig. 2a) or the position of the double bonds (Fig. 2b). The fractional chain length, ($\mathrm{FCL} = (R_{t_x} - R_{t_A})/R_{t_B} - R_{t_A}$) (where x is the target analyte, A the linear compound of the same chemical class eluting just before x and B the linear compound of the same chemical class eluting just after x) was calculated since it has been extensively used to identify branched isomers of fatty acids [34]. It can also be useful to evaluate the position of the first double bond. Fig. 2 shows that, as expected, chromatographic behavior in the first and the second-dimensions is dependent on the chemical structure of a compound. In this way, the number of double bonds is identified in the second-dimension (Fig. 2a) and in the first-dimension, based on the FCL, the position of the double bond is determined (Fig. 2b).

The chromatographic behavior can also be correlated to physicochemical characteristics of the analytes. In an orthogonal system, retention in the second-dimension depends mainly on compound polarity. Since the octanol/water partition coefficient ($\log P$) is also a function of compound polarity, the second-dimension retention time can be correlated with $\log P$. This is mainly true for compounds from the same chemical class, because other parameters which can affect their retention in the second-dimension then are the same. As an example, Fig. 3 shows a strong correlation (Pearson coefficient 0.999; $P < 0.001$) with a negative slope between the second-dimension retention time and $\log P$ for C18 FAME with a different number of double bonds and their positions. It is obvious that this correlation is a useful confirmation tool for identification purposes.

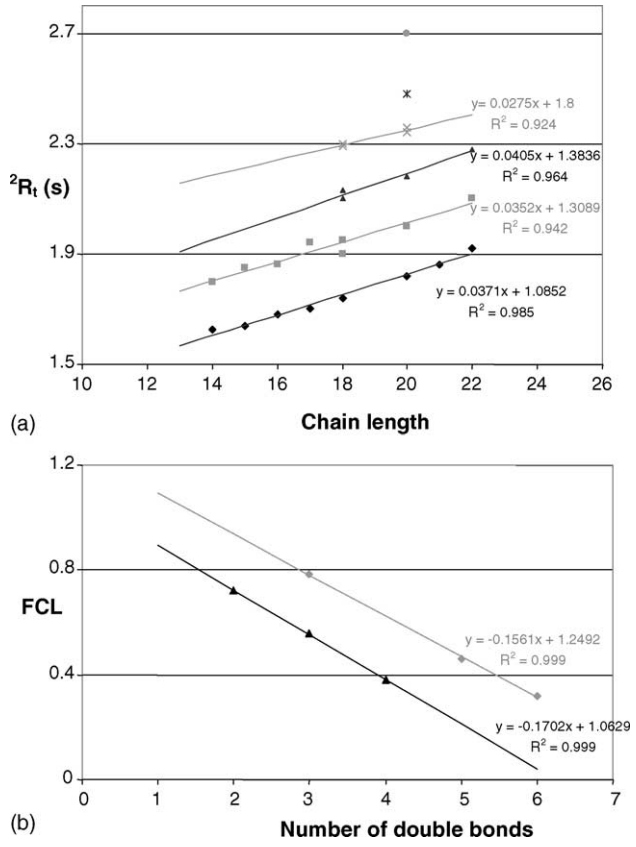


Fig. 2. (a) Dependence of second-dimension retention time ($^2R_t$) of FAME of different chain length and different number of double bonds (DBs) for column set 1 (◆ 0 DB, ■ 1 DB, ▲ 2 DBS, ✕ 3 DBs, ✳ 4 DBs and ● 5 DBs). (b) Dependence of FCL of different positional isomers as a function of their number of DBs using column set 1. For $n-6$, the mean of C18:2, C20:2 and C22:2 was used as compounds with two DBs (RSD < 5%), the mean of C18:3 and C20:3 as compounds with three DBs, and C20:4 as compound with four DBs. For $n-3$, the mean of C18:3 and C20:3 was used as tri-unsaturated compounds, C20:5 as penta-unsaturated compound and C22:6 as hexa-unsaturated compound (◆ $n-3$ and ▲ $n-6$).

