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

Eric Jover

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3. Tècniques monodimensionals

3.1. Caracterització de lípids

En aquest apartat s'han desenvolupat vàries metodologies analítiques monodimensionals per a la caracterització de diferents classes de compostos químics presents a la lanolina. Els dos primers treballs tracten dels FFA, on el primer també s'ocupa dels FALs, diols i hidroxiàcids. En el tercer i el quart treball es presenta, respectivament, la caracterització dels èsters de la lanolina alifàtics i esteroidals.

En cada cas, s'han desenvolupat les metodologies tenint molt presents les característiques fisicoquímiques dels compostos d'interès i de la matriu. Així, per a l'anàlisi de VFAs hem emprat com a tècnica de preconcentració la micro-extracció en fase sòlida en espai de cap (HS-SPME). Aquesta metodologia tan sols extraurà els compostos volàtils de la barreja lipídica que estan en equilibri amb la mostra escalfada. De fet una metodologia per a la determinació de VFAs per SPME en aigües residuals ja s'havia desenvolupat abans al nostre laboratori [Abalos *et al.*, 2000] i es va adaptar a les característiques particulars de la matriu que ens ocupa. Pel que fa referència a la caracterització dels FAs, hem realitzat una saponificació, com a pas previ a la seva determinació, a fi de tenir extractes enriquits en aquests anàlits i així poder identificar els components minoritaris. Aquest pas també ens ha permès de trencar els compostos de massa molecular més elevada que no s'haurien eluït en el sistema de GC-MS utilitzat. Pel que fa a la determinació d'èsters alifàtics, es va realitzar un fraccionament emprant cromatografia d'exclusió estèrica molecular recuperant tan sòls la fracció que conté els compostos de massa molecular més elevada (fracció enriquida en èsters). D'aquesta manera, s'aconsegueix evitar les interferències de compostos més lleugers, però més polars com poden ser els FALs o els FFAs.

En aquest apartat s'ha treballat exclusivament amb acoblaments cromatografia de gasos espectrometria de masses, degut a la gran complexitat de la matriu. S'han emprat analitzadors quadrupolars, però amb diferents tipus de ionització: impacte electrònic i ionització química positiva i negativa. En ionització química, s'han utilitzat diferents gasos reactius (amoníac, metà i isobutà). Encara que aquestes tècniques de MS permetin obtenir molta informació de la mostra, ha estat necessari utilitzar índexs cromatogràfics per a completar la seva caracterització a nivell isomèric. En efecte, els isòmers posicionals *iso* i *anteiso* presenten espectres de masses similars per la qual cosa es necessita d'aquests índexs per a poder-los diferenciar amb facilitat. En aquest cas,

s'han utilitzat l'índex d'equivalència de longitud de cadena (*ECL*) i l'índex de longitud de cadena fraccional (*FCL*). Aquests índexs similars als desenvolupats per Kovats [Kovats, 1958] són emprats usualment en l'anàlisi de lípids i especialment de FFAs [Christie, 1988]. Les seves expressions són les següents.

$$ECL_x = n + \frac{Rt_x - Rt_n}{Rt_{n+1} - Rt_n} \qquad \text{i} \qquad FCL_x = \frac{Rt_x - Rt_n}{Rt_{n+1} - Rt_n}$$

on x és el compost d'interès, n el número de carbonis del compost lineal eluint just abans x, Rt_x el temps de retenció del compost d'interès, Rt_n el temps de retenció del compost lineal eluint just abans x i Rt_{n+1} el temps de retenció del compost lineal eluint just després. Així doncs, tot considerant les informacions dels espectres de masses i dels índexs de retenció es pot caracteritzar aquestes diferents famílies químiques arribant fins i tot a diferenciar els isòmers posicionals.

L'anàlisi d'èsters és d'especial complexitat degut a l'elevada massa molecular d'aquestes substàncies i a la gran quantitat d'isòmers potencials que resulten de la combinació d'un fragment àcid i d'un fragment alcohòlic. Pel que fa a la lanolina, el fragment alcohòlic pot estar format tant per un alcohol gras de diferent longitud com per un esterol (colesterol, lanosterol i dihidrolanosterol). Degut a les diferències de comportament entre aquestes dues famílies de compostos (èsters alifàtics i èsters esteroidals), s'han hagut de desenvolupar metodologies cromatogràfiques específiques per a la seva determinació. Cal destacar sobretot el fet que els èsters de colesterol són termolàbils, la qual cosa dificulta la seva anàlisi per GC. Així doncs, per tal d'evitar llur degradació tèrmica, s'ha decidit analitzar els èsters d'esterol mitjançant la tècnica de la GC a pressió sub-ambient ja que permet eluir els compostos d'interès a temperatures inferiors. En el cas dels èsters alifàtics i vist que no tenim aquesta limitació, s'ha decidit analitzar aquests compostos treballant a alta temperatura (HTGC-MS).

3.1.1 Àcids grassos i altres compostos polars

El primer article *"Gas chromatographic and mass spectrometric methods for the characterisation of long-chain fatty acids. Application to wool wax extracts."* va estar realitzat amb la col·laboració del Dr. Z. Moldovan del National Institute for Research and Development for Isotopic Molecular Technologies de Cluj (Rumania). En aquest treball es realitza una revisió de les metodologies analítiques emprades per a la

caracterització de FFAs i es presenta la caracterització dels FFAs, FALs hidroxiàcids en la lanolina.

El segon article *"Volatile fatty acids as malodorous compounds in wool scouring water and lanolin. Origin and characterisation."* presenta una caracterització dels VFAs ($C<C_7$) de les aigües de rentat de la llana i de la lanolina emprant una metodologia específica per a compostos orgànics volàtils (HS-SPME).

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Gas chromatographic and mass spectrometric methods for the characterisation of long-chain fatty acids Application to wool wax extracts

Z. Moldovan [a,b], E. Jover [b], J.M. Bayona [b,*]

[a] *National Institute for Research and Development for Isotopic and Molecular Technologies, P.O. Box 700, 3400 Cluj-Napoca 5, Romania*
[b] *Instituto de Investigaciones Químicas y Ambientales de Barcelona (C.S.I.C), E-08034 Barcelona, Spain*

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Abstract

A review of the existing methods for the separation, identification and quantification of fatty acids mainly by chromatography and mass spectrometry (MS) techniques is presented. Moreover, a method for the simultaneous determination of the following lipidic classes, which are constituents of the wool wax, namely fatty acids, fatty alcohols, 2-hydroxy fatty acids and 2-hydroxy fatty alcohols have been developed. Every compound class consist of normal-, *iso*- and *anteiso*-branched chain isomers. The separation of all molecular species, in the range of carbon atom number C_8–C_{33} was achieved on a low polar capillary gas chromatograph (GC) column. Compound identification has been made by interpretation of mass spectra of mono- and di-tandem mass spectrometry (TMS) derivatives as well as on the basis of equivalent (ECL) and fractional chain length (FCL) retention parameters. The relative abundance of every compound class has been obtained as a function of carbon atom number.

Keywords: Wool wax; Lanolin; Fatty acids; Fatty alcohols; Hydroxy fatty acids; Hydroxy fatty alcohols; Equivalent chain length (ECL); Fractional chain length (FCL)

1. Introduction

The aliphatic fatty acids (FAs) are an important class of naturally occurring compounds. They are essential parts of most of living cells and cellular fluids [1–7] and can be present as free fatty acids (FFAs), mono- or di-esters, mono-, di-, or triglicerides or phospholipids [8–16].

Many living organisms such as bacteria, sponges and certain plants possess the capability of synthesising a variety of structurally modified FAs [6,17]. Each of these has a distinctive FA profile and, therefore, the FAs are of interest by their utility for characterising the organisms, studies on lipid metabolism and the effect of environmental factors [9,17,18]. The diversity and abundance of FAs among living organisms are very useful for classifying and determining biological interactions between organisms [19].

Living organisms have a diverse structure and quantity of both free and complex lipids. Therefore, the determination of specific FAs from a natural source is difficult because acids must be isolated from complex matrices.

A variety of analytical techniques have been used for the characterisation of FAs. Classical methods include techniques such as thin layer chromatography

* Corresponding author. Tel.: +34-932045904; fax: +34-934006119.
E-mail address: jbtqam@iiqab.csic.es (J.M. Bayona).

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(TLC), high performance liquid chromatography (HPLC), gas chromatography (GC), supercritical fluid chromatography (SFC), mass spectrometry (MS) or hyphenated techniques such as GC/MS, SFC/MS and HPLC/MS. Different ionisation modes namely electron impact (EI), chemical ionisation (CI), electrospray ionisation (ESI) or fast atom bombardment (FAB) have been used.

Some approaches include compound derivatization before analysis. The derivatization and analytical methods strongly depend on the specific problem under study.

1.1. FA containing double bonds

Unsaturated fatty acids (UFAs) have been studied for their impact on human health. Recent studies show a correlation between *trans*-unsaturated fatty acids (TUFAs) ingestion and an increase of blood cholesterol level as well as a relationship between TUFAs and cardiovascular risks [20,21]. On the other hand, conjugated linoleic acid (CLA) shows multiple beneficial effects as anticarcinogenic activity, protection against arteriosclerosis and reduction of body fat. The conjugated linoleic acid has been investigated in animals and human models [22] or food samples [23].

A number of analytical methods have been reported on monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) [3,6,9,17,20,21,24–30]. MUFA regulate the composition of biological membranes and cholesterol metabolism. PUFA as linoleic acid ($C_{18:2n-6}$) and α-linolenic acid ($C_{18:3n-3}$) play very important roles as precursor of long-chain PUFAs (>18 carbons) whose metabolism is regulated by a hepatic competitive enzymatic system [30]. The metabolic pathways have been elucidated by ^{13}C labelled linoleic and linolenic acids [29].

The long-chain fatty acids (LCFAs) derivatives of PUFA are classed as essential FAs [26]. They must be included in any diet because the human body is unable to synthesise them. The eicosapentaenoic acid (EPA, 20:5$n-3$) and docosahexenoic acid (DHA, 22:6) present in fish play major roles in the immune and cardiovascular systems. On the other hand, the erucic acid (22:1$n-9$) leads to high accumulation of fat in the heart muscle and is a factor responsible for cardiac lesions [26]. These facts illustrate the importance of the detailed knowledge of the FA isomeric composition in foods [1], therefore, it is essential to have simple and rapid methods for the determination of FAs profiles.

1.1.1. GC methods for MUFA and PUFAs

Recently, sensitive and rapid GC methods were reported for the determination of MUFA and PUFA in biological samples involving the derivatization of these compounds to fatty acids methyl esters (FAME) using acetylchloride [22] or boron trifluoride–methanol [30,31] as a derivatization agent. FID was used as a detector in all situations. The limited availability of FAME used as standard compounds restricts the application of this method.

1.1.2. Multidimensional chromatography

Baseline separation of *cis*/*trans* $C_{16:1}$ and $C_{17:1}$ FAs isomers was reported using a pre-separation of the FAME by argentation thin-layer chromatography (Ag-TLC) [21]. By using Ag-TLC, the separation is based on the number and configuration of the double bonds. Although the number of double bonds is the governing factor, the *cis*-isomers are retained longer than trans. The separation column used was a highly polar stationary phase (CP-Sil 88, 100% cyanopropyl polysiloxane) capillary column of 100 m length and FID detection. The isomeric identification was performed by mass spectra of different FA derivatives; the method was used for identifying the isomeric distribution of $C_{16:1}$ and $C_{17:1}$ in human milk samples.

Recently, comprehensive multidimensional gas chromatography (GC/GC) has been shown to be very useful for the separation of very complex mixtures of analytes, including PUFA, which are contained in fish and vegetable oils [26]. Using a short non-polar column (methylpolysiloxane, HP-1, 9 m) as a first GC column and a very short moderate polar column (polyethylene glycol, SP-Wax-52, of 0.3 m) as secondary column, a complete profile of FAME (BF_3–methanol) has been determined in edible oils. This technique needs a thermal modulator assembly as a column interface.

Complex mixtures of the geometrical isomers of C_{20} FAs as FAME have been completely determined using three chemically different stationary phases coated on fused-silica capillary columns, running in "off-line" (parallel) mode. The polar cyanopropyl silicone phase (SP-2340), the moderate

polar polyethylene glycol (Supelcowax-10) and a non-polar dimethylpolysiloxane (SPB-1) were found to be a suitable combination for the analysis of 10-monoethylenic and 16-diethylenic C_{20} *cis*/*trans* isomers [25].

1.1.3. Mass spectrometry methods

1.1.3.1. EI mass spectrometry. To locate the double bonds in MUFA and PUFA, very informative mass spectra are obtained from heterocyclic derivatives, which stabilise the ions containing the double bonds [3,4,32]. Analytes must be derivatised with a reagent containing a nitrogen atom [1,4,33,34]. The derivatizing agents are picolinyl esters (3-hydroxymethylpiridinyl) or 4,4-dimethyloxazoline (DMOX) derivatives [35]. The fragmentation of aliphatic side chain of DMOX derivatives of saturated FA results in a series of even mass ions at intervals of 14 mass units starting from *m*/*z* 126. The double bond position in MUFA and PUFA is recognised by a characteristic fragmentation in this homologous mass sequence. The determination of the *cis*/*trans* isomers is limited by small differences between mass spectra [36].

Detailed data on conjugated fatty acids (CFAs) were obtained on biological samples using a polar column and 4-methyl-1,2,4-triazoline-3,5-dione (MTAD) derivatives of FAME by GC/MS methods [37]. The 4-methyl-1,2,4-triazoline-3,5-dione reagent is suitable for the determination of conjugated FAs because with the conjugated double bonds it produces very stable compounds and informative mass spectra.

