DESENVOLUPAMENT DE METODOLOGIES ANALÍTIQUES PER A LA DETERMINACIÓ DE COMPOSTOS ORGÀNICS EN MATRIUS COMPLEXES. APLICACIÓ A L'ESTUARI DEL RIU EBRE

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4. Tècniques multidimensionals

En aquest apartat, s'han desenvolupat metodologies analítiques basades en la cromatografia bidimensional integrada GC×GC. Aquest treball s'ha realitzat en el marc d'una estada al **Analytical Chemistry and Applied Spectroscopy Department** de la **Vrije Universiteit** d'Amsterdam (Holanda), on es va col·laborar amb l'equip investigador del Dr. René Vreuls i del Dr. Udo Brinkman.

La cromatografia bidimensional integrada GC×GC consisteix en connectar en sèrie dues columnes cromatogràfiques de propietats diferents per l'intermediari d'un modulador que permet reinjectar els compostos que elueixen de la primera columna a la segona columna sense perdre la separació obtinguda en el primer sistema cromatogràfic. Els detalls i avantatges d'aquesta tècnica ja han estat a bastament detallats en la secció 1.1.2 d'aquest treball però és interessant tornar-ne a destacar un parell de punts. A més del gran increment de *peak capacity* la tècnica també permet l'obtenció d'estructures ordenades químicament fet que facilita la identificació tant de les diferents famílies químiques ja observades com de nous compostos. Degut a la reduïda amplada dels pics cromatogràfics en la segona dimensió ja s'ha comentat que es necessita emprar detectors que siguin suficientment ràpids per a realitzar-ne el seguiment.

En el primer treball s'optimitza una metodologia basada en l'acoblament a un espectròmetre de masses de temps de vol ToF MS per a caracteritzar la lanolina, que s'utilitza com a referència d'una barreja complexa de lípids. Aquest detector permet d'obtenir una quarta dimensió d'informació que correspon als espectres de masses dels diferents compostos sense les limitacions que encara presenten els quadrupols (reduït interval de masses, manca d'estabilitat dels espectres de masses i velocitat d'escaneig limitada).

En el segon treball s'introdueix per primera vegada el concepte anomenat de la *twin* GC×GC. En l'apartat corresponen se'n presenten les característiques i se'n descriuen les potencialitats. Aquest sistema correspon a l'acoblament en paral·lel de dos sistemes de GC×GC de característiques diferents acoblats a dos FIDs. Aquesta tècnica ens permet obtenir més informació ja que al temps de retenció de les dues dimensions de la GC×GC habitual s'hi afegeixen els temps de retenció de les dues dimensions del sistema en paral·lel i les abundàncies dels dos detectors. La *twin* GC×GC també permet de combinar els beneficis del sistema ortogonal i del sistema no-ortogonal.

4.1 Aplicació de la GC×GC-ToF MS a la caracterització de lípids

En aquest apartat es presenta l'article, *"Characterization of lipids in complex samples using comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry"*, que per primer cop introdueix la tècnica de la GC×GC-ToF MS per a la caracterització de lípids. Com ja s'havia fet per a les tècniques monodimensionals la lanolina ha estat emprada com a matriu de referència degut a la seva complexitat.

En aquest treball es fa palesa la rellevància del procés de derivatització de la mostra en el procés analític. La derivatització, massa sovint, tan sols s'utilitza com un procés per a aconseguir fer cromatografiables substàncies que d'una altre manera no ho serien. Ara bé, realment aquesta etapa, ben utilitzada, pot permetre realitzar un salt qualitatiu important i facilitar-nos molt les fases posteriors de l'anàlisi. En efecte, les tècniques de derivatització poden permetre de derivatitzar grups funcionals específics en funció de l'agent derivatitzant escollit i també poden ajudar a la detecció i identificació dels compostos d'interès. Els compostos derivatitzats poden, d'aquesta manera, presentar una millor resposta amb el detector emprat (obtenint millors LODs i LOQs) i mostrar ions característics (informació estructural) que permetin una més fàcil identificació.

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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).

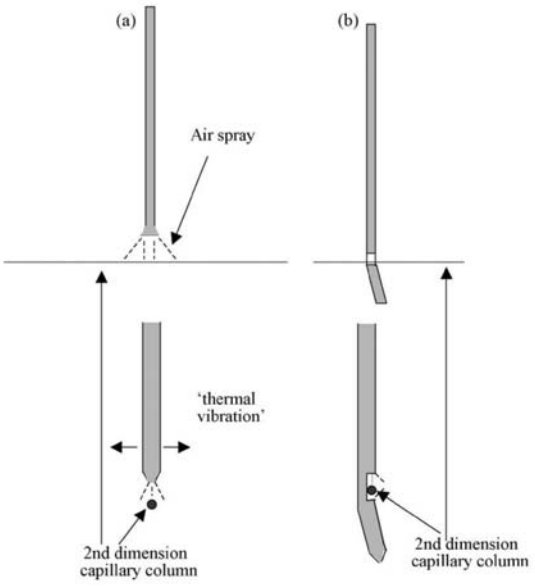

Fig. 1. (a) Original air jet modulator and (b) modification of original air jet modulator (a) to enable proper functioning (no 'thermal vibration') in GC × GC–TOF-MS.

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 ΔR_t being the retention time difference between the two peak maxima and ω_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, (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.

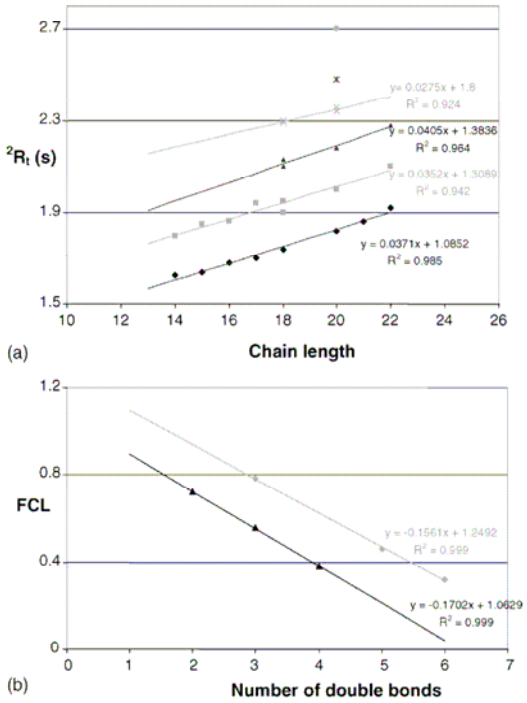

Fig. 2. (a) Dependence of second-dimension retention time (^2R_t) of FAME of different chain length and different number of double bonds (DBs) for column set 1 (♦ 0 DB, ■ 1 DB, ▲ 2 DBS, × 3 DBs, ✳ 4 DBs and ● 5 DBs). (b) Dependence of FCL of different positional isomers as a function of their number 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

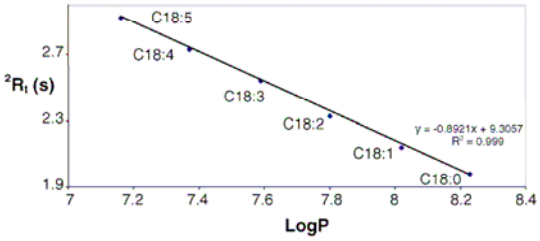

Fig. 3. Correlation between second-dimension retention times (column set 1) of several C_{18} FAME and their log P values.

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.

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.