### 3.4. Derivatization

As is well-known, and was briefly mentioned above, derivatization of FA and FAL is necessary prior to their analysis by GC. The most common derivatization technique for FA is methylation, i.e. analysis as FAME. For the other compounds of interest, silylation, which derivatizes both the alcoholic and acidic functions, is generally preferred. Therefore, lanolin was analyzed in three different ways, after methylation, silylation and methylation + silylation. Such derivatization strongly influences analyte polarity and, consequently, the second-dimension retention times of the target compounds; in addition, the characteristic masses to be used for MS monitoring will change. Relevant information concerning these aspects is presented in Table 3 for $C_{14}$–$C_{16}$ representatives of four main classes of compounds having similar boiling-points. Even if the Pearson correlation coefficient was somewhat lower than in Fig. 3 (0.964, $P < 0.001$) a $\log P$ versus $^2R_t$ correlation similar to the above was found for the data of Table 3. This is a gratifying result if we consider that different classes of compounds and different derivatization techniques (and minor differences in boiling-points) were involved.

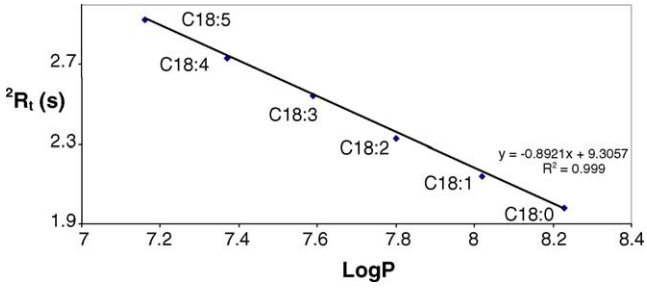


Fig. 3. Correlation between second-dimension retention times (column set 1) of several $C_{18}$ FAME and their log $P$ values.

When using only the methylation step, distinct tailing occurs for the compounds containing hydroxyl groups such as the FAL because they are not derivatized. This is shown in Fig. 4, especially in the insert, which also illustrates that the FAL and FAME have similar second-dimension retention times (cf. Table 3). In other words, with methylation only, there is chemical ordering but, because of the overlap of the bands corresponding to the FAME and to the FAL, the outcome is not really optimal. Above these bands, other mixed structures, corresponding to higher polarity compounds (higher $^2R_t$), are observed but they cannot be easily identified.

Table 3
Calculated log $P$ values [38] and experimental $^2R_t$ (s) of the derivatives of the main classes of compounds with the three derivatization methods[a]

| Analyte | Methylated | | Methylated + silylated | | Silylated | |
|---|---|---|---|---|---|---|
| | log $P$ | $^2R_t$ | log $P$ | $^2R_t$ | log $P$ | $^2R_t$ |
| FA: $C_{15}$ | 6.76 | 4.3 | 6.76 | 4.3 | 8.53 | 3.6 |
| ions ($m/z$) | 74 | 74 | 74 | 74 | 132 | 132 |
| FAL: $C_{16}$ | 6.73 | 4.4 | 9.21 | 3.1 | 9.21 | 3.1 |
| ions ($m/z$) | 83 | 83 | 103 | 103 | 103 | 103 |
| Diol: $C_{15}$ | 5.11 | – | 9.65 | 2.6 | 9.65 | 2.6 |
| ions ($m/z$) | – | – | 147, 103 | 147, 103 | 147, 103 | 147, 103 |
| Hy-A: $C_{14}$ | 4.01 | – | 7.20 | 4.0 | 8.98 | 3.1 |
| ions ($m/z$) | – | – | 103 | 103 | 147, 103, 129 | 147, 103, 129 |

[a] Column set 1; chain lengths selected to have similar boiling-points for the various compounds.