Unsaturated ω-Hy-FAs are the main building blocks of highly cross-linked constituents of the cell walls in which the linear chains of esterified monomer are ether cross-linked at the position of the double bonds.

The double bond position of mono- and di-unsaturated C_{30}–C_{32} (ω-Hy-FA) was identified by comparison of mass spectra of the compounds methylated in the carboxylic group and silylated in the hydroxyl group (tandem mass spectrometry (TMS)-FAME) with the mass spectra of the same compounds derivatised with DMDS (TMS-DMDS-FAME). The mass spectra of the TMS-DMDS-FAME compounds give very characteristic fragments containing the SMe group indicating the position of the original double bond. The long-chain unsaturated ω-Hy-FAs identified were specifically obtained from the cell walls of some fresh green microalgae and were not observed in the cytoplasma [38].

1.1.3.2. ESP mass spectrometry. Recently, a rapid method for the FA determination in plasma or urine samples using new derivatives of heteroatomic compounds was developed [39]. The derivatives were obtained using the dimethylaminoethyl esters (DMAE) by a simple procedure. Compound identification is based on diagnostic ions MH^+ and $[MH - 45]^+$ obtained using an electrospray ion (ESI) source. Despite the simplicity of the method, it is limited by the relative expensive tandem mass spectrometry technique.

A rapid method (6 min) for the analysis of free FAs resulting from the degradation of phospholipids was developed using LC/MS system with an ESI source, running in negative mode [8]. This technique allows the separation and characterisation of intact compounds as oleic, linoleic, linolenic, palmitic and stearic acid in various phospholipid formulations (drug formulations). The low number of visible ions in mass spectra, and the high intensity obtained for diagnostic ion $[MH]^-$ make possible the determination of FA in complex matrices.

1.2. Methyl branched chain determination

The methyl branched FA, particularly *iso*-, *anteiso*- and mid-chain branched acids (MCBA) are often found in bacteria and marine organisms [40] and in natural waxes originating from plants [41] or animals [42–44]. Because it is difficult to discern the *iso*- and *anteiso*-structure from EI mass spectra alone, tandem mass spectrometric methods with special ionisation techniques (FAB or electrospray) [3] or a combination of retention data with EI mass spectral data [41] are necessary.

1.2.1. GC/MS methods

Complex isomeric mixtures of *iso*-, *anteiso*- and mid-chain branched FA were found in a demosponge as derived from specific bacterial symbionts [40]. The respective FAs were converted to FAME with diazomethane and analysed by GC (DB-5 HT capillary column, of 50 m) and GC/MS. The identification of FAs (in the range C_{15}–C_{25}) was based on comparison of the mass spectra and retention parameters with those of published data. The site of methyl branching

is in each case located between the $n-5$ and $n-9$ positions. Partial resolution was obtained for the homologues of low molecular weight (C_{15}–C_{16}) but mid-chain branched acids of increased chain length were typically observed only as a single broadened eluting peak.

1.2.2. Mass spectrometric methods

Branching as well as unsaturation site in FA can be localised by mass spectral correlation after derivation and EI ionisation. The most useful derivatives for determining terminal branching appear to be picolinyl derivatives (β-picolinyl esters) [1,4] and 2-alkyl-4,4-dimethyloxazoline (DMOX) [1,4,32] because it is easy for routine applications in connection with GC/MS in the EI mode.

A new derivative for carboxyl groups, *N*-methyl-2-alkylimidazoline (MIM) was introduced for improving the FA fragmentation [34]. Owing to the strongly basic character and the cyclic structure, strong stabilisation of the charge on ionisation group was obtained so that charge remote fragmentation of the chain can be achieved with electron impact and ESI. The *N*-methyl-2-alkylimidazoline derivatives also have better features with regard to preparation and GC separation.

Some papers reported the branch determination on underivatised [45] FA or FAME [3,46] using advanced mass spectrometry techniques (MS/MS). Branched FA can be distinguished from isomeric straight chain underivatised FA by collisionally activated $[MH]^-$ ions desorbed by using FAB mass spectrometry [45]. The distinguishing element is the loss of the neutral C_nH_{2n+2} as a function of the branched carbon position.

Low-energy collision induced dissociation (CID) of the molecular ions of the FAME obtained by EI decompose in the tandem quadrupole mass spectrometer to yield a regular homologous series of carbomethoxy ions. These ions can be related to the methyl branched position [3,46].

1.3. Determination of very long-chain fatty acids (VLCFAs)

Some recent papers describe rapid and precise methods for the determination of very long-chain fatty acids (VLCFAs, acids with the carbon atom range C_{22}–C_{28}) in human serum (small sample volume) for clinical diagnosis [47–49].

For the determination of VLCFA (as FAME) at clinically significant level of precision (around 50 nmol/l in plasma, equivalent to 20 ng/ml), a GC/MS method in selected ion monitoring (SIM) has been developed [47]. The method is sensitive enough for clinical diagnosis but is time consuming because of the derivative preparation step.

To improve the quantification limits (to 10 ng/ml when 0.5 ml of serum is used), a sensitive method was developed by GC with nitrogen–phosphorous detection (NPD) using cyanomethyl derivation with bromoacetonitrile as a reagent [48]. The derivative separation was performed on a non-polar column (HP-5 of 30 m, 0.32 i.d., 0.25 mm film thickness). The compound identification was made on the basis of EI mass spectra of VLCFA as cyano methyl esters. The method shows very good sensitivity and simplicity suitable for clinical diagnosis of peroxisomal disorder but is limited to determination of only few compounds (saturated C_{22}, C_{24} and C_{26}).

Other authors used a combination of TLC and a short packed column (high-volume injection) with polar stationary phase (OV-225) for elimination saturated/unsaturated FA interference, and FID. Thus it was concluded that in peroxisomal disease a defect in FA desaturation mechanisms (PUFA, C_{20}–C_{22}) can be seen [49].

1.4. FA profiles in taxonomy

Lipids are best characterised by their FA, which differ in chain length, the degree of unsaturation, position of the double bonds and presence of other functionalities. Therefore, the FA profile can be useful for taxonomic purposes.

Microalgae are a major source of lipids in lacustrine and marine environments. Some useful characteristic features have been observed in the abundance of particular polyunsaturated FA. The first group (eustigmatophites and haptophytes) contains high concentration of certain long-chain PUFA such as $20{:}5n-3$ and $22{:}6n-3$. A second group (chlorophytes) have a predominance of C_{18} PUFA such $18{:}2n-6$ and $18{:}3n-3$. A third group (dinoflagellates) have high levels of $20{:}5n-3$, $22{:}6n-6$ and the unusual FA, $18{:}5n-3$. Genetic and environmental factors appear

to be important controls of the relative abundance of various homologous identified [6].

To obtain the FA profile from lichens [50], algae [28,51,52] or bacteria [53,54] the GC/MS methods were used for FAME derivatives. If a low-polar column, as (DB-5, of 30 m) [50,51] is used, the MUFA and PUFA isomers are not completely resolved but the methods have resolution for saturated/unsaturated compounds. For the separation of the compounds with the same carbon atom number but different unsaturation degree, a polar phase column as 007-FFAP [53] or CP-Sil 88 (50 m) [52] has been used.

Based on the fact that modern instrumentation can generate gas-phase ions from non-volatile samples by a variety of ionisation methods, an important number of mass spectrometric methods for FFA analyses were developed to characterise bacteria [3,55].

FFA profiling of whole bacteria was obtained with direct probe mass spectrometry under three different ionisation conditions: EI (70 eV), positive chemical ionisation (PCI) and negative chemical ionisation (NCI), with isobutane as a reactive gas [54]. EI produces spectra that contain molecular ions and fragment ions from various FFAs. The chemical ionisation mass spectra contain molecular ions and other taxonomic information as m/z 173, characteristic for poly-(3-hydroxybutyrate), which has been used as chemotaxonomic marker for bacteria [54]. By negative chemical ionisation conditions, it is possible to observe the M^- ion of dipicolinic acid (m/z 167), a compound used to signal the presence of bacterial spores.

Analysis of untreated samples has been preferred also for confirming the presence of FFA in insect samples [56]. Solid-phase microextraction (SPME) was found to be reliable in combination with a common GC/MS analysis system running in particular conditions (deactivated injection liner) and using a polar stationary phase. By this method, the analysis of more polar compounds like 3-hydroxydecanoic acid and 3-hydroxydodecanoic acid was only partially successful, probably because of the high boiling point of these compounds.

A very selective and sensitive method for the determination of short-chain carboxylic acids, using negative chemical ionisation mass spectrometry with ammonia as a reagent gas was reported [57]. The diagnostic ions $[MH]^-$ and $[2MH]^-$, produced by an ion–molecule reaction, were detected in the SIM mode. Analytes were extracted from aqueous samples by solid-phase microextraction and separated on a polar column (TR-FFAP of 30 m). The method is limited for FA with a chain under C_7.

1.5. Food characterisation by FA profile

An important number of papers report on the analytical methods for the determination of the FA or FAL profiles in different food products as edible oils [58–60], coffee [61], fruit [62] and honey [63]. In many of them, the derivation of free polar compounds to FA-TMS is preferred, followed by GC or GC/MS separation on non-polar capillary columns [58,62–64].

Food is comprised of various mono-, di- or tri-glycerides (MG, DG and TG) differing in the acyl groups. Individual samples have characteristic composition of glycerides and their determination allows us to find out the origin of food or their possible adulteration in the food industry. By transesterification of glycerides in vegetable oils with methanol in alkaline environment, the FAME are produced and their profile can be used as a sample characteristic [65].

Recently, it has been shown that chlorotrimethylsilane (CTMS) used as an acid catalyst (in presence of propanol) allows the transesterification of some fatty esters to propanyl esters (FAPRES) [59], giving more stable compounds. The method can be used for elimination of the degradation of unsaturated compounds observed for FAME.

The determination solely of the acid content is not fully characterising the oil quality. The quality of vegetable oils can be deduced from the ratio of FFA to diacylglycerol using a GC method. The simultaneous separation of FFA (as FA-TMS derivatives) and diacylglycerol was performed on a short non-polar capillary column (DB-1, 10 m) using a FID system. The method is sensitive and precise enough to characterise the edible oils [66]. The FFA/diacylglycerol is correlated with the type of oils and their treatment.

1.6. Wax characterisation by FA profiles

1.6.1. GC/MS methods

Waxes are a class of substances that has been extensively used in many industrial branches as pharmaceutical and cosmetic industry [67]. Waxes produced by animals and plants mainly represent mixtures of esters

containing different percentages of other compounds, as FAs and fatty alcohols [42].

Some early reported methods described the determination of FFA and free fatty alcohols (FFAL), as trimethylsilyl derivatives (TMS) on samples isolated from plant waxes [41,68–70]. The samples were obtained by a multistep extraction process and analysed by GC/MS using fused silica capillary columns of low polarity (SE 54 [68] or DB-5 MS [41]). The acid profile has been obtained as a function of sample origin. The major constituents of FA were in the range of C_{16} and C_{18} carbon atom number (palmitic, linoleic and oleic) in hexane extract of the leaves of *Amaranthus palmeri* [68] and in the range of C_{24} and C_{26} in extract of *Rubus idaeus* [41]. The branched compounds were identified using the retention parameters as equivalent chain length (ECL). The molecular distribution of FFA and FFAL was found to be that in esters [69].

Recently, the chemical composition of epicuticular waxes as chloroform extract from conifers was also reported [70]. The extract was methylated and separated by TLC (on silica gel plates) resulting in few fractions. Some of them were converted to TMS derivatives and analysed by GC/MS on a non-polar capillary column (DB-5). The main class of compounds were characterised by average chain length (ACL) and carbon preference index (CPI). The obtained values suggest humidity adaptation by the coastal conifers, which is evident from the decrease of ACL by two units.

1.6.2. Py-GC/MS

Natural wax composition was investigated by pyrolysis gas chromatography/mass spectrometry (Py-GC/MS) [43,44]. The obtained pyrograms showed very characteristic signal patterns. From the mass spectral data and the retention feature, general information was derived according to the thermal degradation pathways of typical wax constituents. The FA distribution of waxes is incompletely shown by Py-GC/MS, FAs homologues higher than C_{20} were generally lacking [43].

To overcome this, derivatization methods are necessary. Thermally assisted hydrolysis and methylation (THM) in combination with GC/MS was found to be a better technique for the comprehensive analysis of animal and plant waxes [44] or mineral waxes [71]. By thermally assisted hydrolysis and methylation mainly high molecular weight esters were hydrolysed and converted in their methyl derivative in a single step. Tetramethyl ammonium hydroxide (TMAH) as an alkaline derivatization reagent was found to be suitable [44]. By thermally assisted hydrolysis and methylation, the wax ester compounds keep their original structure and are easily recognised by GC/MS analysis but it is not possible to distinguish between FFA and FA resulted from hydrolysis.

The use of different ammonium salts in combination with Py-GC/MS offered a rapid method for the quantitative estimation of free, esterified and total acids [72]. Tetramethyl ammonium acetate (TMAAc) was found to be a selective reagent for the methylation of free acids in presence of esterified FAs. The alkaline reagent tetramethyl ammonium hydroxide was efficient in hydrolysing and derivatising all FAs present, both as free acids and in different esters.

1.7. FFA, FFAL, Hy-FA and Hy-FAL in wool wax samples

Wool wax (lanolin) is a wax obtained from the wool of sheep and is widely used in modern pharmaceutical formulations and cosmetics [73–78]. It is a complex mixture of esters, di-esters and hydroxy esters of high molecular weight alcohols (aliphatic and steroidal) and high molecular weight acids [42–44,75,76,79].