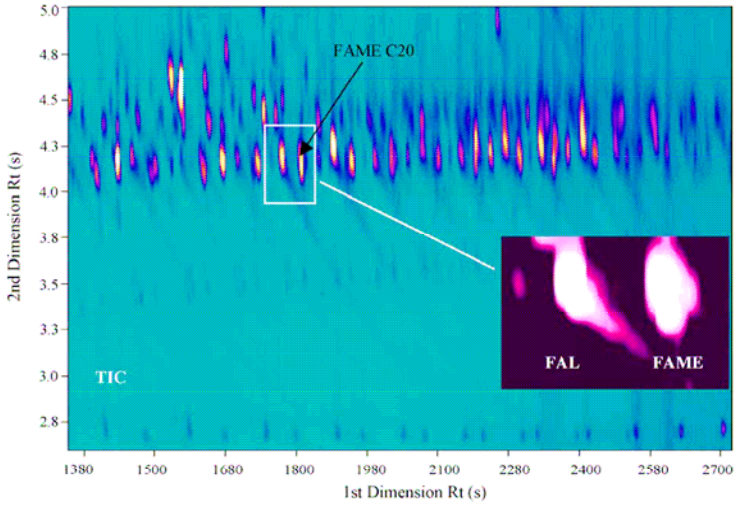

Fig. 4. TIC contour plot obtained by GC × GC–TOF-MS of methylated lanolin. Similar R_{t_2} observed for FAL and FAME. FAL (but not FAME) tailing in first-dimension is shown in insert. Column set 1.

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

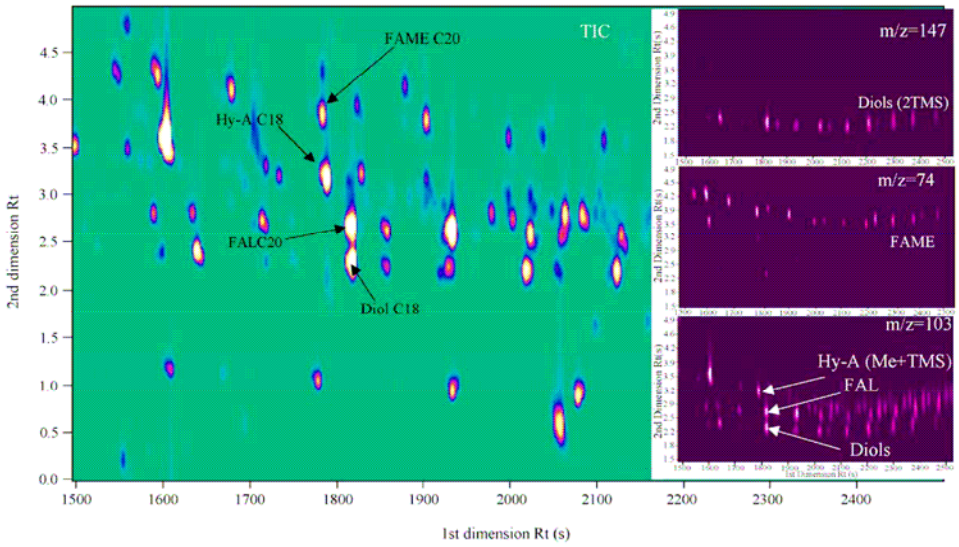

Fig. 5. TIC contour plot obtained by GC × GC–TOF-MS of methylated + silylated lanolin, and inserts of fragmentograms *m/z* 74, 103 and 147. Column set 1.

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.

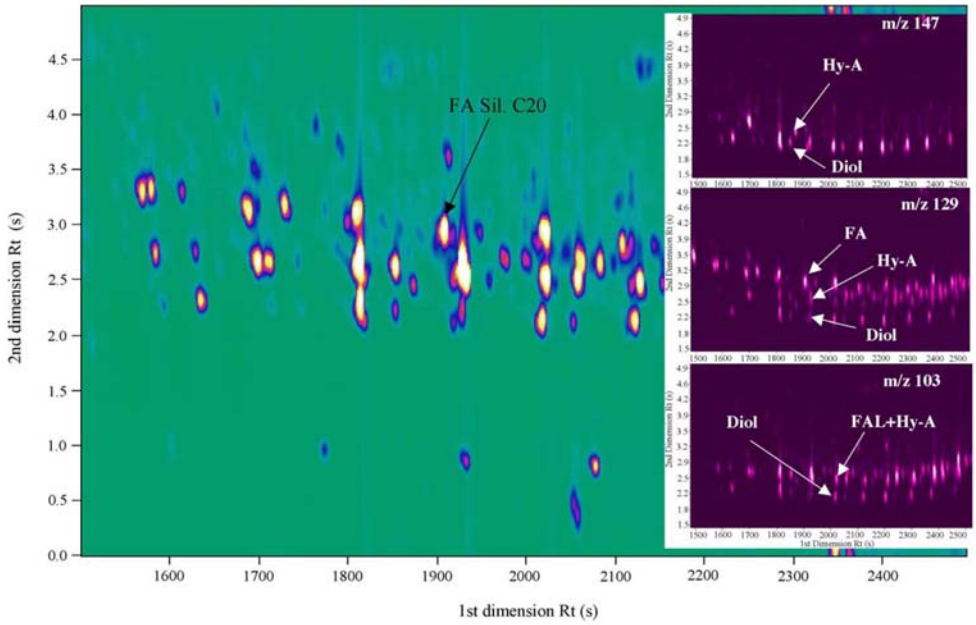

Fig. 6. TIC contour plot obtained by GC × GC–TOF-MS of silylated lanolin, and inserts of fragmentograms *m/z* 103, 129 and 147. Column set 1.

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

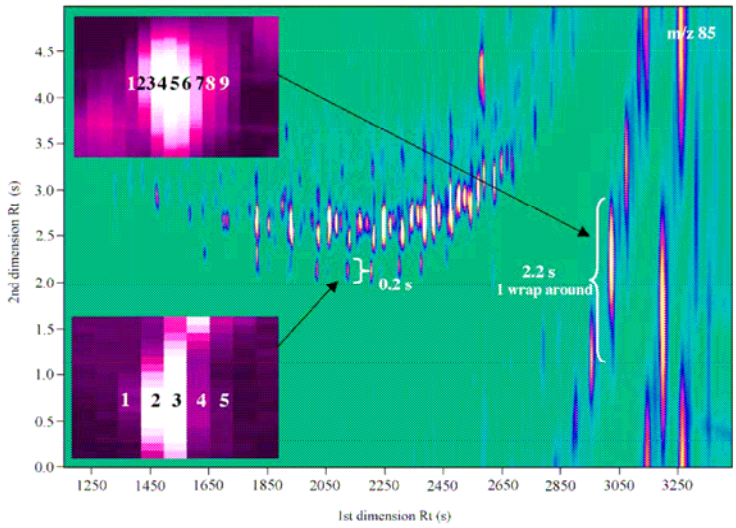

Fig. 7. Contour plot (*m/z* 85) illustrating the differences in peak shape between compounds eluting at low and very high temperatures. Column set 1.

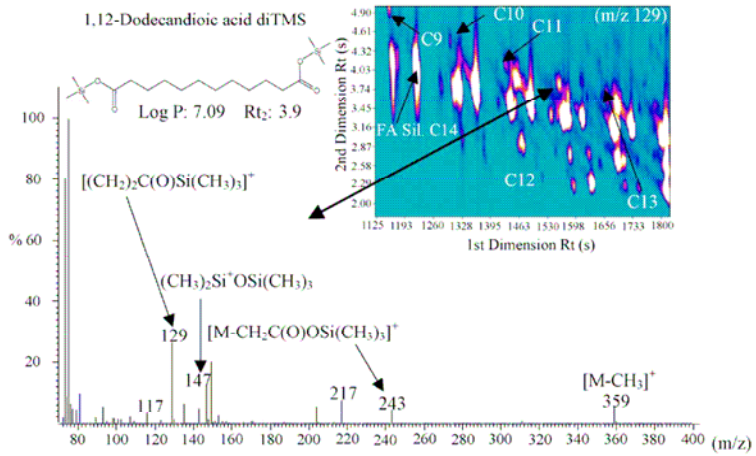

Fig. 8. Contour plot (*m/z* 129) showing the diacids distribution obtained after GC × GC–TOF-MS of a silylated lanolin extract on column set 1 and mass spectrum details of the 1,12-dodecanedioic acid diTMS.