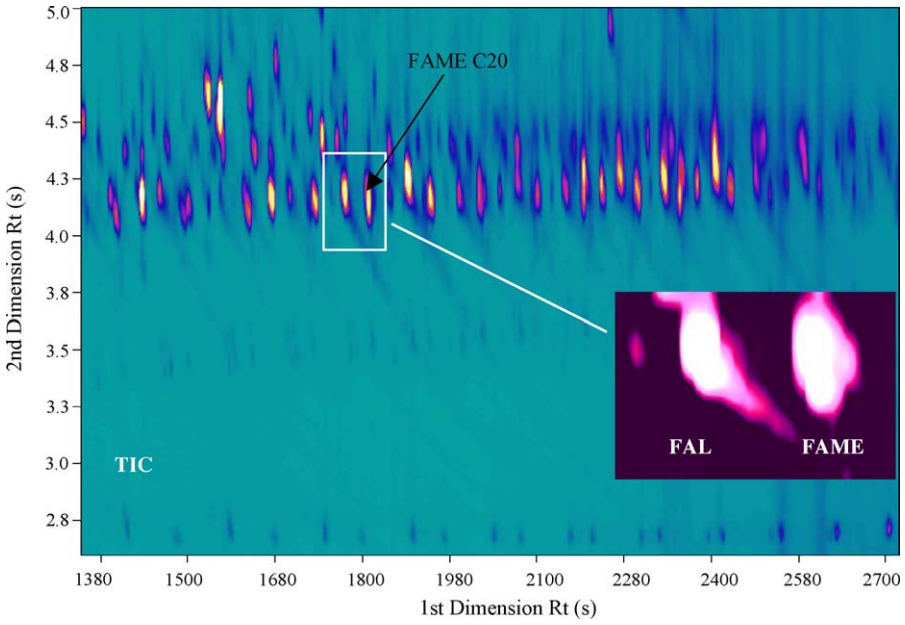


Fig. 4. TIC contour plot obtained by GC × GC–TOF-MS of methylated lanolin. Similar $R_{t_2}$ observed for FAL and FAME. FAL (but not FAME) tailing in first-dimension is shown in insert. Column set 1.

Two-step methylation + silylation is the most time-consuming technique but in principle also the most rewarding one. Fig. 5 shows a GC × GC TIC contour plot for a methylated + silylated lanolin sample, together with several characteristic reconstructed ion chromatograms (*m*/*z* 74, 103 and 147). The most striking observation is that the second-dimension separation is now much better than before due to the polarity change effected by the silylation. The polarity of FAL, diols and Hy-A sharply decreases (cf. Table 3). To quote an example, the log *P* values of the diols increase some four logarithmic units. As a consequence, distinct ordered structures can now be seen for the various chemical classes: they show up as essentially parallel horizontal bands, as the inserts of Fig. 5 indicate. This is most helpful when screening unknown samples. Compared with methylated, lanolin peak shape is improved as especially alcohol tailing