Lanolin alcohols comprise three major chemical groups: aliphatic, sterols (cholesterol and hydrocholesterol) and trimethyl sterols (lanosterols, hydrolanosterol, agnosterol and hydroagnosterol) [42]. The aliphatic series consist of normal-, *iso*-, *anteiso*-alcohols and normal-, *iso*-, *anteiso*-2-hydroxyalcohols (1,2-diols series).

The lanolin acids consist also of normal-, *iso*-, *anteiso*-acids and normal-, *iso*-, and *anteiso*-2-hydroxyacids (2-hydroxyacids series). The products of all wool wax acids and alcohols represent the theoretical maximum number of monoesters, assuming no preferential esterification in the biosynthesis of wool wax from the sheep sebaceous glands (resulting >10,000 compounds) [42].

Wool wax contains also FFA and FFAL, which are released by a slow hydrolysis mechanism, operating whilst the wool wax is still spread on the sheep's fleece. The quantity of every type of compound released can be deduced from the thermodynamics of hydrolysis [75].

As was shown in current applications, using GC and GC/MS techniques the FAs carboxyl groups are converted to different types of esters [2]. The most common derivation methods involve esterification to FAME [2,61] using acid (BF_3, HCl, H_2SO_4, BCl_3) or base (NaOH, KOH, $NaOCH_3$) catalysts [9].

In multifunctional compounds, the carboxylic group is usually derivatised first to FAME and then the other functional groups can be converted to more volatile form group as TMS ethers [2]. The use of FAME have several limitations such as the instability of the reagent, loss of polyunsaturated and volatile FA, complete destruction of epoxy, hydroepoxy, cyclopropenyl, cyclopropyl and hydroxy groups [80]. In the EI mass spectra of FAME, low intensity ions in the molecular mass range can also be shown.

TMS derivatization is known as a common method to overcome the limitations of methyl esterification in spite of thermal instability and possibility of hydrolysis of derivatives in the presence of very small amounts of water [80]. Recently, it has been shown that for the identification and quantification of acid homologues, their analysis in the form of TMS derivatives is the method of choice [27,41,63,64,81]. The EI fragmentation of different TMS members of various homologous series of acids or hydroxy acids provided ions of high intensity in the molecular mass region with very informative characteristics [82,83]. The average reported reproducibility as RSD was under 3.6% for alcohols and sterols [58] and under 6.7% for FAs [62,63].

The early published papers on wool wax, reported the FA and FAL only as a product of ester hydrolysis [42–44,75,76,84]. Little is known about the free compounds comprised in wool wax.

The present paper describes a rapid method for the simultaneously determination of some chemical classes of free compounds from wool wax samples as TMS derivatives. The analytes consist of FA, FAL, hydroxy fatty acids (Hy-FA) and hydroxy-fatty alcohols (Hy-FAL) in the range of C_9–C_{33}. All three isomeric structures (normal-, *iso*- and *anteiso*-) can be found for every chain of a given number of carbon atoms (*N*). The identification of the compounds was made on the basis of the EI mass spectra as well as retention parameters as ECL [24,25,41,85,86] or fractional chain length (FCL). The comparison of the chromatographic behaviour in the terms of ECL values is widely used for isomer identification [24,25,41].

We found it useful to represent the dependence of FCL versus integer part of ECL, denoted as I(ECL), for every homologous series data for performance separation characterisation. The ECL values were obtained by using an earlier reported equation [86]. Based on the cross-points of this graph, the coeluting compounds can be deduced.

We used a low polarity capillary column (polymethylsiloxane, 5% phenyl), which have some advantages such as high thermal stability, leading to a wide range of operating temperatures and chemical inertness. The capillary columns with polymethylsiloxane as stationary phase are amongst the most widely used for FA analysis [9,27].

Samples were obtained by supercritical fluid extraction (SFE). SFE presents an attractive alternative to Soxhlet extraction because the process is commonly performed with carbon dioxide, a relative inert and non-toxic solvent. Extraction of wool wax by supercritical fluid extraction offers advantages over the current methods because a colourless, low-odour product may be obtained by properly adjusting the extraction temperature and pressure [87,88]. Changes in the lipid composition of wool wax collected fractions are attributed to percentage of modifier and to extraction time [89,90].

2. Experimental

2.1. Samples

Wool wax samples were isolated from raw wool by supercritical fluid extraction with carbon dioxide (CO_2) in the presence of modifier, according to a previously described procedure [90]. Wool wax (lanolin) samples (100 mg) were dissolved in 2.5 ml of an ethylacetate/cyclohexane(1:1) mixture. In order to obtain a FFA enriched fraction, the solution was extracted three times with 5 ml of alkaline water pH 8 (with NaOH). The aqueous extract contains an emulsion due to FAs, the micelles contain wool wax greasy compounds. Then, the aqueous extract was acidified at pH 2 with HCl to obtain the FFAs. The solution was extracted three times with 5 ml of dichloromethane. The organic phase was evaporated to dryness under gentle nitrogen flow.

The TMS derivatives were obtained by reaction with *N*,*O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) (Merck, Darmstadt, Germany) at 70 °C for 1 h.

2.2. Instrumentation

GC/MS analysis was performed using a Fisons MD 800 Mass Spectrometer (Fisons Scientific Equipment, Loughborough, England). The instrument was operated in the EI mode at 70 eV.

The following chromatographic conditions were used. Injection was in the splitless mode at 280 °C. The GC column was a phenylmethylsilicone (5% phenyl) HP-5MS of 30 m length, 0.25 mm i.d. and 0.25 mm thickness (J&W Scientific columns, Folsom CA, USA). Column temperature was programmed from 90 °C (1 min) to 120 °C at 10 °C min^{-1} and then to 240 °C at 6 °C min^{-1} and then to 315 °C at 5 °C min^{-1} and holding the final temperature for 31 min (total time 70 min). Helium was the carrier gas at a flow rate of 1 ml/min. Transfer line and ion source temperatures were held at 270 and 230 °C, respectively.

3. Results and discussion

The identification of compounds was based on the following information: (A) interpretation of the mass spectra of the individual compounds, as TMS derivatives, obtained by GC/MS in the EI mode and (B) retention chromatographic data as ECL and FCL.

3.1. Mass spectra

The *m*/*z* and structure of the ions used for compound identification are shown in Table 1. For every class of compounds, the characteristic homologue series (*m*/*z* 117, 129, 132 and 145 for FA, *m*/*z* 103 for FAL and *m*/*z* 147 for 2Hy-FA and 2Hy-FAL) and diagnostic ions ($[M]^+$ and $[M-15]^+$ for FA, $[M-15]^+$ for FAL, $[M-117]^+$ for 2Hy-FA and $[M-103]^+$ for 2Hy-FAL) can be seen. The peaks used for quantification are underlined ($[M-15]^+$ for FA and FAL, $[M-117]^+$ for 2Hy-FA and $[M-103]^+$ for 2Hy-FAL).

Compounds of different structures were described by the ions a and g (Table 1) with *m*/*z* $14n+75$ for FAL, Hy-FA and Hy-FAL but *m*/*z* $14n+89$ for acids. Therefore, all compounds in the range C_9–C_{33} can be determined by the ion series: 187, 201, 215, 551.

3.2. Separation parameters

Compound separation in the range of C_{12}–C_{33} is shown in Fig. 1 by characteristic ion chromatogram. The quantification has been made by diagnostic ions for every compound, ion a for FA and FAL, ion g for 2Hy-FA and 2Hy-FAL.

To the same *m*/*z* number, 12 corresponding compound structures are detected (denoted from S_1 to S_{12}

Table 1
Diagnostic ions used for the identification of FA, FAL, Hy-FA and Hy-FAL as TMS derivatives[a]

Ion	Structure	*m*/*z*	FA	FAL	Hy-FA	Hy-FAL
M	$M^{\bullet+}$	$14N+104$	+	−	−	−
		$14N+90$	−	+	−	−
		$14N+192$	−	−	+	−
		$14N+178$	−	−	−	+
a	$[M\text{-}CH_3]^+$	**$14N+89$**	+	−	−	−
		$14N+75$	−	+	−	−
		$14N+177$	−	−	+	−
		$14N+163$	−	−	−	+
b	$O^+{\equiv}COSi(CH_3)_3$	117	+	−	−	−
c	$CH_2{=}C(O^{\bullet+})OSi(CH_3)_3$	132	+	−	−	−
d	$[(CH_2)_2C(O)OSi(CH_3)_3]^+$	145	+	−	−	−
e	$[(CH_2)_2C(O)Si(CH_3)_3]^+$	129	+	−	+	+
f	$CH_2{=}O^+Si(CH_3)_3$	103	−	+	+	+
g	$H(CH_2)_{N-1}CH{=}O^+TMS$	**$14N+75$**	−	−	+	+
h	$(CH_3)_2Si^+OSi(CH_3)_3$	147	−	−	+	+

[a] *N* is the carbon atom number. The bold *m*/*z* values has been used for area calculation.

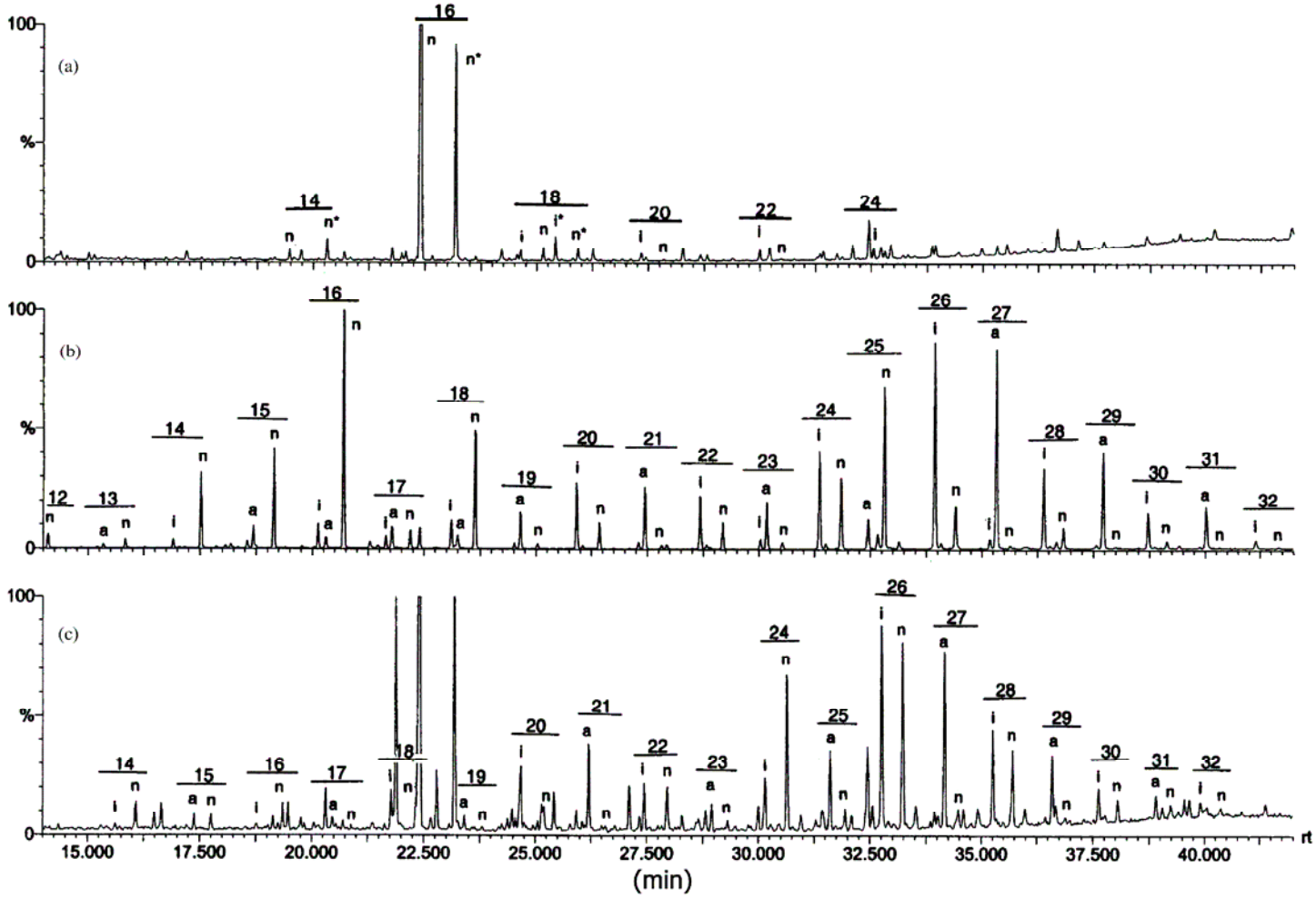

Fig. 1. Characterisation of wool wax lipids as TMS derivatives by ion chromatogram (a) *m/z* 147: 2-hydroxy-fatty acids (2Hy-FA) (∗) and 2-hydroxy-fatty alcohols (2Hy-FAL); (b) *m/z* 132: fatty acids (FA), and (c) *m/z* 103: fatty alcohols (FAL).