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.

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4.2 *Twin* comprehensive GC×GC

El concepte de *twin* GC×GC s'ha presentat en l'article, *"Twin comprehensive two-dimensional gas chromatographic system: concept and applications."*. Aquesta tècnica permet de combinar la GC×GC ortogonal (primera dimensió apolar – segona dimensió polar) amb la GC×GC no-ortogonal (primera dimensió polar – segona dimensió apolar). D'aquesta manera aconseguim obtenir en un sola anàlisi les estructures químicament ordenades dels dos sistemes. En l'article es descriu tant els aspectes més tècnics d'aquest sistema cromatogràfic com diferents aplicacions que en demostren el seu interès emfatitzant molt especialment la complementarietat de la informació obtinguda pels dos sistemes cromatogràfics en paral·lel.

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Twin comprehensive two-dimensional gas chromatographic system: concept and applications

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Abstract

A twin GC × GC system has been designed which enables the analysis of a sample by means of two different and independent column combinations simultaneously. Both combinations are incorporated in the same oven, using the same temperature programme, and are fed using a 50:50 column-entrance-split. It is demonstrated that, employing combinations of a conventional non-polar × polar and a reversed-type polar × non-polar column set, the information content is as high, and the analytical performance is as good as when using two separate GC × GC systems. That is, there is an appreciable gain of time and a reduction of costs without any loss of quality. The general usefulness of performing, and comparing two mutually different GC × GC runs is further illustrated with FAMEs in olive oil, and pollutants in a sediment sample.

Keywords: Comprehensive two-dimensional gas chromatography; Twin system; Orthogonality; Column combinations; Food extracts

1. Introduction

In the food industry but also in other disciplines, highly complex extracts have to be analysed in order to determine traces of (semi-) volatiles. They are often present at the low-ng/g level, especially those responsible for the odour. One-dimensional capillary gas chromatography (1D-GC) generally does not provide sufficient separation for a complete qualitative, let alone, quantitative, analysis — not even when identification/confirmation techniques such as those based on mass spectrometry (MS) are used. Even after careful sample preparation, such extracts often contain high concentrations of matrix constituents that can easily obscure the analytes of interest. Experience shows that this is frequently true, even though complicated sample preparation techniques such as solvent-assisted flavour evaporation (SAFE), various high-vacuum distillation (HVD) methods, steam distillation and/or fractionation are used in order to create sufficient separation of the analytes of interest from the matrix [1].

To solve problems such as the above, comprehensive two-dimensional gas chromatography (GC × GC) is an extremely useful technique, since it enhances separation of analytes of interest from each other and, more importantly, from the matrix background. In the past few years, GC × GC has been shown to provide the capability to considerably improve the analysis of complex samples [2–4]. However, as demonstrated in recent studies on food analysis, where sample extracts contain many types of semi- and highly polar classes of compounds such as aldehydes, ketones, lactones, acids and alcohols, both orthogonal and non-orthogonal approaches have to be used [5–7]: depending on the polarity of the analytes of interest, either one or the other technique may well fail to give a good-quality GC × GC result. In a previous study the two approaches were used with marked success [6]. Next to better overall chromatographic behaviour and separation of the polar and non-polar analytes, interesting ordered structures, which play such a prominent role in group-type identification, were observed in both instances.

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However, for acquiring the desired information on a variety of sample constituents the use of two separate GC × GC systems is required. We then concluded that combining the two approaches in one system is highly desirable and might improve sample throughput considerably. With such a 'twin' GC × GC system, one may expect that the desired information on all the sample constituents of interest can be obtained in a single GC-run, i.e. in a relatively short time and at acceptable costs. The aim of this study is to show the potential of such a system for the trace-level determination of flavour compounds in food extracts and of pollutants in sediment.

2. Experimental

2.1. Analytes and samples

Three standard mixtures were used in the present study. Mixture 1 (for compound names, see Table 1 below) contained *n*-alkanes in hexane. For quantitative aspect a diesel oil sample was obtained from a local service station. Mixture 2, containing 40 flavour compounds found to be responsible for the odour of olive oil, was dissolved in freshly distilled methyl acetate [6]. All 95–99% pure standards were from the Unilever Research Laboratory, which also provided various olive oil samples and extracts in diethyl ether. High-vacuum degassing (HVD) extraction was used to isolate the volatile flavour compounds from these extracts (see [6]). Mixture 3 containing 37 fatty acid methyl esters (FAMEs), with different chain lengths and number of double bonds, was purchased from Supelco (Supelco Park, PA, USA). In this work, the fatty acids will be designated as $C_{a:bnc}$, where a is the number of carbon atoms, b the number of double bonds and c the position of the first double bond beginning at the methyl terminal group. A sediment sample was taken from a bay in the Southeast of Spain on the Mediterranean coast.

Methylation of fatty acids was done using a 5 mM solution of trimethylsulphonium hydroxide (TMSH) from Fluka (Buchs, Switzerland) in methanol. Equal volumes of reagent and sample were mixed and the mixture was held at room temperature for 30 min. Under these mild conditions, *trans*-esterification of esters is minimal [8].

2.2. Twin GC × GC system

The twin GC × GC system consists of a Hewlett-Packard HP 6890 (Agilent Technologies, Palo Alto, CA, USA) gas chromatograph with a split/splitless injector, a short 20 cm × 0.53 mm i.d. retention gap, a glass Y-shaped press-fit connector and two GC × GC column combinations set up in parallel, with one being orthogonal and the other non-orthogonal. The first column set was a CPSil 8 (Varian-Chrompack, Middelburg, The Netherlands; 10 m × 0.25 mm i.d. × 0.25 μm d_f) × BP20 Wax (SGE Europe, Milton Keynes, UK; 1 m × 0.1 mm i.d. × 0.1 μm d_f) combination, and the second one a BP20 Wax (25 m × 0.32 mm i.d. × 0.25 μm d_f) × BPX35 (SGE Europe; 1 m × 0.1 mm i.d. × 0.1 μm d_f) combination. The second-dimension columns were connected to two FIDs which can produce a digital signal at a rate of 200 Hz and were monitored

Table 1
Determination of *n*-alkanes and flavour compounds with twin GC × GC system using orthogonal (A) and non-orthogonal (B) column combinations[a]