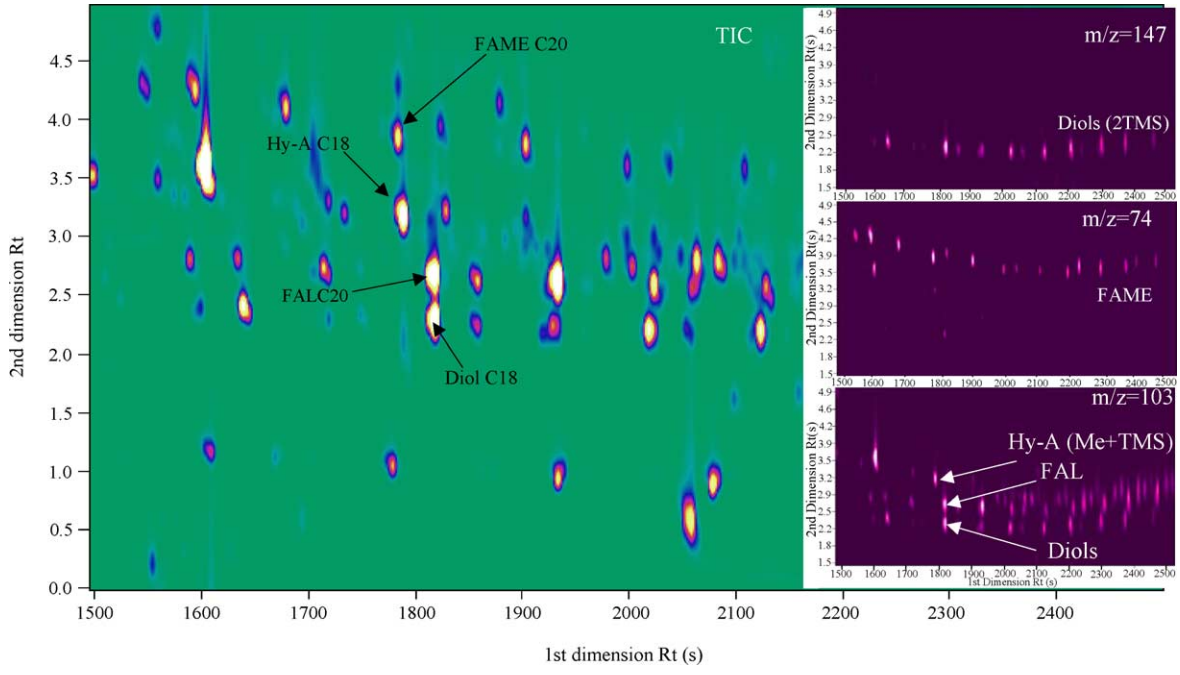


Fig. 5. TIC contour plot obtained by GC × GC–TOF-MS of methylated + silylated lanolin, and inserts of fragmentograms *m*/*z* 74, 103 and 147. Column set 1.

is now avoided. Baseline separation is obtained for various compounds for which coelution has been reported in the first-dimension [34]. Several coelution problems are also solved in the second-dimension, for example between FAL and diols (i.e. FAL C20 and diol C18) and between Hy-A and FAME (i.e. Hy-A C18 and FAME C20) (Fig. 5). The two-step derivatization permits a rapid identification of the diols and Hy-A, which is more complex when using silylation only, because of the identical mass fragments then observed. In the present case, only the diols have two trimethylsilyl (TMS) groups and show the characteristic *m/z* 147 fragment $[(CH_3)_2Si^+OSi(CH_3)_3]$.

Silylation, which is the technique most frequently used for lanolin characterization, enables analysis of all target compounds after a single derivatization step. This method was tested to check if similar results, than those presented for the methylation + silylation, could be obtained in less time. As Fig. 6 shows there is a clearly visible chemical ordering also here; however, the various bands are quite close to each other and the result is less than optimal. This is due to the decrease in polarity of FA and Hy-A compared to the (methylation + silylation) approach, as can be read from Table 3. Also, when using this technique compound identification is more problematic due to the similarity of the ion patterns obtained for the various classes of compounds. For example, *m/z* 117, which is a characteristic ion for FA and Hy-A $[O^+ \equiv COSi(CH_3)_3]$, is also obtained with the 1,2-diols; further, *m/z* 147, which is characteristic of the diols in the two-step procedure, now is found also for Hy-A.

### 3.5. Identification

In order to identify the various compounds, an automated method based on the deconvolution software of the Pegasus system was used. The program gave 9999 (the maximum number) hits, but from among these hits only 113 had a similarity of over 800. Furthermore, one should consider that each peak is modulated some four or five times and is, therefore, identified as many times by the software. That is, the number of compounds that were identified automatically was on the order of thirty. While this may seem a somewhat disappointing result in view of the fact that lanolin contains thousands of compounds, the result is not unlike that of an earlier study on another complex matrix, cigarette smoke [16]. As for an explanation, first of all, the NIST library is rather incomplete as regards high-molecular-weight derivatives and odd-chain-length compounds. In addition, the Leco system was initially thought to be mainly directed at the analysis of volatile compounds; as a consequence it presents somewhat poor-quality mass spectral data for the heavier ions with a sharp decrease in ion intensity [17]. This is a major problem since heavier ions are usually more selective and permit to obtain molecular-weight information. For example, for cholesterol, which is a major constituent of lanolin and was correctly identified (similarity 896), the decrease of the relative abundances of the higher masses was considerable, with mass *m/z* 368 being three times lower than expected (compared to the NIST library) and *m/z* 458 (molecular mass) even four times.