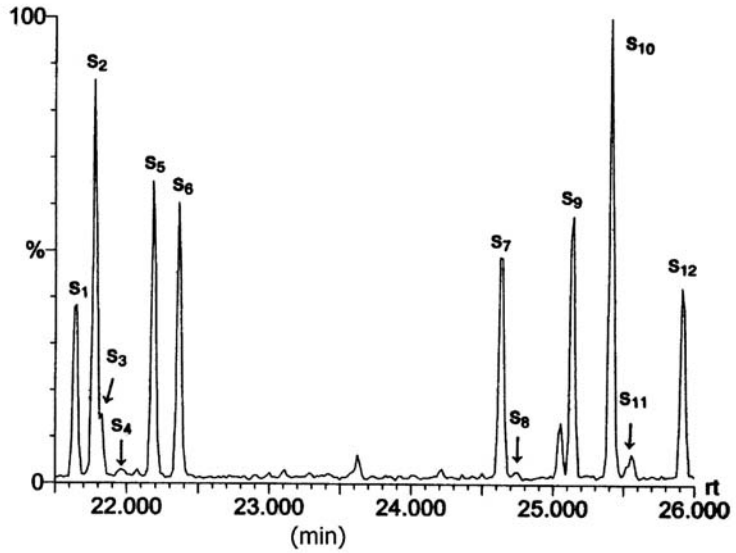

Fig. 2. Compound structures (S_1–S_{12}) described by ion chromatogram with *m/z* 327. The S_1–S_{12} symbols correspond to the following TMS compounds: S_1, S_2, S_5 are FA C_{17} with structure *i*, *a*, *n*; S_3, S_4, S_6: FAL C_{18} *i*, *a*, *n*; S_7, S_8, S_9: 2Hy-FAL C_{18} *i*, *a*, *n*; S_{10}, S_{11}, S_{12}: 2Hy-FA C_{18} *i*, *a*, *n*.

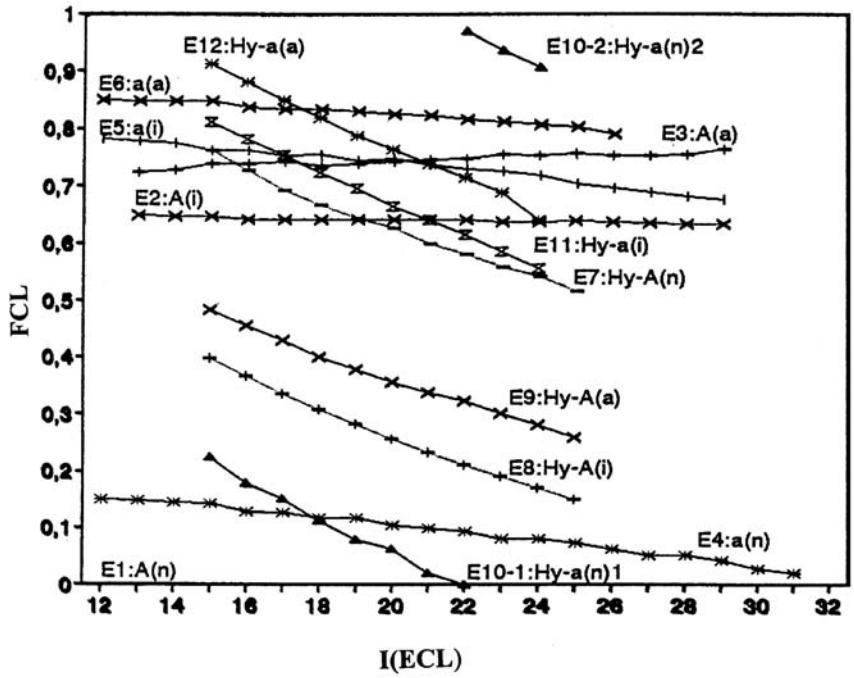

Fig. 3. The variation of FCL vs. I(ECL) for 12 groups of lipids. E1, *n*-FA; E2, *i*-FA; E3-*a*-FA; E4, *n*-FAL; E5, *i*-FAL; E6, *a*-FAL; E7, 2Hy-*n*-FA; E8, 2Hy-*i*-FA; E9, 2Hy-*a*-FA; E10-1, 2Hy-*n*-FAL ($N \leq 21$); E10-2, 2Hy-*n*-FAL ($N = 22$); E11, 2Hy-*i*-FAL; E12, 2Hy-*a*-FAL (see also Table 5).

Table 2
Retention time (t_R), ECL and relative concentration of the fatty acids in the range of N: 9–33[a]

N	M (I_b)	t_R	ECL	%
9*i*	216 (201)	–	–	–
a		–	–	–
n		8.775	9.000	0.797
10*i*	244 (229)	9.876	9.649	0.102
a		10.001	9.717	0.023
n		10.539	10.000	0.940
11*i*	258 (243)	11.661	10.656	0.045
a		11.788	10.726	0.202
n		12.303	11.000	0.207
12*i*	272 (257)	13.438	11.650	0.190
a		13.583	11.728	0.045
n		14.098	12.000	1.071
13*i*	286 (271)	15.195	12.647	0.079
a		15.330	12.723	0.298
n		15.833	13.000	0.605
14*i*	300 (285)	16.902	13.646	0.565
a		17.037	13.724	0.090
n		17.520	14.000	4.456
15*i*	314 (299)	18.543	14.645	0.432
a		18.677	14.726	1.213
n		19.134	15.000	5.202
16*i*	328 (313)	20.126	15.645	1.474
a		20.304	15.757	0.512
n		20.695	16.000	13.986
17*i*	342 (327)	21.638	16.640	0.677
a		21.772	16.728	1.298
n		22.189	17.000	0.988
18*i*	356 (341)	23.100	17.640	1.146
a		23.247	17.741	0.287
n		23.630	18.000	6.374
19*i*	370 (355)	24.508	18.636	0.212
a		24.649	18.735	1.660
n		25.026	19.000	0.275
20*i*	384 (369)	25.908	19.638	3.113
a		26.050	19.738	0.120
n		26.423	20.000	1.204
21*i*	398 (383)	27.298	20.639	0.289
a		27.439	20.740	2.436
n		27.806	21.000	0.156
22*i*	412 (397)	28.667	21.636	2.251
a		28.817	21.745	0.144
n		29.172	22.000	1.016
23*i*	426 (411)	30.026	22.640	0.358
a		30.157	22.737	1.890
n		30.517	23.000	0.217
24*i*	440 (425)	31.301	23.636	3.730
a		31.507	23.753	0.178
n		31.838	24.000	2.560
25*i*	454 (439)	32.650	24.635	0.422
a		32.802	24.752	6.291
n		33.127	25.000	0.202
26*i*	468 (453)	33.929	25.637	6.297
a		34.082	25.757	0.131
n		34.395	26.000	1.378
27*i*	482 (467)	35.174	26.636	0.347
a		35.317	26.751	7.712
n		35.628	27.000	0.094
28*i*	496 (481)	36.383	27.634	3.057
a		36.525	27.752	0.077
n		36.826	28.000	0.795
29*i*	510 (495)	37.564	28.633	0.093
a		37.705	28.753	3.626
n		37.998	29.000	0.035
30*i*	524 (509)	38.716	29.632	1.279
a		38.866	29.762	0.037
n		39.141	30.000	0.262
31*i*	538 (523)	39.867	30.619	0.045
a		40.016	30.745	1.561
n		40.350	31.000	0.015
32*i*	552 (537)	41.151	31.620	0.328
a		–	–	–
n		41.651	32.000	0.041
33*i*	566 (551)	–	–	–
a		42.835	32.630	0.454
Total (%)				(*i*) 26.831, (*a*) 30.285, (*n*) 42.876

[a] The symbols: *i*, *a*, *n* denote the *iso*-, *anteiso*- and normal-isomer, respectively; M is molecular mass and I_b the ion used for area calculation.

as a function of retention time): FA containing N carbon atoms of isomeric structure *i*-, *a*-, *n*- (S_1, S_2 and S_5, respectively) FAL with $N + 1$ carbon atoms of isomeric structure *i*-, *a*-, *n*- (S_3, S_4 and S_6, respectively), Hy-FA with $N + 1$ carbon atoms of isomeric structure *i*-, *a*-, *n*- (S_7, S_8 and S_9, respectively) and Hy-FAL with $N + 1$ carbon atoms of isomeric structure *i*-, *a*-, *n*- (S_{10}, S_{11}, and S_{12}, respectively). The used diagnostic ions for every class of compounds are: m/z 132 (FA), m/z 103 (FAL) and m/z 147 (Hy-FA and Hy-FAL).

Table 3
Retention time (t_R), ECL relative to *n*-acids and relative concentration of the fatty alcohols in the range of *N*: 12–34[a]

N	*M* (I_b)	t_R	ECL	%
12*i*	258 (243)	–	–	–
a		–	–	–
n		12.573	11.161	1.229
13*i*	272 (257)	–	–	–
a		–	–	–
n		14.348	12.152	0.275
14*i*	286 (771)	15.438	12.783	0.044
a		–	–	–
n		16.074	13.150	1.432
15*i*	300 (285)	–	–	–
a		–	–	–
n		17.745	14.145	0.270
16*i*	314 (299)	–	–	–
a		–	–	–
n		19.349	15.143	1.324
17*i*	328 (313)	20.313	15.763	0.012
a		20.455	15.852	0.724
n		20.881	16.129	0.114
18*i*	342 (327)	21.825	16.763	0.065
a	–	–	–	–
n		22.367	17.127	1.588
19*i*	356 (341)	23.265	17.753	0.134
a		23.406	17.849	0.633
n		23.791	18.118	0.146
20*i*	370 (355)	24.676	18.754	7.120
a		24.810	18.849	0.122
n		25.178	19.111	1.489
21*i*	384 (369)	26.050	19.744	0.090
a		26.192	19.838	5.099
n		26.566	20.106	0.232
22*i*	398 (383)	27.439	20.740	2.232
a		27.573	20.835	0.094
n		27.940	21.100	2.429
23*i*	412 (397)	28.808	21.738	0.430
a		28.940	21.834	1.200
n		29.296	22.094	0.507
24*i*	426 (411)	30.148	22.730	3.560
a		30.827	22.832	0.143
n		30.632	23.089	9.353
25*i*	440 (425)	31.437	23.727	0.510
a		31.603	23.825	3.991
n		31.941	24.081	1.000
26*i*	454 (439)	32.760	24.719	11.339
b		32.895	24.823	0.387
n		33.220	25.075	10.066
27*i*	468 (453)	34.014	25.704	0.536
a		34.158	25.816	9.594
n		34.417	25.057	0.396
28*i*	482 (467)	35.250	26.697	5.218
a		35.393	26.812	0.135
n		35.695	27.057	3.528
29*i*	496 (481)	36.450	27.689	0.204
a		36.592	27.807	3.574
n		36.885	*m/z*	0.276
30*i*	510 (495)	37.621	28.682	1.191
a		37.772	28.803	0.227
n		38.048	29.043	1.381
31*i*	524 (509)	38.766	29.677	0.155
a		38.900	29.791	1.120
n		39.174	30.028	0.162
32*i*	538 (523)	39.908	30.654	0.805
a		40.058	30.778	0.210
n		40.367	31.019	0.447
33*i*	552 (537)	41.101	31.657	0.084
a		41.368	31.785	0.905
n		41.701	–	0.187
34*i*	566 (551)	42.660	–	0.027
Total (%)				(*i*) 33.986, (*a*) 28.158, (*n*) 37.856

[a] The symbols: *i*, *a*, *n* denote the *iso*-, *anteiso*- and normal-isomer, respectively; *M* is the molecular mass and I_b the ion used for area calculation.

In Fig. 2, the S_1–S_{12} structures, corresponding to the *m/z* 327 are shown. A good resolution ($R > 1$) has been obtained for all the isomers (*i*, *a*, *n*) from the same family but for compounds from different families, the resolution changes when *N* increases.

The retention parameters were calculated in terms of the ECL [24,85,86] with respect to FA. The ECL for *n*-FA is an integer equal to their carbon atom number (*N*) but ECL for the other compounds contains a fractional part (FCL), which depends on the relative distance from the ECL of the *n*-FA compound, situated in the chromatogram immediately before it. The experimental values obtained for ECL and relative molar concentration for FA, FAL, Hy-FA and Hy-FAL detected in the range of C_9–C_{33} are shown in Tables 2, 3, 4 and 5, respectively.

We found that FCL is a diagnostic parameter in the separation configuration. Taking into account

Table 4
Retention time (t_R), ECL relative to *n*-acids and relative concentration of 2-hydroxy-fatty acids in the range of *N*: 14–24[a]

N	*M* (I_b)	t_R	ECL	%
14*i*	388 (271)	19.741		0.445
a		–	–	–
n		20.313	15.763	6.086
15*i*	402 (285)	–	–	–
a		21.363	16.456	0.973
n		21.772	16.728	3.691
16*i*	416 (299)	22.662	17.336	1.167
a		–	–	–
n		23.178	17.693	68.535
17*i*	430 (313)	–	–	–
a		24.178	18.400	1.938
n		24.552	18.667	1.863
18*i*	444 (327)	25.410	19.281	7.849
a		–	–	–
n		25.917	19.644	3.691
19*i*	458 (341)	–	–	–
a		26.906	20.355	0.542
n		27.280	20.626	0.148
20*i*	472 (355)	28.118	21.233	0.543
a		–	–	–
n		28.616	21.599	0.069
21*i*	486 (369)	–	–	–
a		29.599	22.322	0.258
n		29.947	22.581	0.289
22*i*	500 (383)	30.763	23.190	0.124
a		–	–	–
n		31.248	23.558	0.167
23*i*	514 (387)	–	–	–
a		32.192	24.279	0.570
n		32.530	24.542	0.041
24*i*	528 (411)	33.314	25.150	0.821
a		–	–	–
n		33.777	25.517	0.199
Total (%)				(*i*) 10.949, (*a*) 4.281, (*n*) 84.774

[a] The symbols: *i*, *a*, *n* denote the *iso*-, *anteiso*- and normal-isomer, respectively; *M* is the molecular mass and I_b the ion used for area calculation.