Analyte	R.S.D. (%) (n = 5)				Peak areas (%) for			
	A		B		Standards (R.S.D. (%), n = 5)		Sample (R.S.D. (%), n = 3)	
	^1t_R	^2t_R	^1t_R	^2t_R	A	B	A	B
n-Alkanes								
C_{14}	0.1	0.6	0.3	1.5	14.6 (7)	14.7 (6)	20.3 (7)	20.5 (7)
C_{15}	0.1	0.7	0.2	1.7	14.4 (5)	14.6 (5)	18.9 (6)	18.8 (7)
C_{17}	0.1	0.5	0.2	1.2	14.4 (6)	14.3 (4)	20.3 (6)	20.5 (6)
C_{18}	0.1	0.2	0.3	1.3	14.5 (6)	14.4 (7)	13.6 (7)	13.6 (8)
C_{19}	0.1	0.3	0.3	1.1	14.1 (4)	14.2 (7)	12.3 (5)	12.3 (9)
C_{20}	0.1	0.2	0.3	1.2	14.0 (4)	13.8 (4)	7.6 (5)	7.5 (6)
C_{21}	0.1	0.2	0.2	1.4	13.9 (5)	14.0 (6)	7.0 (6)	6.9 (7)
Flavour compounds								
Octanal	0.1	0.6	0.2	0.5	6.5 (3)	6.4 (6)	6.5 (4)	6.5 (7)
Nonanal	0.1	0.7	0.2	0.6	7.3 (2)	7.2 (7)	25.0 (3)	24.9 (7)
trans-2-Octenal	0.2	0.5	0.1	0.4	11.8 (3)	11.8 (4)	4.7 (3)	4.7 (6)
trans-2-Decenal	0.2	0.2	0.1	0.5	7.0 (4)	7.0 (4)	9.1 (5)	9.1 (5)
trans,*trans*-2,4-Nonadienal	0.1	0.3	0.1	0.5	7.5 (4)	7.5 (3)	4.7 (4)	4.7 (4)
trans,*trans*-2,4-Decadienal	0.1	0.2	0.1	0.6	7.3 (5)	7.4 (5)	7.8 (6)	7.9 (5)
Acetic acid-*cis*-3-hexenyl ester	0.1	0.2	0.2	0.8	12.4 (2)	12.5 (7)	6.3 (4)	6.4 (6)
Cyclohexylacetic acid ethyl ester	0.1	0.2	0.1	0.6	10.3 (3)	10.3 (6)	5.8 (3)	5.8 (7)
1-Octen-3-one	0.2	0.3	0.1	0.4	15.0 (4)	15.0 (3)	15.9 (5)	15.8 (4)
3-Octen-2-one	0.1	0.4	0.1	0.3	14.8 (3)	14.8 (3)	14.2 (4)	14.1 (5)

R.S.D. (%) in brackets.
[a] *n*-Alkanes in diesel oil; flavour compounds in olive oil extract.

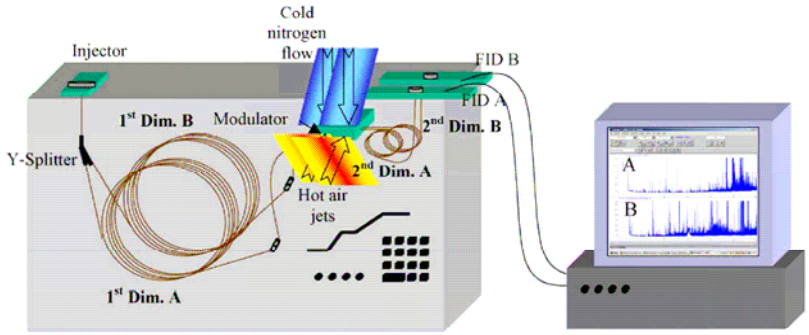

Fig. 1. Schematic of the twin GC × GC system with its two column sets, A and B.

by the same computer using HP Chemstation software (Agilent). One four-jet two-stage modulation system (KT2001) [9] was used to simultaneously modulate on both column sets. The two second-dimension columns are led through the modulation chamber using two-hole ferrules and a short section of each of the columns are placed in parallel close to each other inside the modulator. A schematic of the twin GC × GC system is shown in Fig. 1.

The carrier gas was helium (99.999% purity; Hoekloos, Schiedam, The Netherlands). The temperature of the two GC column sets, which were housed in the same oven, was programmed from 50 °C (2 min hold) to 250 °C (10 min hold) at 5 °C/min. The modulation time was 4 s; the modulator temperature was kept 100 °C below the oven temperature.

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

3. Results and discussion

3.1. Twin system performance

In order to fully profit from the advantages provided by GC × GC, it has recently been recommended to use both orthogonal and non-orthogonal systems because – contrary to earlier belief – next to improved separation and enhanced detectability, structured chromatograms can also be obtained in the latter case [5,6]. Furthermore, to improve sample throughput, it will be advantageous if both approaches can be integrated in one GC system. Therefore, a twin system was built as described in Section 2.2 and shown in Fig. 1.

The twin system was tested using flavour compounds in olive oil extracts. This is a relevant sample type which has complex, but known composition. Compared to a previous study [6], where the orthogonal and non-orthogonal approaches were used separately, similar chromatograms were obtained, and their valuable complementarity was again observed. As an example, the GC × GC chromatograms of the flavour compounds with the less well-known non-orthogonal approach are displayed in Fig. 2. They compare very well

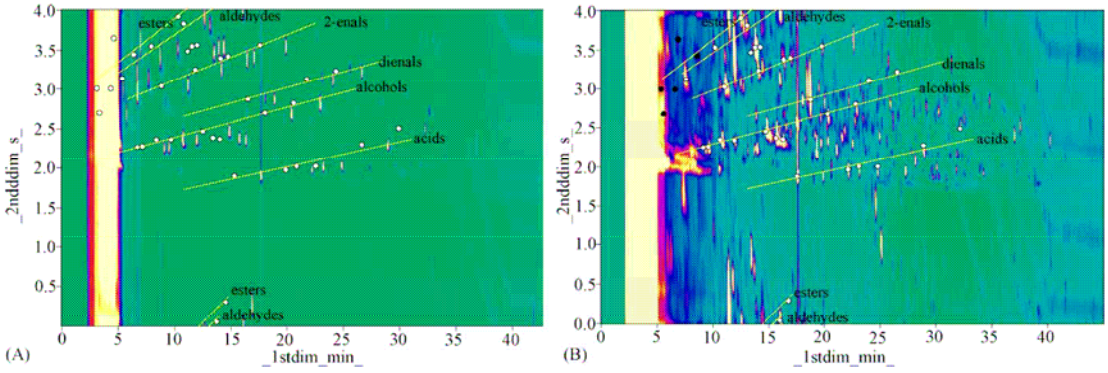

Fig. 2. Non-orthogonal GC × GC-FID chromatograms of 37 olive oil flavour compounds (A) and an olive oil extract (B). White and black spots correspond to non-problematic and problematic flavour compounds, respectively (see text for details). For experimental conditions, see text.

with Figs. 2 and 5 of ref. [6]. The main benefit derived from the non-orthogonal run is the excellent separation of polar analytes such as alcohols and acids. Their tailing and wrap-around phenomena, typically observed in orthogonal GC × GC and extremely problematic not only in crowded regions, but also in non-complex chromatograms [6], has completely disappeared. The fact that, mainly non-polar, analytes eluting close to the solvent peak may cause detection problems in the non-orthogonal approach (Fig. 2B), is not important. These compounds are detected quite easily in the orthogonal run (data not shown). In other words, these preliminary experiments show that, in the twin system, sample throughput can be two-fold increased without any loss of information.

In order to explore the potential and practical usefulness of the twin system in more detail, parameters such as repeatability of retention times and peak areas, which are important for both qualitative and quantitative analysis, were studied. Repeatabilities in both the orthogonal and non-orthogonal systems were calculated as the relative standard deviations (R.S.D.s) of five consecutive injections of the standard mixtures 1 and 2. Results for selected analytes are summarized in Table 1. For all compounds, the R.S.D.s were found to be below 0.3 and 1.7% ($n = 5$) for the first- and second-dimension retention times, respectively.

Table 1 also includes data on peak area measurements. With both standards as well as real-life samples the relative areas of the selected compounds are equal. This indicates that the split at the junction point feeding both column sets does not change during temperature programming. The R.S.D.s for the peak area measurements were also satisfactory: they were below 9% for all compounds. It should be noted that, despite the relative complexity of the twin system, the present results are similar to those reported previously for the two separately used approaches [6]. Similar results were observed for all other analytes. The small differences of the peak areas in the orthogonal versus the non-orthogonal analyses are probably due to the automated integration process. Finally, the split between the two column sets (cf. Fig. 1) is close to 50:50. This is according to expectations because (i) the splitter was designed to affect a 50:50 split, and (ii) the short piece of column (20 cm) between the injector and the splitter will have only minimal influence.