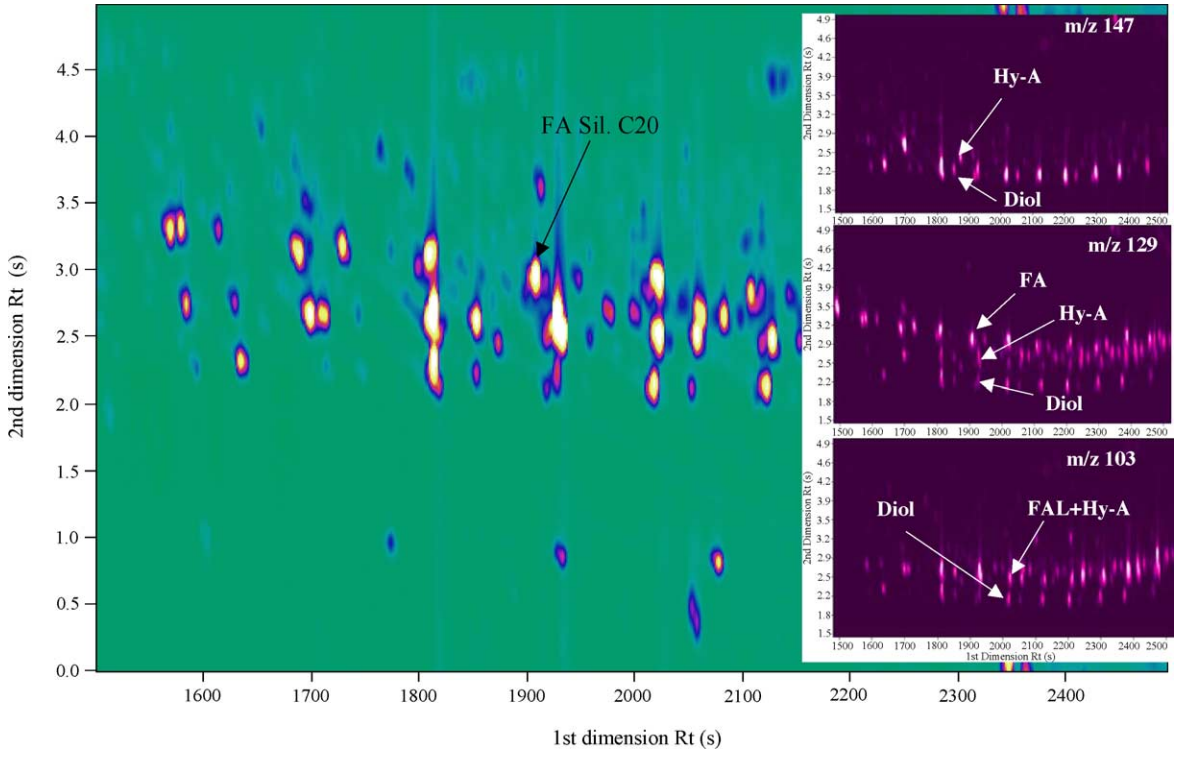


Fig. 6. TIC contour plot obtained by GC × GC–TOF-MS of silylated lanolin, and inserts of fragmentograms *m/z* 103, 129 and 147. Column set 1.

The presence of ordered structures can be used for confirmation purposes or, alternatively, for provisional identification. Unknown spots showing up within the ordered bands attributed to, for example, FAME, can be provisionally identified as additional FAME compounds. Their chain length, number of double bonds and position of the double bonds can be found by using the regression procedure shown in Figs. 2 and 3, with the MS spectrum being used for final confirmation. Actually, one may well say that the present refined procedure (for earlier attempts, see [7,28]) enables identification without the use of standards even for complex mixtures and in the case of compounds with similar mass spectra.

For the high-molecular-weight compounds which elute at the isothermal final temperature of the programme, i.e. above 360 °C, next to the problem of low mass-spectral quality, there is also wrap-around. In fact, one of the drawbacks of GC × GC is that until now, modulation cannot be optimized simultaneously for low- and high-molecular-weight compounds. Also due to the peak broadening (first-dimension, isothermal broadening which increases number of modulations per peak; second-dimension, wrap-around broadening) (Fig. 7), detectability is considerably decreased. Consequently, no minor constituents can be detected in the isothermal final part of the run. This adverse effect can be minimized by increasing the modulation time, which will reduce the number of modulations per peak. However, this is not a proper solution because compounds eluting earlier will now be modulated only two to three times, and their first-dimension separation will be affected.