Table 5
Retention time (t_R), ECL relative to *n*-acids and relative concentration of 2-hydroxy-fatty alcohols in the range of *N*: 14–24[a]

N	*M* (I_b)	t_R	ECL	%
14*i*	374 (271)	–	–	–
a		–	–	–
n		19.474	15.225	3.534
15*i*	388 (285)	–	–	–
a		–	–	–
n		20.953	16.178	0.085
16*i*	402 (399)	–	–	–
a		–	–	–
n		22.401	17.151	67.372
17*i*	416 (313)	–	–	–
a		23.406	17.849	0.168
n		23.782	18.112	0.214
18*i*	430 (327)	24.632	18.724	4.314
a		–	–	–
n		25.133	19.079	4.588
19*i*	444 (341)	25.988	19.695	0.259
a		26.121	19.788	0.682
n		26.487	20.062	–
20*i*	458 (355)	27.333	20.664	2.556
a		–	–	–
n		27.833	21.020	0.865
21*i*	472 (369)	–	–	–
a		28.809	21.738	2.250
n		29.172	22.000	0.342
22*i*	486 (383)	29.991	22.614	4.539
a		–	–	–
n		30.474	22.969	0.956
23*i*	500 (397)	31.282	23.584	0.136
a		31.419	23.688	2.771
n		31.752	23.936	0.050
24*i*	514 (411)	32.547	24.555	4.153
a		32.658	24.641	0.071
n		33.006	24.905	0.094
Total (%)				(*i*) 15.957, (*a*) 5.942, (*n*) 78.100

[a] The symbols: *i*, *a*, *n* denote the *iso*-, *anteiso*- and normal-isomer, respectively; *M* is the molecular mass and I_b the ion used for area calculation.

that each of the four families of compounds (FA, FAL, Hy-FA and Hy-FAL) contains three different isomer families (*i*, *a*, *n*) resulting in 12 homologue series to be studied (*i*-, *a*-, *n*-acids, *i*-, *a*-, *n*-alcohols, 2Hy-*i*-, 2Hy-*a*-, 2Hy-*n*-acids, and 2Hy-*i*-, 2Hy-*a*-, 2Hy-*n*-alcohols, respectively), the FCL behaviour is a function of integer ECL (denoted I(ECL)) for every homologous series (Fig. 3). The parameters of the function FCL versus I(ECL) are shown in Table 6. For each homologue series, the ΔN parameter (defined as

difference between I(ECL) and N) is calculated. In other words, ΔN describes the chromatogram position of each compound relative to the position of the normal fatty acid, having the same number of carbon atoms. For $\Delta N < 0$, the compounds will appear on the chromatogram before the n-FA with the same atom number (I(ECL) is lower than N_C). For $\Delta N > 0$, the compounds will appear on the chromatogram after the n-FA with the same atom number (I(ECL) is higher than N_C).

As can be seen, all of the functions (FCL = A-BI (ECL)) are, with a good approximation, a straight line ($r^2 > 0.87$, see Table 6 and Fig. 3). The function for the n-, i- and a-FA (E1, E2 and E3) are parallel lines FCL = 0.00, FCL $\cong$ 0.64 and 0.74, respectively, the retention parameter FCL being the same when N increases. The FCL function of n-, i- and a-FAL (E4, E5 and E6) also are approximately parallel but with a negative slope suggesting that the compound polarity is decreasing, faster than the acids, when N is increasing.

The straight lines corresponding to i-, a-, n-Hy-FA (E7, E8 and E9) and to i-, a-, n-Hy-ALC (E10-1, E10-2; E11 and E12) are also parallel for the same family, but the slope for Hy-ALC is highest. The unexpected negative slope for Hy-FA and Hy-FAL is due probably to a lower polarity of these compounds given by the screening effect of the two TMS groups on the oxygen atoms. The change of ΔN from +1 to 0 for 2Hy-nFAL with $N_C = 21$ (I(ECL) = 22) is to be noted. This is equivalent to the fact that 2Hy-nFAL with $N_C = 21$ will appear in the chromatogram superimposed with the n-FA, having the same number of carbon atoms.

From the intersection points of the curves (Fig. 3), the chromatographic positions with superimposed

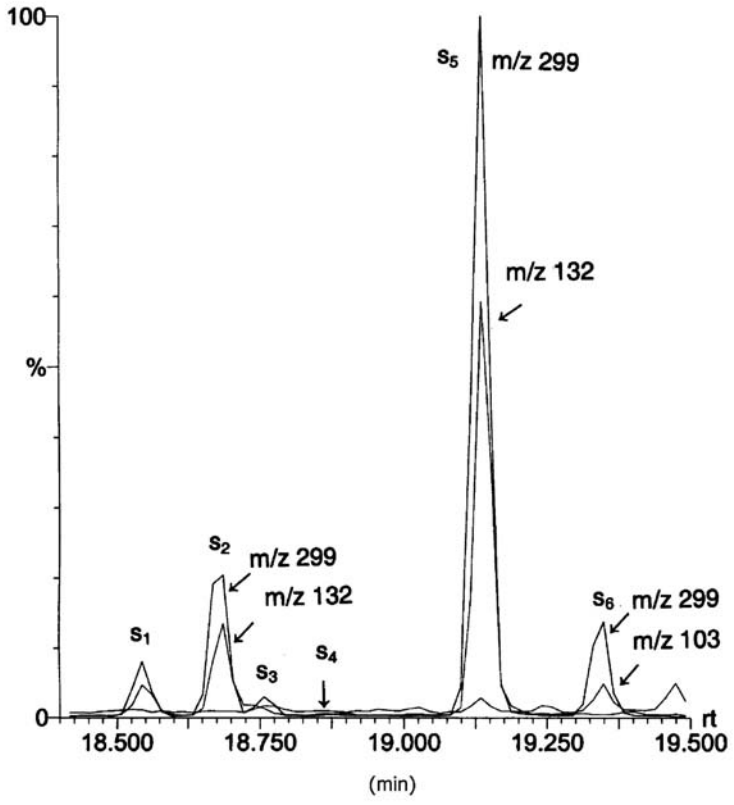

Fig. 4. Ion chromatogram of FA and FAL in the I(ECL) range of 14–16 with m/z 299 (FA + FAL), m/z 132 (only FA), m/z 103 (only FAL). Symbols: S_1, i-FA C_{15}; S_2, a-FA C_{15}; S_3, i-FAL C_{16}; S_4, a-FAL C_{16}; S_5, n-FA C_{15}; S_6, n-FAL C_{16}.

compounds can be deduced. We will discuss some situations, which involve the coelution of compounds of major concentration (S_1–S_6 structures).

In the range of I(ECL) 12–16 the curves corresponding to acid and alcohol isomers (E1–E6 in Fig. 3) do not intersect and therefore, the corresponding chromatogram peaks (structures S_1–S_6) are completely resolved (resolution > 1). The elution sequence is the following: *i*-acid (S_1), *a*-acid (S_2), *i*-alcohol (S_3), *a*-alcohol (S_4), *n*-acid (S_5) and *n*-alcohol (S_6). The acids have N carbon atoms but the alcohols have $N + 1$. Figs. 4–6 shows the separation of acids C_{15} and alcohol C_{16} (structures S_1–S_6), by mass fragmentogram m/z 299.

If I(ECL) increases above 16, the distance between the curves E3 (*a*-acids) and E5 (*i*-alcohols) decreases and chromatographic resolution for a-acids (S_2) and *i*-alcohols (S_3) decrease as results from Fig. 3. For I(ECL) 20, the intersection of the curves E3 and E5 can be observed. This corresponds to the *a*-acid C_{21} (E3) and the *i*-alcohol C_{22} (E5) which are superimposed in the chromatogram ($S_2 + S_3$).

For I(ECL) 24 the elution sequence for the structures S_2 (*a*-acids) and S_3 (*i*-alcohols) is changed. The *i*-alcohol C_{26} (S_3) is situated before the *a*-acid C_{25} (structure S_2).

As resulting from Fig. 3 for I(ECL) 32 three pairs of FCL curves intersect: E3 (*a*-acids) with E6 (*a*-alcohols); E2 (*i*-acids) with E5 (*i*-alcohols) and E1 (*n*-acids) with E4 (*n*-alcohols). Therefore, it is to be expected that the compounds of structures S_1–S_6 to interfere as pairs of acids and alcohols of the same isomer type (*i*-acid + *i*-alcohol, *a*-acid + *a*-alcohol, *n*-acid + *n*-alcohol) producing three chromatographic peaks ($S_1 + S_3$), ($S_2 + S_4$) and ($S_5 + S_6$). In Fig. 7 the detection of all acids (m/z 132) and alcohols (m/z 103) isomers corresponding to m/z 509 (acids C_{30} + alcohols C_{31}) are shown. Knowing the positions

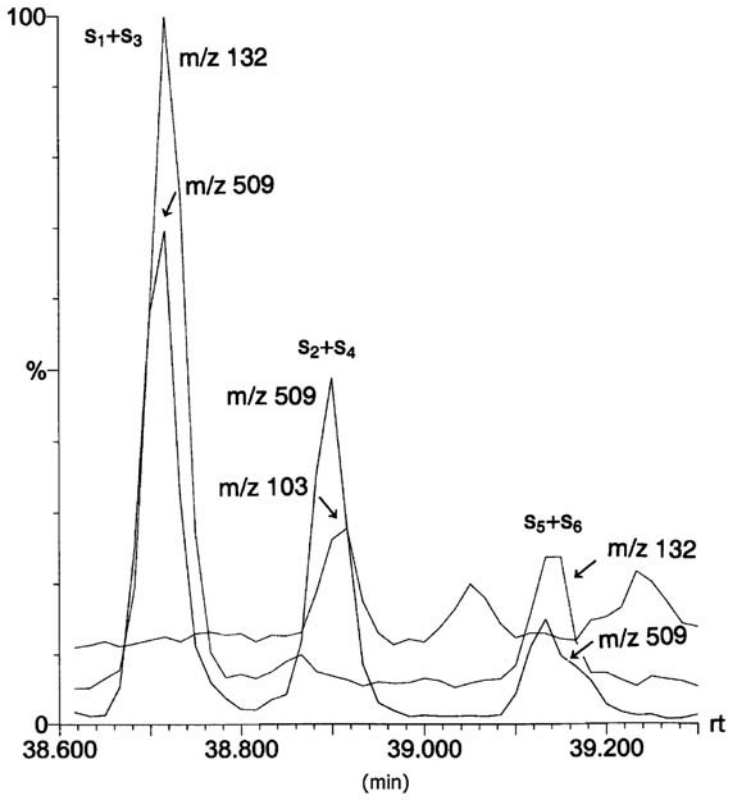

Fig. 5. Ion chromatogram of FA and FAL in the I(ECL) range of 29–30 with m/z 509 (FA + FAL), m/z 132 (only FA), m/z 103 (only FAL). Symbols: S_1, *i*-FA C_{30}; S_2, *a*-FA C_{30}, S_3, *i*-FAL C_{31}; S_4, *a*-FAL C_{31}; S_5, *n*-FA C_{30}; S_6, *n*-FAL C_{31}.

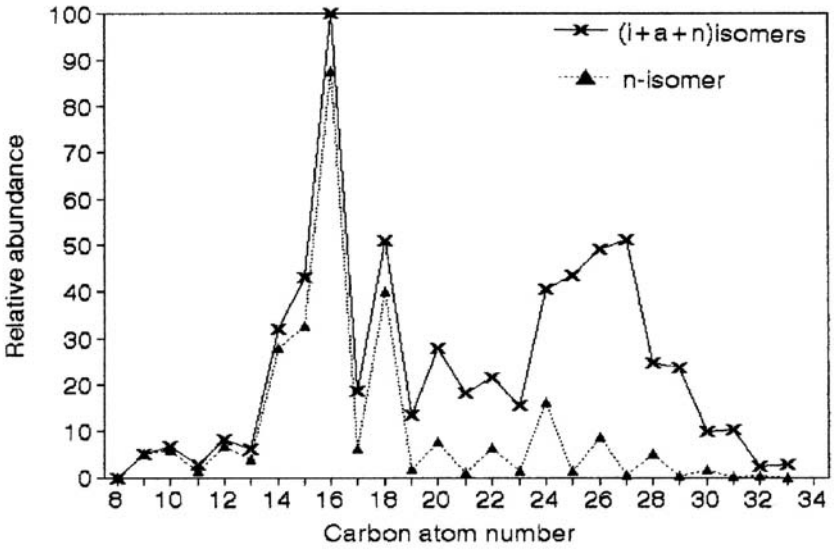

Fig. 6. The molecular distribution of the FFA in the wool wax sample.

with superimposed structures, the compound determination can be performed by mass spectrometric diagnostic ions.

3.3. Molecular distribution

3.3.1. Wool wax FFA distribution

The distribution of FFA (containing *n*-, *i*- and *a*-isomers) as relative abundance (mol%) versus carbon atom number in the range of C_9–C_{33} is shown in Fig. 8. The same carbon atom range was reported for other natural waxes originating from plant [41], desmosponges [40] or microalgae [6]. The *i*- and *a*-FA were found also in lanolin samples after hydrolysis [42,91] or after pyrolysis [44]. In the range of C_9–C_{18} the major contribution is from *n*-chain compounds and two distinct maxima were observed at C_{16} and C_{18}. These maxima reflect probably the utilisation of

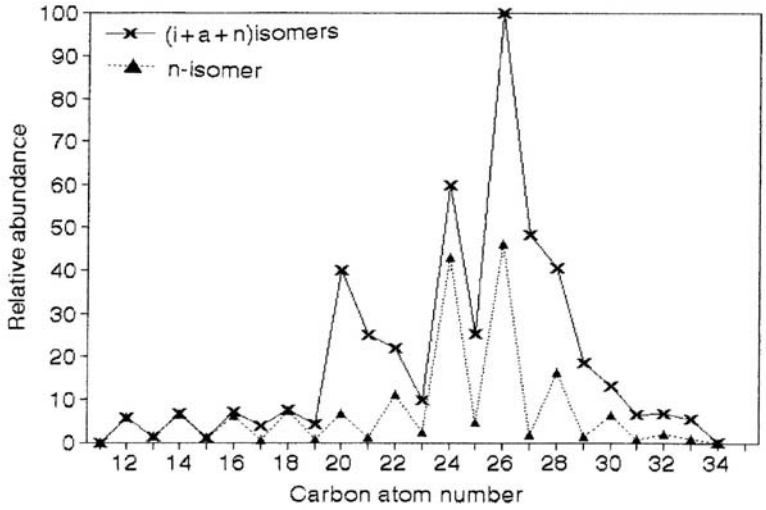

Fig. 7. The molecular distribution of the FFAL in the wool wax sample.