The results presented here allows the conclusion that the twin system can be used for analyte determination and char-

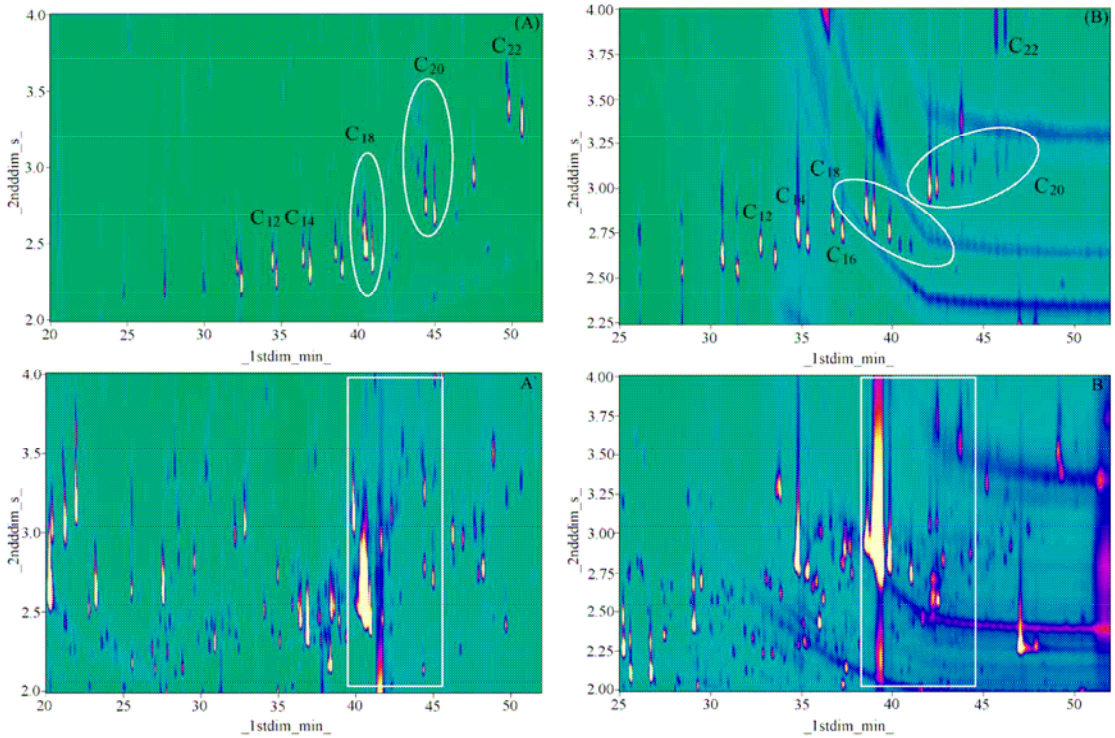

Fig. 3. Twin GC × GC-FID chromatograms of 37 FAME compounds (A, B: orthogonal and non-orthogonal, respectively) and a methylated olive oil extract (A′, B′: orthogonal and non-orthogonal, respectively). The marked C_{18}–C_{20} FAME regions are shown in more detail in Fig. 4. Note that the C_{20} FAs elute in the isothermal part of the chromatogram.

acterisation of complex mixtures. These aspects will be discussed in the next section.

3.2. Applications

3.2.1. FAMEs in olive oil

Next to the fundamental role they play regarding maintaining good health, fatty acids (FA) are very important in olive oil analysis as they are used to detect oil adulterations, i.e., to identify the cultivar and its origin [10]. A large number of FAs, with different chain lengths and different degrees of unsaturation, are present in olive oil. As is usual, we analysed them as FAMEs after methylation of an olive oil extract. In these samples, FAs with even carbon numbers are dominant, while odd-numbered FAs are present as minor components. Because of this, and also because of the general complexity of the FA profile in oils, the odd-numbered FAs often remain undetected in 1D-GC. Actually, even in GC × GC analysis, rather serious problems are encountered [11]. Consequently, it is of interest to use a twin GC × GC system with its two mutually different, but simultaneously run, chromatograms as final outcome. As an example, Fig. 3 shows 'the twin chromatograms' of the FAME standards and of an olive oil extract. Next to the considerably improved overall separation due to the use of a comprehensive 2D-separation technique, the differently ordered structures are clearly visible.

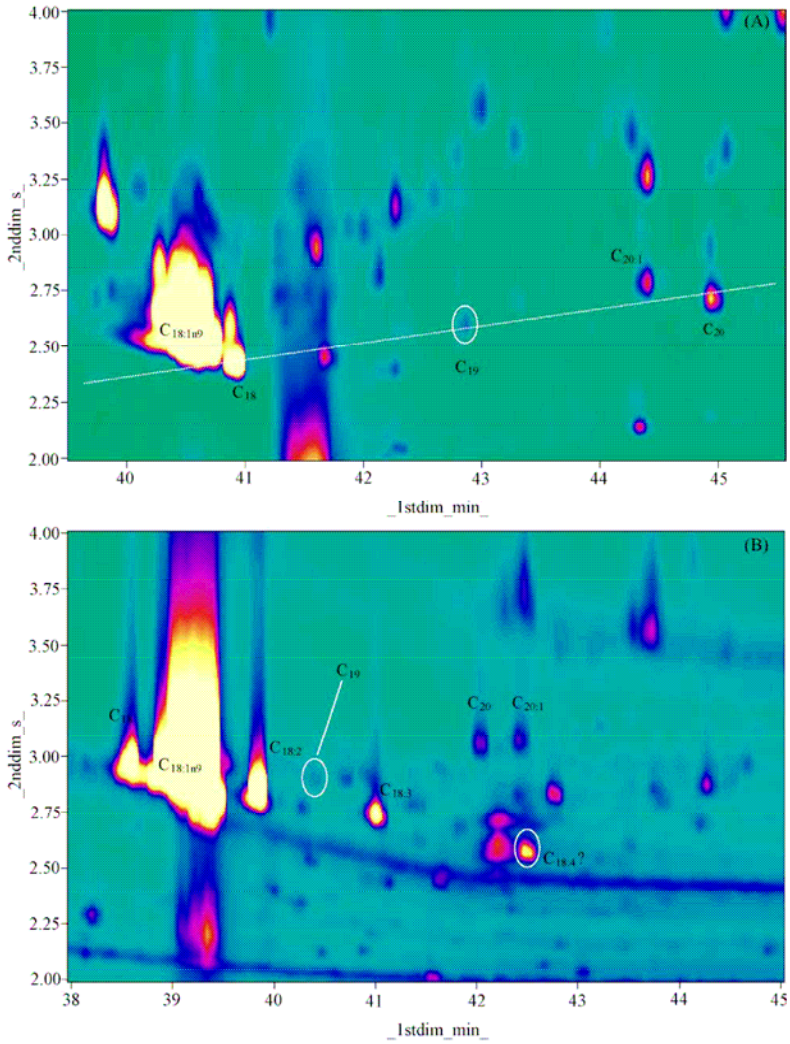

Fig. 4. Details of C_{18}–C_{20} regions of the olive oil extract marked in Fig. 3: A, orthogonal; B, non-orthogonal. Note that, for reasons of convenience, the rectangle dimensions differ from those in Fig. 3.

As regards the general separation, some further improvement would have been possible by optimising the temperature programme. This rather time-consuming operation was not performed because the main goal was to demonstrate the applicability of the system.