Even if it was not the goal of this work to carry out a complete lanolin characterization, the increased resolution provided by the comprehensive separation enabled the identification of several minor constituents of lanolin. For example, next to linear aliphatic diols ranging from $C_{14}$ to $C_{24}$ that have been reported in another work [34], the present study enabled the detection and identification of diol $C_{25}$ anteiso, $C_{26}$ iso and $C_{27}$ anteiso. Above that, a new class of compounds was identified – to our knowledge – for the first time in lanolin. Its origin probably is the degradation of FA. These compounds were discovered when looking for FA using *m*/*z* 129, which is characteristic for FAs, in the rather crowded area in the contour plot between 1125 and 1800 s. While FAs are easily recognized by their specific arrangement of the fragment ions *m*/*z* 73, 75, 117, 129, 132 and 145, five peaks had distinct different spectra. In the spectra of these five compounds *m*/*z* 132 and 145 were absent or with a negligible abundance, while ions *m*/*z* 147, 204 and 217 had a relative high abundance. The ion *m*/*z* 147 indicates the presence of two trimethylsilyl groups, $[(CH_3)_2Si^+OSi(CH_3)_3]$. After close examination of the spectra, all appeared to have two abundant ions in the region above *m*/*z* 200 with a difference of 116 amu, which could be attributed to $[M\text{-}CH_2C(O)OSi(CH_3)_3]^+$ and $[M\text{-}CH_3]^+$. In Fig. 8, this is illustrated for diTMS derivative of dodecandioic acid with specific ions at *m*/*z* 243 and 359, respectively. All five compounds were identified as linear dicarboxylic acids with chain lengths of 9–13 carbon atoms. These diacids were observed only when the silylation step was chosen as derivatization technique. With the other two procedures, they would have been derivatised into the more polar dimethyl diesters. Consequently, they would have eluted with a larger second-dimension retention time, even larger than the modulation time (wrapped around; ${}^2R_t >$ modulation time) as a rather broad peak with a much lower signal-to-noise-ratio. Due to the low concentration

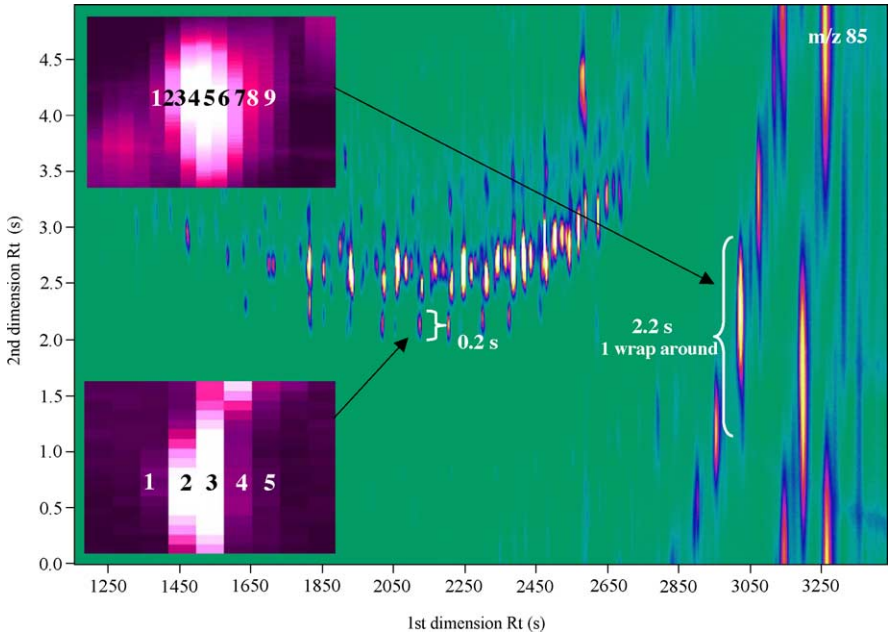


Fig. 7. Contour plot (*m*/*z* 85) illustrating the differences in peak shape between compounds eluting at low and very high temperatures. Column set 1.