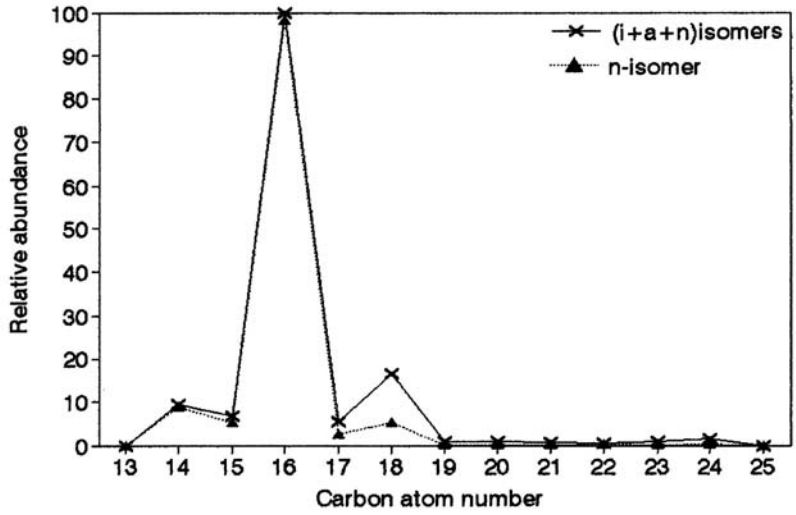

Fig. 8. The molecular distribution of the 2Hy-FA in the wool wax sample.

acyl chains up to C_{16}–C_{18} as produced initially by synthesis de novo. In the range of C_{19}–C_{33}, *iso*- and *anteiso*-isomers predominate and a broad maximum in the range of C_{24}–C_{27} can be seen. This maximum can be explained by the hypothesis that the acyl chain produced is utilised in subsequent elongation [41].

The FFAs are quantitatively distributed between the three groups of isomers as follows: normal-FA 42.88%, *iso*-FA 26.83% and *anteiso*-FA 30.28%. The almost equal quantity of the *iso*- and *anteiso*- isomers is to be noted.

3.3.2. *Free fatty alcohols*

The molecular distribution of the free wool wax fatty alcohols is shown in Fig. 7 (mol% versus carbon atom number). The carbon atom range of the FFAL

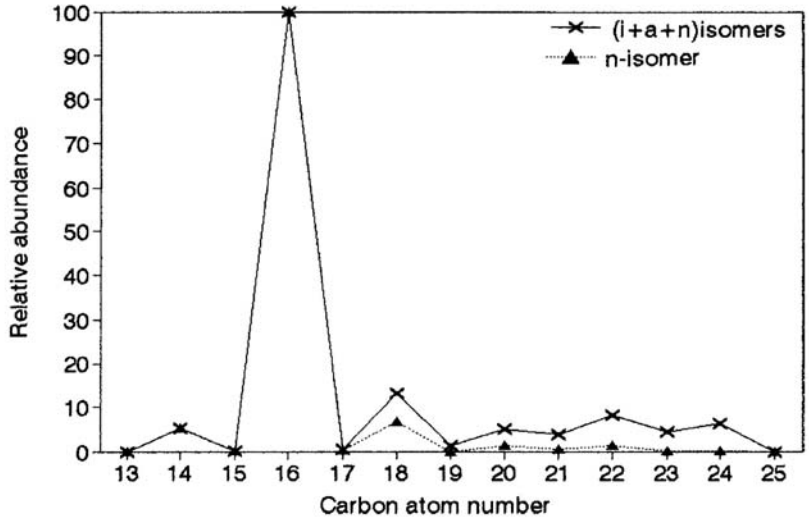

Fig. 9. The molecular distribution of the 2Hy-FAL in the wool wax sample.

is C_{12}–C_{34} of which C_{24}–C_{28} homologues were most abundant. The species with even carbon atom are predominant.

The overall range of FFAL was similar to that found for free acids, but the distribution was different having a maximum at C_{26} rather than C_{16}, as found for acids. An equiponderance of the *i*-, *a*- and *n*-isomers was found (34, 28 and 38%, respectively, Table 3).

Recently, it was reported that some free alcohols from wool wax samples as *i*-hexadecanol and *a*-heptadecanol to have a remarkable antitumor activity [92]. This activity was attributed to the molecular hydrophobicity of specified alkyl moiety bulkiness balanced with a hydroxyl group.

3.3.3. Free 2Hy-fatty acids and 2Hy-fatty alcohols

The molecular and isomer distribution of 2Hy-FA and 2Hy-FAL are shown in Figs. 8 and 9, respectively. The overall range is practically C_{14}–C_{18} for Hy-FA and C_{14}–C_{24} for Hy-FAL. In every situation, the normal compounds were dominant (>78%, *n*-compounds). In the range of C_{20}–C_{24} of Hy-FAL the branched isomers ($i + a$) dominate. This distribution can be sustained by the fact that they derive from the biosynthesised diesters as product of the lanolin hydrolysis occurring naturally on the wool after secretion by sebaceous glands [74,75]. The great concentration of the Hy-FA and Hy-FAL with 16 carbon atoms (>67%) is due probably to high value of hydrolysis rate constant of the esters of 2Hy-FA with C_{16} [75].

4. Conclusions

(1) The overall range of FFA and FFAL in samples isolated from wool wax is C_9–C_{33}. The range of the free 2-hydroxy fatty acids and 2-hydroxy fatty alcohols is more narrow, C_{14}–C_{24}. All classes of compounds consist of *i*-, *a*- and *n*-isomers.

(2) The TMS derivatives appear to be suitable for the determination of FA, FAL, Hy-FA and Hy-FAL by GC/MS methods for the overall carbon atom range. The mass spectra of TMS derivatives show diagnostic fragments of high intensity $[M - 15]^+$ for FA and FAL and $[M - 117]^+$ and $[M - 103]^+$ for 2Hy-A and 2Hy-AL, respectively.

(3) The separation of FA, FAL, Hy-FA and 2Hy-FAL as TMS derivatives (each class including *i*-, *a*- and *n*-isomers) can be performed on a low polarity capillary column coated with 5% phenylmethyl polysiloxane. The coeluting compounds can be foreseen from the curves of FCL versus I(ECL) for homologues series (cross-points).

(4) In 2Hy-FA and 2Hy-FAL family, the *n*-isomers dominate with >78% (the 2Hy-FA and 2Hy-FAL with C_{16} being in concentration around of 68% each).

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VOLATILE FATTY ACIDS AS MALODOROUS COMPOUNDS IN WOOL SCOURING WATER AND LANOLIN. ORIGIN AND CHARACTERISATION

E. Jover*, M. Ábalos, L. Ortiz and J. M. Bayona

Environmental Chemistry Department I.I.Q.A.B.-C.S.I.C., Jordi Girona 18-26, E-08034 Barcelona, Spain

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ABSTRACT

Volatile fatty acids (C_2-C_7) analysis in wool scouring water and lanolin is presented. These substances are of major interest as malodorous compounds in urban and industrial wastewaters. In this work, they have been analysed in wool scouring water by headspace solid-phase microextraction followed by gas chromatography negative chemical ionisation mass spectrometry. Most of the volatile fatty acids have been identified at $\mu g\ g^{-1}$ levels. In addition, since lanolin is a major impurity of raw wool, volatile fatty acid patterns of wool scouring water and lanolin have been compared in order to establish the origin of these compounds in the wastewater. Finally, the efficiency of the deodorization step, mandatory to obtain commercial lanolin, has been assessed taking into account the decrease in volatile fatty acid content from the raw wool to the lanolin.

Keywords: Wool scouring water, lanolin, volatile fatty acids, solid phase microextraction

INTRODUCTION

In the wool scouring process raw wool is treated with hot water containing sodium carbonate and non-ionic surfactants to obtain clean fibers appropriate for the textile industry. The final effluent of this process is a dark, highly polluted and malodorous slurry with a complex composition [1]. Wool grease in emulsion is among the major components of this wastewater (i.e. concentration of 0.5% or higher) [2]. This material has been characterised as a mixture of sterols, esters and straight and branched chain fatty acids and alcohols [3-5].

The high organic content of wool scouring effluents may cause major pollution problems. For this reason, several methods have been proposed for their treatment (i.e. anaerobic biodegradation [6,7] or sequential anaerobic-aerobic digestion [8] and ultrafiltration [9, 10]). In addition, odorous emissions from the wool scouring wastewater could give rise to a serious annoyance at the workplace itself and in its vicinities. The high-temperature of this wastewater favour the water/air equilibrium of the volatile substances present, enhancing odour concentration.

Among the large number of substances that are related to odour problems, volatile fatty acids (VFAs) might be the principal responsible for odour generation in wool scouring water. These compounds are biodegradation products of the fermentation of sugars, proteins and fats. In this way, they have been identified as the main odorous components of a wide range of industrial wastewaters, such as distilleries [11], sugar refineries [12, 13] or dairy industries [14] effluents. In the case of wool scouring water, the VFAs will originate from the degradation of higher molecular weight fatty acids of the wool grease. Moreover, large amounts of VFAs have been found in animal faeces [15], which are usually present in raw wool and therefore they also become an important component of the considered effluent.

The main objective of this study was to assess the presence of VFAs in wool scouring waters and to compare the levels with those that are usually found in urban wastewaters. VFAs have also been analysed in wool grease (lanolin) in order to confirm whether this matrix is the origin of these compounds in the effluent or not. For this reason, the correspondent patterns have been compared. In addition, determination of the VFA content in lanolin completes a previous study carried out by our group on the characterisation of long-chain fatty acids in wool wax [16]. To the best of our knowledge, there are no previous studies trying to identify the main substances responsible for odour generation either in lanolin or in wool scouring wastewater. Headspace solid phase microextraction (SPME) followed by gas chromatography negative chemical ionisation mass spectrometry (GC-NCI-MS) has been used as the method to

determine VFAs in the two different matrices. This methodology was previously developed in our group [17] and its suitability in the analysis of wastewater samples has been proved [18].

EXPERIMENTAL

Standards and Reagents

Sodium chloride 99.5 % was provided by Carlo Erba (Milan, Italy). Hydrochloric acid 25 % was from Merck (Darmstadt, Germany). Acetic, propionic, butyric, valeric, iso-valeric, hexanoic, heptanoic and ethyl butyric acid, all >99%, were provided by Aldrich (Steinheim, Germany). Ethyl butyric acid was used as internal standard (I.S.). Raw wool was generously gifted by Peinajes del Llobregat (Spain). Lanolin was Corona Lanolin, a refined wool wax from Croda, (Snaith Goole, UK). Mature urban wastewater came from Les Franqueses del Vallés (North East Catalonia, Spain), a sample was collected following a sedimentation tank working without sludge removal. Helium 5.0 grade from Air Liquide (France) was used as carrier gas. Reagent gas for ionisation was ammonia electronic grade from Air Liquide (France). SPME fiber was a Carboxen™ polydimethylsiloxane of 75 μm thickness from Supelco (Bellefonte, PA, USA).

Sample Preparation

A stock mixture was prepared containing 2500 $\mu g\ g^{-1}$ of acetic, propionic and butyric acids and 500 $\mu g\ g^{-1}$ of valeric, isovaleric, hexanoic and heptanoic acids. Standard solutions were prepared by dilution of the stock mixture; and both solutions were stored at 4°C.

Simulated wool scouring water was prepared as follows. Samples were weighed (~0.5 g of raw wool) in a 40 ml vial; around 25ml Milli-Q water and a magnetic stir bar were added. Each sample was mixed at 1200 rpm for 10 min at 80°C using an electronically-controlled magnetic stirring Ikamag® RCT Basic with heating and an electronic contact thermometer Ikatron® ETS-D4 fuzzy, provided by Ika (Staufen, Germany).

Around 10 ml of the resulting solution were transferred through a septum to another 40 ml vial containing a magnetic stirring bar and 3.75 g of sodium chloride, using a 10 ml syringe. The sample was cooled at 25°C, and 25 μl of I.S. (60 $\mu g\ \mu l^{-1}$) and 50 μl of hydrochloric acid 2 M were introduced through a septum. Headspace SPME extraction was carried out for 20 min at 25°C in headspace as reported elsewhere [17, 18].

For lanolin VFAs analysis, ~0.35 g of lanolin was weighed in a 40 ml vial sample and was mixed with 25 ml of Milli-Q water at 1200 rpm for 10 min at 80°C. Thereafter, the same procedure as for raw wool samples was carried out.

For the analysis of urban wastewater 2.5 g of wastewater were directly weighed in a 40 ml vial containing 3.75 g of sodium chloride and 7.5 g of Milli-Q water, then I.S. and hydrochloric acid were added and the sample was directly extracted by SPME.

Instrumental Analysis

A TR-FFAP capillary column coated with ethyleneglycol esterified with nitroterephtalic acid of 0.25 μm of film thickness, 0.25 mm of I.D. and 30 m of length was provided by Tecknokroma (Sant Cugat del Vallès, Spain).