In the present instance, the orthogonal separation appears to be superior to its non-orthogonal counterpart. However, also here there is valuable information that can be derived from the non-orthogonal approach. As an example, Fig. 4 gives blow-ups of the C_{18}–C_{20} areas of the olive oil extract marked in Fig. 3; note that the rectangle dimensions (marked areas) in the former figure differ from those in the latter one. Quantitatively, the C_{18} fatty acids are the main constituents of this sample, with an over 85% contribution on the basis of FID-based peak area measurements. The most abundant single constituent is $C_{18:1n9}$ (>75%) which is, therefore, overloading both dimensions of both GC × GC sets. As a consequence, the $C_{18:1n9}$ peak obscures minor peaks, including target compounds such as $C_{18:2}$ and $C_{18:3}$ which virtually co-elute in the first-dimension column of the orthogonal approach (Fig. 4A). With the twin system, this problem is easily solved in the alternative run: now, they are nicely separated in the first dimension and display fairly satisfactory peak shapes (Fig. 4B). It is even possible to provisionally identify $C_{18:4}$. Rather concentrated extracts had to be injected to enable the detection of the minor

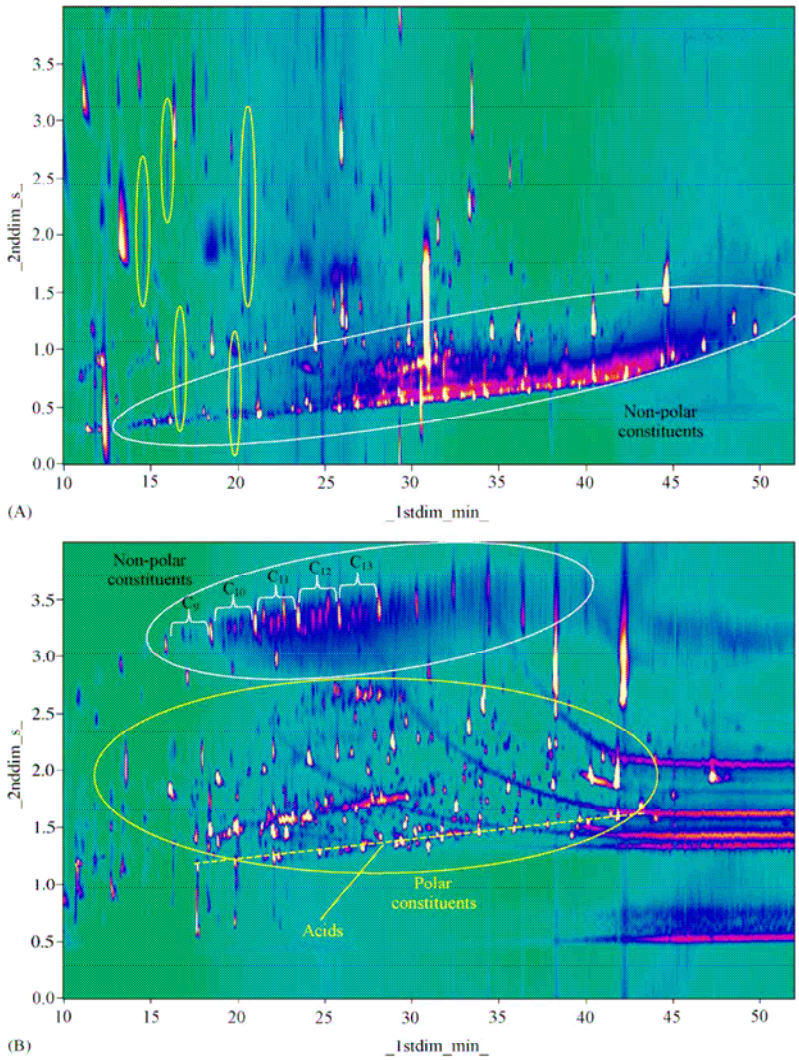

Fig. 5. Twin GC × GC-FID chromatogram of a marine sediment extract: A, orthogonal; B, non-orthogonal. For more details, see text.

FAMEs. One beneficial result of this procedure is the detection of the faint spot at $^1t_R/^2t_R$ of 42.9 min/2.6 s in Fig. 4A. Interpretation on the basis of retention times and ordered structures allows its provisional identification as C_{19}, a fatty acid not included in several recent studies [7,12]. By using proper shading techniques (cf. Jens et al.), the C_{19} spot could also be observed in the non-orthogonal GC × GC chromatogram (Fig. 4B). Further confirmation will require the use of mass spectrometric detection and/or a standard becoming available.

Finally, one should note that the stability of the stationary phases is also important: column bleed of the first column will also be modulated and may cause system peaks in the second-dimension chromatograms [6,13], and co-elution with target compounds will adversely affect their detectability. Polar stationary phases such as, e.g., Carbowax suffer more from this problem than non-polar ones such as, e.g., CPSil 8. If these phases are used in the first-dimension column, as is the case in the non-orthogonal approach, bands of modulated bleed material may show in the contour plots. This is clearly seen in Figs. 3 and 4; the Carbowax column of the non-orthogonal approach starts to bleed at a temperature of about 200 °C (Figs. 3B, B′, and 4B), while such bleeding does not occur in the case of CPSil 8 (Figs. 3A, A′, and 4A). Fortunately, this did not cause any real problems in the present study.

3.2.2. Sediment analysis

Sediment samples can be quite complex, especially if taken from a bay in which much shipping activity exists — and even more if in addition, wastewater treatment plants discharge into it. In such a case, a large number of pollutants widely different in polarity and chemical nature, can be expected to be present [14]. As an example, Fig. 5 shows twin GC × GC-FID chromatograms of such a marine sediment extract. The constituents of the extract are seen to be separated into two main groups. One group, with mainly non-polar analytes which are circled in Fig. 5, contains linear alkylbenzenes (LABs). In the orthogonal run, they are found at ^2t_R of ca. 0.5 s and show severe overlap. As a consequence, their ordered structure is obscured and cannot be seen in the contour plot. In the non-orthogonal run, however, the LAB are grouped at 3.0–3.5 s second-dimension retention times, and their ordered structure is clearly visible. As an illustration, the C_9–C_{13} LAB homologous series is indicated in Fig. 5B.

In both Fig. 5A and B, the more polar compounds take up the larger part of the GC × GC plane. While they are rather scattered (at ^1t_R = 15–20 min) in the orthogonal run, as exemplified by the linear carboxylic acids marked out in Fig. 5A, with wrap-around phenomena causing overlap and adding to identification problems — they are nicely grouped at ^2t_R = 1.0–2.5 s, without any wrap-around, in the non-orthogonal approach of Fig. 5B. The dashed line indicates the small and intense spots of the acids: contrary to what is observed in Fig. 5A, neither qualitative nor quantitative analysis will now cause any real problems.

4. Conclusions

It has previously been demonstrated that performing a complementary set of analyses on both a conventional (non-polar × polar) and a reversed (polar × non-polar) column combination yields higher-quality results than a single GC × GC analysis. Especially for, samples containing polar analytes such an approach yields more information for the entire sample. With the twin GC × GC system presented in this study, these complementary sets of results can be acquired simultaneously — without any loss of quality compared with the earlier technique of using two separate instruments. In other words, there is a considerable time gain and, thus, an increased sample throughput. The various examples shown in the introductory [6] as well as the present paper moreover demonstrate that the 'twin approach' is valuable for many different classes of compounds and/or sample types.