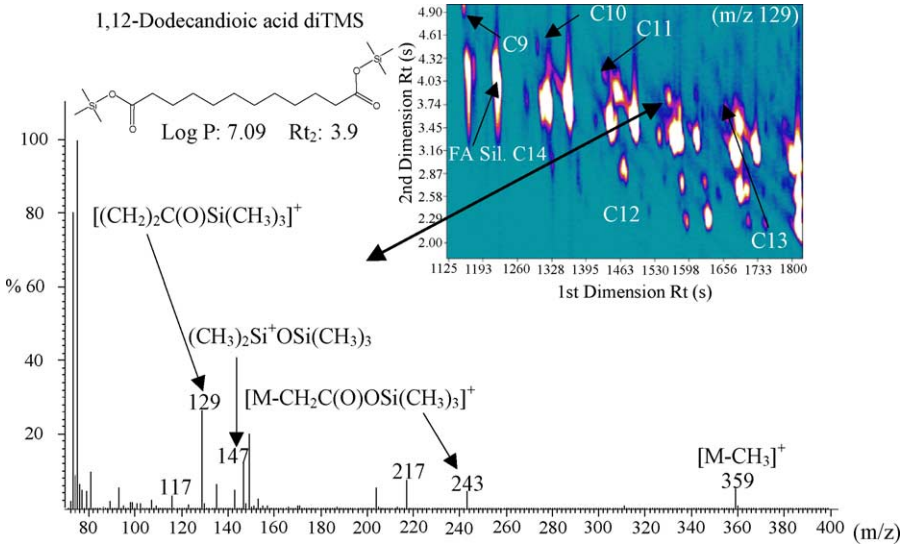


Fig. 8. Contour plot (*m*/*z* 129) showing the diacids distribution obtained after GC × GC–TOF-MS of a silylated lanolin extract on column set 1 and mass spectrum details of the 1,12-dodecanedioic acid diTMS.

of these acids and the probable coelution with other sample components, the diacids could not be detected at all.

A much more selective contour plot (data not shown) was obtained when using the $[M\text{-}CH_3]^+$ ions (*m*/*z* 317 + 331 + 345 + 359 + 373) and two remarkable aspects could be deduced. Regarding the position in the contour plot, it appeared that all diTMS derivatives of the diacids eluted 65 s earlier from the first-dimension column than the silylated FA *n* + 5 (that is, five carbon atoms more than the diacid), and with a slight higher, though not constant, second-dimension retention time. In case of nonandioic acid the difference was 0.9 s, while for tridecanedioic acid only 0.4 s was observed. For the $C_{12}$ diacid the log *P* value was calculated, and it correlated with its second-dimension retention similarly to what was observed for the compounds indicated in Table 3, thereby confirming its identification. These compounds, due to their level of similarity, did not permit an automated identification. For the $C_{12}$ di-acid (Fig. 8), the obtained similarity 698, below 800, can be explained by the low abundance of the highest ion, *m*/*z* 359.

## 4. Conclusions

In this paper, the suitability of GC × GC–TOF-MS to characterize lipids in complex samples has been studied. Lanolin was used as model matrix, but the nature of the developed methodology as well as the type of conclusions drawn clearly show that the approach will be useful for a wide variety of lipid mixtures. One main conclusion is that a two-step derivatization – i.e. methylation plus silylation – is the preferred option to improve the chromatographic properties of the classes of compounds of interest, FA/FFA, FAL, Hy-A and diols, and to obtain easily distinguishable ordered structures for each of these. The use of such ordered structures, especially when using properly selected mass traces, and the correlation with phisicochemical properties of the analytes, all are most valuable tools for many GC × GC applications. The recognition, and identification, of several minor sample constituents by means of this approach is a convincing illustration of its potential.

For the rest, although TOF-MS is an excellent tool which is fully compatible with GC × GC, automated identification still has its problems, primarily because of technical deficiencies. This again indicates that future work should increasingly be directed at improving identification procedures and, actually, all aspects of data handling. At the same time, one should also stress that, even with the somewhat imperfect tools available today, the combination of comprehensive gas chromatography and mass spectrometric confirmation has an impressive potential.

## References

[1] A.R. Zimmerman, E.A. Canuel, Estuar. Coast. Shelf Sci. 53 (2001) 319.
[2] R.G. Ackman, Anal. Chim. Acta 465 (2002) 175.
[3] E.S. Lima, D.S.P. Abdalla, Anal. Chim. Acta 465 (2002) 81.
[4] F.J. Señoráns, E. Ibañez, Anal. Chim. Acta 465 (2002) 131.
[5] K. Blau, J. Halket, Handbook of Derivatives for Chromatography, Wiley, Chichester, 1993.
[6] K. Grob, C. Mariani, in: J.H.P. Tyman, M.H. Gordon (Eds.), Developments in the Analysis of Lipids, Royal Society of Chemistry, Cambridge, UK, 1994, pp. 73–81.
[7] J. Dallüge, J. Beens, U.A.Th. Brinkman, J. Chromatogr. A 1000 (2003) 69.
[8] J.M.D. Dimandja, Anal. Chem. (2004) 167A.
[9] G. Schomburg, J. Chromatogr. A 703 (1995) 309.
[10] P.J. Marriott, P.D. Morrison, R.A. Shellie, M.S. Dunn, E. Sari, D. Ryan, LC.GC Eur. 16 (2003) 23.
[11] R.B. Gaines, G.S. Frysinger, J. Sep. Sci. 27 (2004) 380.
[12] R. Shellie, P. Marriott, P. Morrison, Anal. Chem. 73 (2001) 1336.