A GC 6890A from Agilent Technologies (Palo Alto, CA, USA) coupled to an MS 5973N was used. Gas chromatographic was run at a constant flow rate of 1 ml min^{-1} of helium. Quadrupole and transfer line temperatures were held at 150°C and 180°C respectively. Reagent gas was maintained at a pressure of $14.8 \cdot 10^{-5}$ torr in the analyser. MS was run in the negative polarity using SIM-mode. SPME fiber was desorbed at 300°C in the splitless mode activating the injector purge at 5 min. Initial column temperature was held at 70°C for 1 min and then programmed at 10°C min^{-1} to 200°C and was held for 1 min.

Identification and Quantification Methods

Identification of target compounds have been carried out using diagnostic ions in SIM-mode and comparing obtained retention times with standard ones.

Quantification of wool scouring water VFAs was carried out by external calibration in SIM-mode because no matrix effect was observed for I.S. response. R^2 for calibration were between 0.988 and 0.999 for all the target compounds.

VFAs from lanolin have been quantified by standard addition because I.S. showed a lower response compared to standards analysis. For quantification five point addition standards plots were used obtaining R^2 of 0.98 for acetic acid due to its poor response, for the other compounds better results were obtained $0.993<R^2<0.999$.

RESULTS AND DISCUSSION

VFA Levels in Wool Scouring Water

To simulate the common wool scouring conditions, raw wool was treated with water at at 80°C. This high temperature could be an important parameter to make VFAs available to participate in water/air equilibrium since it allows wool grease to melt and therefore, releases compounds from this grease, which give rise to odour annoyance. In order to calculate the VFA amounts in wool scouring wastewater, it was assumed that the wool/water ratio in the cleansing process is 1/10 (w/w) [8]. Levels of mg l^{-1} were found for the majority of VFAs or of $\mu g\ g^{-1}$ if referred to by wool weight, acetic and iso-valeric acids being the compounds which showed the highest concentrations. A summary of the VFA results obtained for the analysed wool scouring water is presented in Figure 1. These concentrations, if considered in

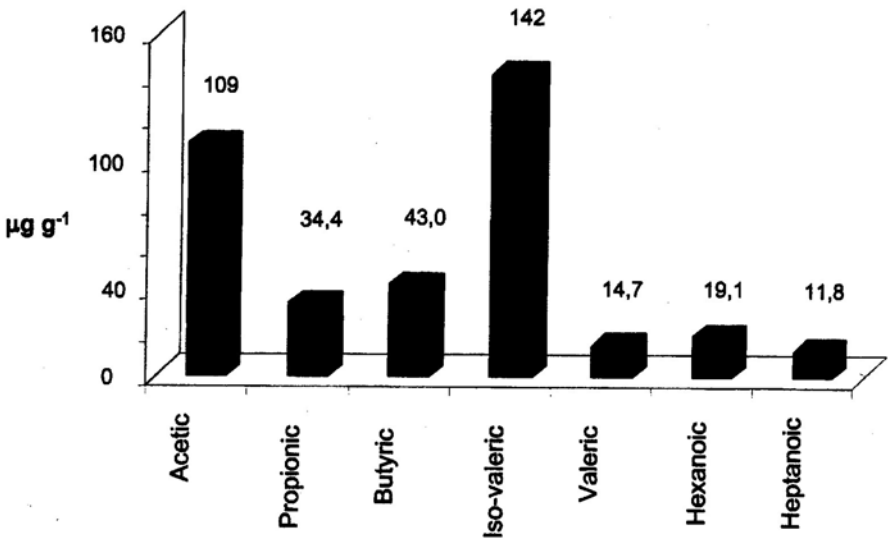

Figure 1. VFA content in wool scouring water in $\mu g\ g^{-1}$ of raw wool.

mg l^{-1} were similar to the ones observed in fresh and mature urban wastewater for acetic acid and higher in the case of iso-valeric acid.

VFA Pattern in Wool Scouring Water

It was expected that VFAs present in wool scouring water came from lanolin. In a previous work our group studied the distribution pattern of free fatty acids (FFAs), ranging from 9 to 33 carbon atoms, in lanolin. The FFA pattern was characterised for a monomodal distribution, where the highest percentages observed belong to FFAs with 16 and 18 number of carbons. Therefore, on the assumption that VFAs originate from lanolin, a decrease in the concentration was expected with the decrease of carbon atoms in the acids, apart from the exceptionally high acetic acid content coming from long chain fatty acids degradation. In this sense, hexanoic and heptanoic acids contribution to the VFA pattern was found to be around 15 times higher than in mature urban waste water (Figure 2), these VFAs could be considered as coming from lanolin. However, a high iso-valeric content was also observed. This could be attributed to

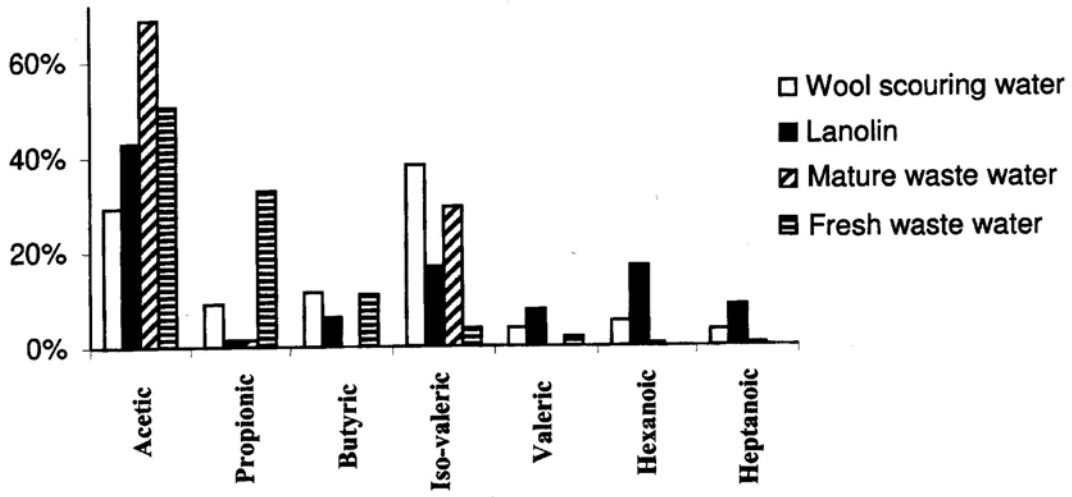

Figure 2. Comparison between VFAs patterns in different samples, fresh urban wastewater, mature urban wastewater, lanolin and wool scouring water.

the biological degradation of proteins and to the lower biodegradation rate of iso-valeric itself. The VFA pattern obtained for wool scouring water was more similar to mature urban wastewater than to fresh urban wastewater. High iso-valeric and acetic acids levels and low butyric, propionic and longer normal acids also characterised mature wastewater. On the contrary, fresh wastewater showed a very regular pattern similar to an inverse exponential function with higher amounts of the smallest VFAs [19].

VFA Pattern in Lanolin

The VFA lanolin pattern showed some differences compared to the wool scouring water pattern, although acetic acid was also the most important VFA (Figure 2). Apart from this fact, the obtained pattern was consistent with the behaviour previously found for the higher molecular weight FFAs evaluated. Thus, in general the relative contribution of each acid increases with the carbon number. However, iso-valeric acid proportion was still too high and the ratio between the concentration of valeric and iso-valeric acids was below the unit. In contrast, for longer odd FFA, the opposite situation was observed. The origin of large amounts of the iso isomeric form of the C-5 acid could be protein degradation, coming from cross faecal contamination. Even thought the analysed lanolin is a refined wool wax, the refining process cannot completely remove the original contamination due to sheep living conditions; therefore, other sources of VFAs should be considered, apart from degradation of long-chain fatty acids present in wool grease, in order to correctly explain the pattern of these compounds in lanolin.

Lanolin Deodorising Step

During lanolin processing, a deodorization step is usually carried out in order to eliminate bad odour compounds. One of the aims of this work was to check if the deodorization step gives rise to a decrease in the VFA levels present in lanolin. The lanolin content in wool varies depending on sheep species, sheep origin and also the part of the body from where the wool originates, in any case grease can represent between 20% and 50% of whole wool weight. In order to calculate the minimum and maximum decrease in lanolin VFA levels for both extreme conditions were considered.

A decrease, ranging from 10% to 98%, was observed for all VFAs, being more important when the carbon chain becomes shorter. This could be related to the fact that shorter chain fatty acids are more volatile than longer ones. VFAs reduction data are shown in Figure 3.

It can be concluded that VFAs are eliminated in part during the wool scouring process itself, due to the high temperatures applied, and also in a second stage, in the deodorization lanolin process, which seems to act as a stripping or distillation process improving loss of the lower molecular weight compounds. However, the behaviour of acetic acid did not agree with this assumption because its

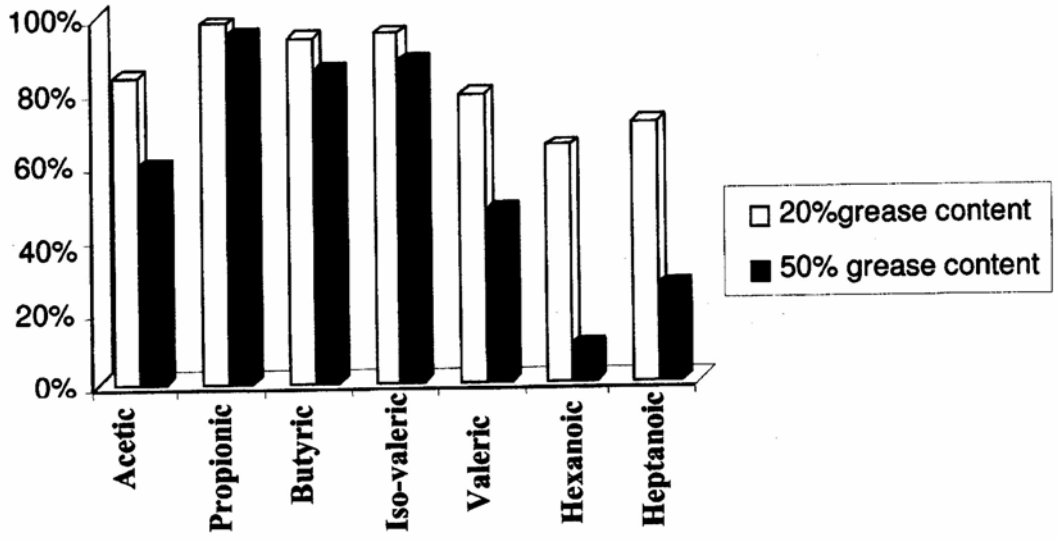

Figure 3. Rate of VFA decrease from wool to lanolin correcting by the amount of lanolin in wool (considering the two extreme cases 20% and 50%).

percentage of decrease was lower than theoretically expected (i.e. 60% to 85% instead of 95%). These results suggest that acetic acid is formed from FFA biological degradation after the lanolin refining process. A deodorization step will eliminate the VFAs depending of their volatility, so a significant acetic acid decrease is expected to take place during the process. However, after the deodorization step FFA biodegradation continues with the generation of acetic acid by β-oxidation, the other VFAs needing much more time to appear, therefore their decreasing rates are more according to the theory.

CONCLUSIONS

In this work, VFAs of wool scouring water and lanolin have been determined. Origin of bad odours during wool cleansing have been confirmed to be due to high amounts of VFAs, mainly acetic and iso-valeric acid. Also wool scouring water VFAs pattern have been explained by means of biochemical reactions and comparison with other VFAs patterns from fresh and mature urban waste water. Lanolin is obtained from the refining process of wool grease. During this refining process wool grease follows a deodorization step, and the efficiency of this step has been evaluated for VFAs elimination obtaining mean decreasing rates of 71%. Finally, this work has also completed an earlier study about FFA characterisation of lanolin [16] contributing to the determination of this complex mixture.

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3.1.2 Èsters alifàtics i esteroidals

El primer article, "*Systematic characterisation of long-chain aliphatic esters of wool wax by gas chromatography-electron impact ionisation mass spectrometry*", presenta la caracterització dels èsters alifàtics per HTGC-MS posant especial èmfasi en comprendre el comportament dels diferents isòmers presents.

El segon article, "*Complete characterisation of lanolin steryl esters by sub-ambient pressure gas chromatography-mass spectrometry in the electron impact and chemical ionisation modes.*", descriu la caracterització dels èsters esteroidals de la lanolina, sabent que aquests compostos són termolàbils. Per aquest motiu, es va decidir utilitzar la cromatografia de gasos a pressió subambient acoblada a un espectròmetre de masses.

Aquests dos treballs s'han realitzat amb la col·laboració del Dr. Z. Moldovan del National Institute for Research and Development for Isotopic Molecular Technologies de Cluj (Rumania).

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Systematic characterisation of long-chain aliphatic esters of wool wax by gas chromatography–electron impact ionisation mass spectrometry☆

Zaharie Moldovan[a], Eric Jover[b], José Maria Bayona[b,*]

[a]National Institute for Research and Development for Isotopic and Molecular Technologies, P.O. Box 700, 3400 Cluj-Napoca 5, Romania

[b]Environmental Chemistry Department (I.I.Q.A.B.–C.S.I.C), Jordi Girona 18–26, E-08034 Barcelona, Spain

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Abstract

A detailed structural characterisation of the aliphatic high-molecular-mass esters extracted from raw wool based on high-temperature gas chromatography–electron impact ionisation mass spectrometry is described. The raw wool esters extracted are in the range of C_{37} to C_{54} (i.e., molecular mass 550–788). The selected ion chromatogram exhibited four isomers for the esters with an odd number of carbon atoms (*i*:*a*, *i*:*n*, *a*:*n* and *n*:*n*) and five for those with an even number of carbon atoms (*i*:*i*, *a*:*a*, *i*:*n*, *a*:*n* and *n*:*n*). Isomeric structural elucidation is discussed with respect to the long-chain fatty acid and long-chain fatty alcohol structures, on the basis of chromatographic retention behaviour and mass spectral information.