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4.3 Discussió de resultats

La GC×GC ha permès a les tècniques multidimensionals realitzar un salt qualitatiu molt important No obstant això, dels resultats obtinguts en aquest apartat, és interessant ampliar alguns trets rellevants. Dels sistemes de columnes provats, la columna de 50 μm ID és la més innovadora tot i que que la utilització d'aquestes columnes de petit diàmetre en GC×GC ja s'havia realitzat [Adahchour *et al.*, 2003]. Per analitzar els resultats obtinguts és interessant referir-se a un treball excel·lent, que avaluava els efectes de la pressió del gas portador en el número de plats cromatogràfics d'un sistema de GC×GC i on es veu que el model clàssic de les corbes de van Deemter de la 1DGC no pot ser immediatament extrapolat a la GC×GC [Beens *et al.*, 2005]. Normalment, s'hauria d'esperar d'una columna de 50 μm ID més resolució cromatogràfica degut a que es redueix l'alçada teòrica del plat cromatogràfic i aquest fet hauria de compensar la menor longitud de la columna (0.5 m × 50 μm ID (*column set 2*) *vs* 1 m × 100 μm ID (*column set 1*)) (Taula 1 pàgina 108 [Jover *et al.*, 2005]). No obstant això, varem obtenir millor resolució cromatogràfica amb la columna de 100 μm ID que amb la de 50 μm ID (Taula 2 pàgina 109 [Jover *et al.*, 2005]). Encara que la fase estacionària no és la mateixa i per tant, la selectivitat hi entra en joc, aquests resultats, poden ser explicats per la velocitat lineal del gas portador en la segona dimensió que s'allunya molt de l'òptim. En efecte, en el *column set* 2, la velocitat lineal, degut al menor diàmetre, és 25 cops superior en la segona dimensió que la velocitat lineal en la primera dimensió. De fet aquest comportament coincideix amb el que descriu Beens (Taula 3), on s'observa que la columna de 50 μm ID té un número de plats inferior (3800) a la de 100 μm ID (7100).

Taula 3. Extreta de [Beens *et al.*, 2005].

Comparison of performance parameters of six GC × GC column-dimension combinations operated at the optimum flow settings of the first-dimension column

Dimensions of second-dimension column[a] (m × mm I.D., μm d_f)	p_{in} (kPa)	p_{mid} (kPa)	$^1\bar{u}$ (cm s^{-1})	$^2\bar{u}$ (cm s^{-1})	^1N	^2N	$^1N/s$ (s^{-1})	$^2N/s$ (s^{-1})	$^2t_R/^1\sigma$
0.5 × 0.05, 0.05	655	625	8	310	131 000	3800	360	23 300	0.2
1.0 × 0.10, 0.10	305	240	19	180	130 000	7100	820	12 900	1.3
	210[b]	*170*[b]	*11*[b]	*95*[b]	*91 500*[b]	*10 500*[b]	340[b]	*10 150*[b]	*1.2*[b]
1.8 × 0.18, 0.18	235	135	28	85	127 000	10 000	1190	4800	7.0
	210[b]	*130*[b]	*23*[b]	*70*[b]	*123 400*[b]	*10 600*[b]	930[b]	*3800*[b]	*7.3*[b]
2.5 × 0.25, 0.25	225	115	31	50	126 000	10 700	1280	2120	18
	280[c]	*120*[c]	*39*[c]	*75*[c]	*126 000*[c]	*9600*[c]	*1460*[c]	*2830*[c]	*14*[c]
	320[c]	*125*[c]	*42*[c]	90[c]	*126 000*[c]	*8600*[c]	*1420*[c]	*3050*[c]	*11*[c]
3.2 × 0.32, 0.32	215	110	31	30	125 000	10 300	1240	930	38
5.3 × 0.53, 0.53	215	100	31	10	125 000	8200	1290	175	175

[a] First-dimension column, 30 m × 0.25 mm I.D. × 0.25 μm d_f. For abbreviations and symbols, see Fig. 1 and Section 2.
[b] Performance parameters of the column set operated at the optimum flow settings of the second-dimension column.
[c] 33.6 and 37.3 m First-dimension columns operated slightly above their optima to keep ^1N constant at 126 000. For details, see text.

En aquest aspecte una de les conseqüències de la utilització de la GC×GC és que no es pot estar treballant en condicions òptimes de velocitat lineal en les dues dimensions simultàniament. Això es pot veure bé a la Figura 8 on els màxims en número de plats per a la primera i la segona dimensió corresponen a pressions en cap de columna molt diferents Així doncs, és necessari, en funció de l'aplicació, triar unes condicions que permetin optimitzar la separació o bé en la primera dimensió o bé en la segona. Finalment, cal destacar que quan menys diferència hi hagi entre el diàmetre intern de les dues dimensions menys allunyats estaran els òptims respectius. Per tant, en el nostre cas s'han obtingut millors resultats quan la columna de la segona dimensió era una 100 μm ID. Així, amb les columnes de 50 μm ID obtenim separacions més ràpides però amb una pitjor resolució. El següent pas seria de disminuir el diàmetre intern de la primera dimensió a fi i efecte de que els óptims de les velocitats lineals d'ambdues columnes sigui el més proper possible.

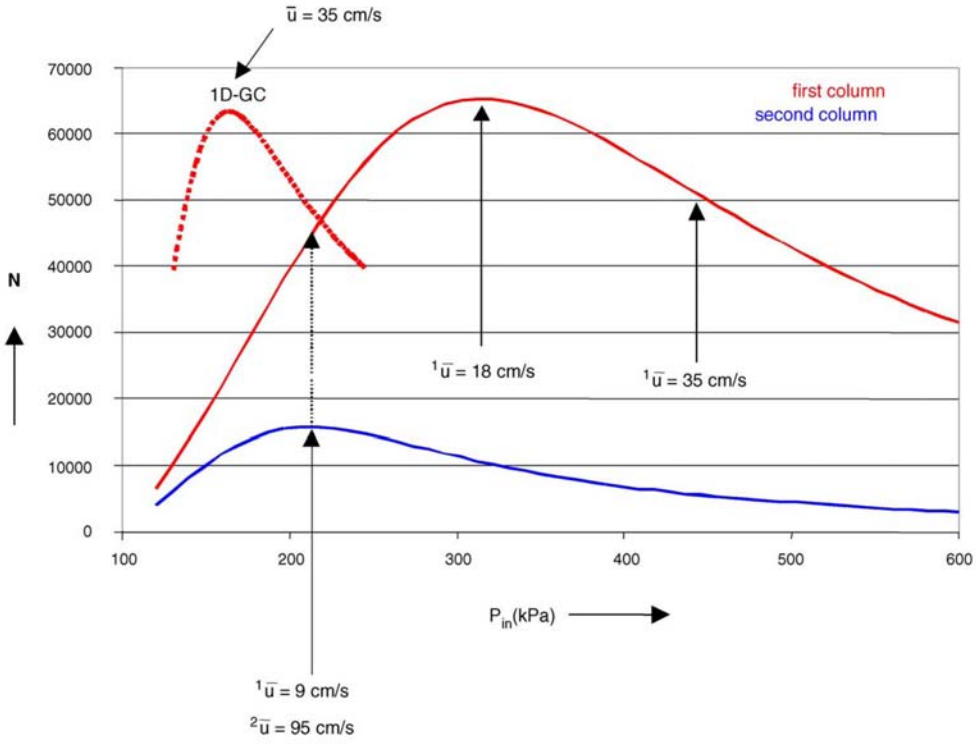

Fig. 3. N vs. p_{in} curves for (15 m × 0.25 mm I.D., 0.25 μm d_f) × (1.5 m × 0.1 mm I.D., 0.1 μm d_f) column combination. Carrier gas, helium. The red line is the curve for the first, and the blue line that for the second-dimension column. The dotted red line is the curve for the first column when used as a 1D-GC column. The arrows depict the points where the average linear velocities in the first column were calculated as indicated.

Figura 8. Extreta de [Beens *et al.*, 2005].