[13] J. Wu, X. Lu, W. Tang, H. Kong, S. Zhou, G. Xu, J. Chromatogr. A 1034 (2004) 199.
[14] M.T. Roberts, J.-P. Dufour, A.C. Lewis, J. Sep. Sci. 27 (2004) 473.
[15] J.M.D. Dimandja, Am. Lab. (2003) 42.
[16] J. Dallüge, L.L.P. van Stee, X. Xu, J. Williams, J. Beens, R.J.J. Vreuls, U.A.Th. Brinkman, J. Chromatogr. A 974 (2002) 169.
[17] X. Lu, J. Cai, H. Kong, M. Wu, R. Hua, M. Zhao, J. Liu, G. Xu, Anal. Chem. 75 (2003) 4441.
[18] X. Lu, M. Zhao, H. Kong, J. Cai, J. Wu, M. Wu, R. Hua, J. Liu, G. Xu, J. Sep. Sci. 27 (2004) 101.
[19] M. Adahchour, L.L.P. van Stee, J. Beens, R.J.J. Vreuls, M.A. Batenburg, U.A.Th. Brinkman, J. Chromatogr. A 1019 (2003) 157.
[20] W. Welthagen, J. Schnelle-Kreis, R. Zimmermann, J. Chromatogr. A 1019 (2003) 233.
[21] J. Hamilton, P. Webb, A. Lewis, J. Hopkins, S. Smith, P. Davy, Atmos. Chem. Phys. 4 (2004) 1393.
[22] M.M. Van Deursen, J. Beens, J.C. Reijenga, P.L. Lipman, C.A.M.G. Cramers, J. Blomberg, J. High Resolut. Chromatogr. 23 (2000) 507.
[23] P. Korytar, L.L.P. van Stee, P.E.G. Leonards, J. de Boer, U.A.Th. Brinkman, J. Chromatogr. A 994 (2003) 179.
[24] J. Dallüge, R.J.J. Vreuls, J. Beens, U.A.Th. Brinkman, J. Sep. Sci. 25 (2002) 201.
[25] J. Dallüge, M. van Rijn, J. Beens, R.J.J. Vreuls, U.A.Th. Brinkman, J. Chromatogr. A 965 (2002) 207.
[26] J. Zrostlikova, J. Hajslova, T. Cajka, J. Chromatogr. A 1019 (2003) 173.
[27] M. Harju, A. Bergman, M. Olsson, A. Roos, P. Haglund, J. Chromatogr. A 1019 (2003) 127.
[28] H.J. de Geus, I. Aidos, J. de Boer, J.B. Luten, U.A.Th. Brinkman, J. Chromatogr. A 910 (2001) 95.
[29] R.J. Western, S.S.G. Lau, P.J. Marriott, D. Nichols, Lipids 37 (2002) 715.
[30] L. Mondello, A. Casilli, P.Q. Tranchida, P. Dugo, G. Dugo, J. Chromatogr. A 1019 (2003) 187.
[31] L. Mondello, A. Casilli, P.Q. Tranchida, R. Costa, B. Chiofalo, P. Dugo, G. Dugo, J. Chromatogr. A 1035 (2004) 237.
[32] T. Hyötyläinen, M. Kallio, M. Lehtonen, S. Lintonen, P. Peräjoki, M. Jussila, M.-L. Riekkola, J. Sep. Sci. 27 (2004) 459.
[33] T.T. Truong, P.J. Marriott, N.A. Porter, R. Leeming, J. Chromatogr. A 1019 (2003) 197.
[34] Z. Moldovan, E. Jover, J.M. Bayona, Anal. Chim. Acta 465 (2002) 359.
[35] J. Thewlis, Cosmet. News 106 (1996) 27.
[36] R. Ong, P. Marriott, P. Morrison, P. Haglund, J. Chromatogr. A 962 (2002) 135.
[37] M. Adahchour, J. Beens, R.J.J. Vreuls, A.M. Batenburg, U.A.Th. Brinkman, J. Chromatogr. A 1054 (2004) 47.
[38] SRC, Syracuse Research Corporation, http://www.syrres.com/esc/kowdemo.htm.