En el mateix article es comenta que una altre de les limitacions de les columnes de 50 μm és la poca capacitat d'introducció de mostra però això tan sols és cert si es treballa en una dimensió. La capacitat d'introducció de mostra és proporcional a d^3, sent d el diàmetre intern de la columna, i per tant passant d'una columna de 250 μm ID a una de 50 μm, la capacitat de mostra en 1DGC es veu reduïda d'un factor 125. Però en GC×GC, no tota la mostra injectada en el sistema arriba simultàniament a la segona dimensió, cal recordar que cada pic cromatogràfic està modulat entre 4 i 5 cops i que es solucionen la majoria de coelucions, per això cal matisar aquesta limitació. Resumint, la capacitat d'introducció de mostra en GC disminueix molt significativament al disminuir el diàmetre intern de la columna, no obstant, aquesta limitació és més important en 1DGC que no en GC×GC.

En la introducció de la secció 4.1 i en la introducció general ens referíem a la importància que poden tenir els processos de derivatització en les metodologies analítiques. Aquest fet es veu perfectament reflectit en el treball presentat on depenent de la metodologia emprada s'aconsegueix una millor o pitjor separació en la segona dimensió. El fet que, com s'ha demostrat, la retenció en la segona dimensió depengui de les propietats fiscoquímiques dels compostos d'interès i més concretament del seu log K_{OW} fa que es pugui preveure l'efecte de l'agent derivatitzant en funció de veure com modifica el log K_{OW} dels compostos d'interès.

En aquest apartat, també s'ha observat la qualitat deficient per les masses grans dels espectres obtinguts amb el ToF MS Pegasus II (LECO, St. Joseph, MI, USA). Però, sembla ser que aquest problema, ja ha estat corregit en la última versió de l'aparell (Pegasus IV) que utilitza un sistema de calibració en continu. Això hauria de permetre d'aprofitar millor les potencialitats de l'equip pel que fa la identificació i quantificació automàtica dels compostos desconeguts.

Cal comentar també que s'ha observat que quan treballem en isoterma tenim un eixamplament del pic en les dues dimensions (Figura 7 pàgina 113 [Jover *et al.*, 2005]). L'eixamplament en la primera dimensió és responsable de que els pics cromatogràfics siguin modulats més cops perdent així sensibilitat. L'eixamplament en la segona dimensió provoca el fenomen dels *wrapped around* (temps de retenció en la segona dimensió superior al temps de modulació). Aquest fenomen, provoca també una pèrdua de sensibilitat i augmenta el risc de coelucions. Actualment no es pot solucionar aquest problema, però hi han dues possibles vies de treball. La primera consistiria a millorar la estabilitat tèrmica de les columnes cromatogràfiques fet que permetria d'eluir tots els

compostos d'interès en la part programada de la rampa de temperatures. Així, s'evitaria l'eixamplament dels pics cromatogràfics. La segona opció consistiria en poder anar variant el temps de modulació durant el programa cromatogràfic. A l'arribar a l'isoterma final, el temp de modulació s'aniria perllongant fins modular 4 o 5 cops els pics, més amples, que eluiríen de la primera dimensió. Aquest fet també minimitzaria els *wrapped around* encara que no evitaria una pèrdua de sensibilitat deguda a l'eixamplament dels pics cromatogràfics a la segona dimensió.

El desenvolupament del sistema de Twin GC×GC, presentat a l'article de la secció 4.2, representa combinar els avantatges dels sistemes ortogonals amb els dels sistemes no-ortogonals. Quan es treballa en GC×GC s'acostuma a triar la primera dimensió com sent la que es triaria si s'estigués realitzant la determinació per 1DGC. Així, per exemple, si volguéssim determinar compostos polars es triaria una primera dimensió polar. El problema sorgeix quan es desitgen analitzar simultàniament compostos de característiques fisicoquímiques diferents. És en aquest cas que el sistema de Twin GC×GC permet obtenir, al mateix temps, resultats cromatogràfics adequats per compostos de diferents polaritats.

Un dels altres avantatges de la Twin GC×GC que cal destacar és el fet que permet de confirmar la identificació dels diferents compostos sense necessitat d'emprar una tècnica de MS. En efecte com ja s'ha comentat tant en el sistema ortogonal com en el sistema no-ortogonal s'obtenen estructures químicament ordenades. Si un compost s'identifica emprant un dels sistemes cromatogràfics com sent el membre d'una família química (tenint el temps de retenció en les dues dimensions que el fan aparèixer en la estructura química d'aquesta família), per a confirmar la seva identitat, en l'altre sistema cromatogràfic haurà d'haver-hi la mateixa correspondència. De fet, en aquest cas, s'està utilitzant l'alternativa que sempre s'ha tingut per a confirmar la identificació d'un compost que és analitzar-lo en una columna de polaritat diferent.

S'ha comentat que un dels defectes de la GC×GC era la complexitat del tractament de dades que implica. Aquest mateix defecte es veu augmentat en el cas de la Twin GC×GC ja que estem generant el doble d'informació.

Però potser, el defecte més important d'aquesta tècnica és la seva pèrdua de versatilitat, ja que sumem les limitacions dels dos sistemes cromatogràfics. En aquest sentit, la temperatura màxima que podem assolir ens ve marcada per la columna cromatogràfica que tingui una menor estabilitat tèrmica entre les quatre de que està format el sistema. També, cal considerar que el sistema està constituït d'un sol

modulador, tenint doncs, de ser el mateix temps de modulació en els dos sistemes. Això, fa més difícil de mantenir un bon número de modulacions per pic cromatogràfic (l'amplada de pic de la primera dimensió serà diferent per a cada sistema) i els *wrapped-around* són menys evitables (els temps de retenció en la segona dimensió també seran diferents per a cada sistema).

4.4 Conclusions

Les tècniques multidimensionals permeten obtenir una resolució cromatogràfica més gran que les tècniques monodimensionals encara que impliquen una major complexitat del sistema cromatogràfic i una menor robustesa. En aquesta Tesi Doctoral s'ha desenvolupat una metodologia per a la caracterització de barreges lipídiques complexes, emprant la lanolina com a matriu de referència. Com a resultat d'aquest desenvolupament metodològic cal destacar els punts següents:

- S'han avaluat diferents sistemes de columnes obtenint els millors resultats en emprar una columna de 5% fenilpolidimetil siloxà (10 m 0.25 mm ID) com a primera dimensió i una BGB-WAX polietilè glicol (0.5 m 0.10 mm ID) com a segona dimensió.
- S'ha desenvolupat un sistema de modulació per aire que permet modular correctament els compostos amb un punt d'ebullició superior al del FA C_{14}.
- S'ha constatat que un sistema de derivatització seqüencial basat en una metilació seguida d'una sililació, permet obtenir una bona separació de les diferents famílies químiques analitzades.
- La tècnica desenvolupada s'ha aplicat a la caracterització de diferents famílies químiques de lípids (FAs, FALs, hidroxiàcids, diols i diacids).
- Degut a les millores obtingudes en la resolució cromatogràfica i en el soroll s'han pogut identificar per primera vegada a nivell molecular els diacids en una mostra de lanolina.

El sistema de *twin* GC×GC desenvolupat en aquesta memòria permet combinar les propietats dels sistemes de GC×GC ortogonal i no-ortogonal. Del treball realitzat cal destacar els punts següents:

- S'ha concebut un sistema de twin GC×GC emprant dos FIDs per a la detecció i utilitzant un sistema de 2 fases quatre jets KT2001 com a modulador.

- El sistema desenvolupat ha demostrat tenir una bona repetitivitat tant pel que fa als temps de retenció en les dues dimensions com en el tractament quantitatiu de les dades.
- La utilitat d'aquesta tècnica s'ha demostrat en diferents aplicacions (fragàncies en olis d'oliva, FAMEs en olis d'oliva i en la caracterització dels contaminants orgànics de sediments).