Keywords: Waxes; Retention behaviour; Lanolin; Fatty acids; Fatty alcohols; Esters

1. Introduction

Wool wax, known as lanolin, is a complex mixture of high-molecular-mass lipidic compounds [1–8]. It is an important ingredient for the manufacture of cosmetics, toiletries and pharmaceuticals [2–5,7,9,10]. Its specific use can be determined only by the characterisation of its individual classes of components.

Previously published papers dealing with lanolin characterisation have mainly focused on either the individual fatty acids and alcohols obtained by complete hydrolysis of the lanolin ester mixture or the global lipidic classes [6,11–14]. Only limited data have been reported on aliphatic esters structures because of their extremely high complexity. More than 10 000 mono-esters may result from the combination of lanolin alcohols and lanolin acids, assuming a completely randomised combination and no preferential esterification in lanolin biosynthesis in the sheep sebaceous gland [7].

The aim of the present work is to study by gas chromatography–mass spectrometry (GC–MS), the

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*Corresponding author. Tel.: +34-93-4006-100; fax: +34-93-2045-904.

E-mail address: jbtqam@iiqab.csic.es (J.M. Bayona).

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specificity of the ester biosynthesis process in which high-molecular-mass fatty acids combine with fatty alcohols. Therefore, we have developed a high-temperature capillary gas chromatography–electron impact ionisation mass spectrometry (HT-GC–EI-MS) method for the structural characterisation and quantification of the long-chain aliphatic esters obtained from lanolin by supercritical fluid extraction (SFE) combined with gel permeation chromatography (GPC) fractionation. Ester structures were elucidated using chromatographic retention data and the EI-MS information obtained by GC–MS.

2. Experimental

2.1. Samples

Lanolin samples were isolated from raw wool by SFE with carbon dioxide (CO_2) in the presence of a modifier, according to a previously described procedure [15]. Real samples enriched in aliphatic esters were selected for this study. The SFE extract was collected in an ethyl acetate–cyclohexane (1:1) mixture and filtered through a 0.45 μm nylon membrane. Filtered samples were fractionated using an LC system (see Section 2.2).

2.2. Instrumentation

The LC system used was from Shimadzu (Kyoto, Japan) equipped with a Rheodyne high-pressure valve with a 100 μl loop, LC-10AT pumps, a UV detector SPD-10AV, SCL-10A and Class-VP software. The GPC column used was 450 mm×10 mm I.D. and packed with Bio Beads SX-3, 200–400 mesh from Bio-Rad (Hercules, CA, USA). Ethyl acetate–cyclohexane (1:1) at a 2 ml min^{-1} flow-rate as the mobile phase was used and the first fraction was collected in the time interval from 0 to 8 min.

The HT-GC–MS analyses were performed using a Fisons MD 800 mass spectrometer (Fisons, Loughborough, UK). The instrument was operated in the EI mode at 70 eV. Chromatographic conditions were as follows: injection was in the splitless mode at 330 °C. The GC column was a polyimide-clad polycarborane–dimethylsiloxane column (HT 5, SGE, Ringwood, Victoria, Australia) of 10 m×0.32 mm I.D. and 0.10 μm film thickness. Column temperature was programmed from 90 °C (1 min) to 240 °C at 10 °C min^{-1} and then to 360 °C at 5 °C min^{-1} and holding the final temperature for 5 min (total analysis time 45 min). Helium was the carrier gas at an inlet pressure of 12 p.s.i. (1 p.s.i=6894.76 Pa) (i.e., 1 ml min^{-1} flow-rate). Transfer line and ion source temperatures were held at 320 and 230 °C, respectively.

3. Results and discussion

The identification of the long-chain esters was based on (a) the interpretation of mass spectra of the individual compounds obtained by GC–MS, and (b) the chromatographic retention data.

The characterisation of the wool long-chain esters presents great difficulty because of the high number of compounds, as aliphatic or steryl ester type, which may occur in the samples [2–5,7,8,10,16]. The wool wax aliphatic alcohols and acids consist of (*normal*) (*n*) *iso* (*i*) and *anteiso* (*a*) series [2,4,5,10,16]. The *iso* series contains an isopropyl terminal group and the *anteiso*, a secondary butyl terminal group. Compounds containing an odd carbon number comprise *n* and *a* structures and the ones with an even carbon number, *n* and *i* structures. As a consequence, in the ester biosynthesis process, there is a distinct possibility of aliphatic ester formation by the combination of acids and alcohols of different structure and size.

We propose the following nomenclature:

(1) [O], [E] for the aliphatic esters containing an odd or even number of carbon atoms, respectively.

(2) [O_i], [E_i] for the aliphatic acid and alcohol moiety containing an odd or even number of carbon atoms, respectively, where the *i* value can be 1 or 2 and denotes the acid or alcohol moiety, respectively.

(3) a_i, i_i, n_i the *anteiso*, *iso* and *normal* structures, where $i=1$ for the acid chain and $i=2$ for the alcohol chain.

(4) [x_1:y_2] denoting the isomeric structures of esters, where *x* and *y* can be *a*, *i* or *n* for the acid ($i=1$) or the alcohol ($i=2$) moiety, respectively.

(5) *N* (*k*:*m*) for an ester with *N* total carbon atoms, *k* atoms in the acid and *m* in the alcohol moiety.

The general structural scheme of all the isomeric

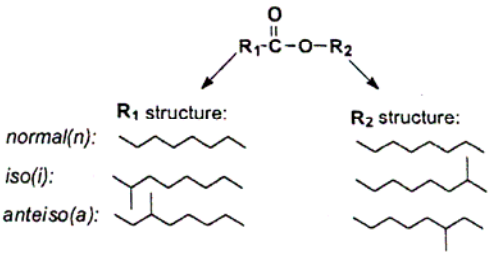
aliphatic esters and the notation used are shown in Fig. 1.

Taking into account that an acid or alcohol of the $[O_i]$ type can have an a_i or n_i structure and that an acid or alcohol of the $[E_i]$ type can have an i_i or n_i structure, the resultant ester formed by biosynthesis can be deduced.

An aliphatic ester [O], with an odd number of carbon atoms, can be formed from an acid $[O_1]$, with an odd number of carbon atoms, and an alcohol $[E_2]$, with an even number of carbon atoms; or from an acid $[E_1]$, with an even number of carbon atoms, and an alcohol $[O_2]$, with an odd number of carbon atoms, as follows:

$$[O] = [O_1]:[E_2] = [a_1, n_1]:[i_2, n_2] = [a_1:i_2] + [a_1:n_2] + [n_1:i_2] + [n_1:n_2] \quad (1)$$

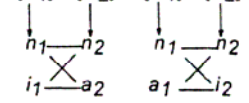
$$[O] = [E_1]:[O_2] = [i_1, n_1]:[a_2, n_2] = [i_1:a_2] + [i_1:n_2] + [n_1:a_2] + [n_1:n_2] \quad (2)$$



Symbols:
k and **m** are the number of carbon atoms in acid and alcohol moiety, respectively.
[E1](if **k** is even) and **[O1]**(if **k** is odd) for acid moiety,
[E2](if **m** is even) and **[O2]**(if **m** is odd), for alcohol moiety,
[E](if **N** is even) and **[O]**(if **N** is odd) for the intact esters.
N(=k+m) is the number of carbon atoms in the intact ester;

Isomeric structures of **[O]** type esters:

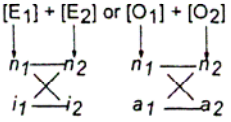
[O] = [E1] + [O2] or [O1] + [E2]



[n1:n2]+ [n1:a2]+ [i1:n2]+ [i1:a2]
[n1:n2]+ [n1:i2]+ [a1:n2]+ [a1:i2]

Isomeric structures of the **[E]** type esters:

[E] = [E1] + [E2] or [O1] + [O2]

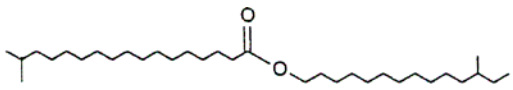


[n1:n2]+ [n1:i2]+ [i1:n2]+ [i1:i2]
[n1:n2]+ [n1:a2]+ [a1:n2]+ [a1:a2]

Ester notation:

-by the carbon atom number: **N(k:m);**
-by the sort of carbon atom number: **[O]=[E1:O2], [O]=[O1:E2], [E]=[E1:E2], [O]=[O1:O2];**
-by the isomer type of acid and alcohol moiety: ***[x1:y2]***, where ***x,y*** can be ***i, n*** or ***n*** isomers, for acid and alcohol moiety, respectively; the indice **1** is for acid and **2** for alcohol moiety.

Example



k=18, m=15, N=33
Symbols: 33(18:15); [O]=[E1:O2]; [i1:a2]

Fig. 1. The general structure scheme of aliphatic ester isomers from lanolin sample and the symbolism used.

In the same way, an ester [E], having an even number of carbon atoms, can be synthesised from an acid [O_1] and an alcohol [O_2], or from an acid [E_1] and an alcohol [E_2], as:

$$[E] = [O_1]:[O_2] = [a_1, n_1]:[a_2, n_2] = [a_1:a_2] + [a_1:n_2] + [n_1:a_2] + [n_1:n_2] \quad (3)$$

$$[E] = [E_1]:[E_2] = [i_1, n_1]:[i_2, n_2] = [i_1:i_2] + [i_1:n_2] + [n_1:i_2] + [n_1:n_2] \quad (4)$$

As can be seen from Eqs. (1)–(4), eight different isomeric structures for a given molecular mass are theoretically possible.

3.1. Mass spectra of aliphatic long-chain esters

The base peaks in the mass spectra of the long-chain esters are produced by a rearrangement process involving the transfer of 2H atoms from the alcohol chain to the acid chain giving a protonated acid ion [17,18]. The molecular ion M^+ has an intensity in the range of 20–25%. The base peak gives the number of carbon atoms in the acid moiety, $k=(m/z-33)/14$ and the M^+, the total number of carbon atoms, $N=(M-32)/14$. The mass spectrum of the *normal* ester 38 (16:22) (docosanoylhexadecanoate, $N=38$, $M=564$) in the EI mode (70 eV) is shown in Fig. 2. The base peak is m/z 257 corresponding to the protonated hexadecanoic acid. The other ions in the mass spectrum are: M^+, m/z 564 (21.4%); $O^+{\equiv}C{-}(CH_2)_{21}{-}CH_3$, m/z 353 (12.1%) produced by α-fission of the molecular ion and the ion $[CH_2{=}CH{-}(CH_2)_{19}{-}CH_3]^+$, m/z 308 (5.7%) corresponding to the deprotonated alcohol moiety. Some common ions for saturated hydrocarbons from the series $[C_nH_{2n+1}]^+$ with m/z 57, 71 and 85 can be detected with an abundance of 10–25%.

It is possible to determine the individual contribution of esters to every chromatographic peak by mass spectrometric determination of the molecular ion and the base peak, correlated with the retention parameters. We analysed the wool wax (lanolin) aliphatic esters with N in the range 37–54 (molecular mass range 550–788). The chromatograms are shown in Fig. 3 ($N=37$–42), Fig. 4 ($N=43$–48) and Fig. 5 ($N=49$–54).

3.2. Chromatographic separation

3.2.1. Effect of the acid or alcohol moiety structure

A complete resolution for N (total carbon atoms), as is shown in Figs. 3–5, was obtained. To study the branching effects of the acid or alcohol moiety, the ester pairs of the N (k:m) and N (m:k) types were analysed by MS. The latter compound contains the same type of branched chain but in reversed position (the acid chain structure is replaced by the alcohol

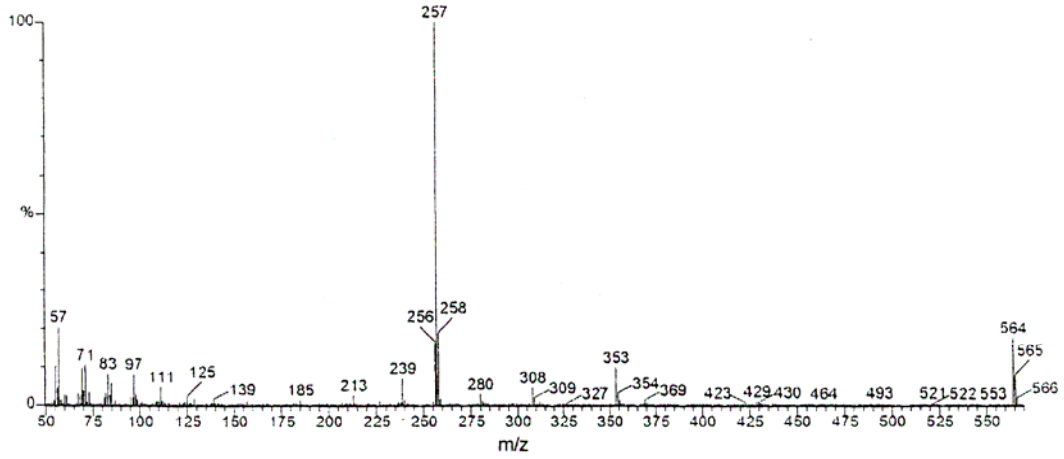


Fig. 2. EI mass spectrum (70 eV) of docosanoyl hexadecanoate, 38 (16:22), $M=564$.

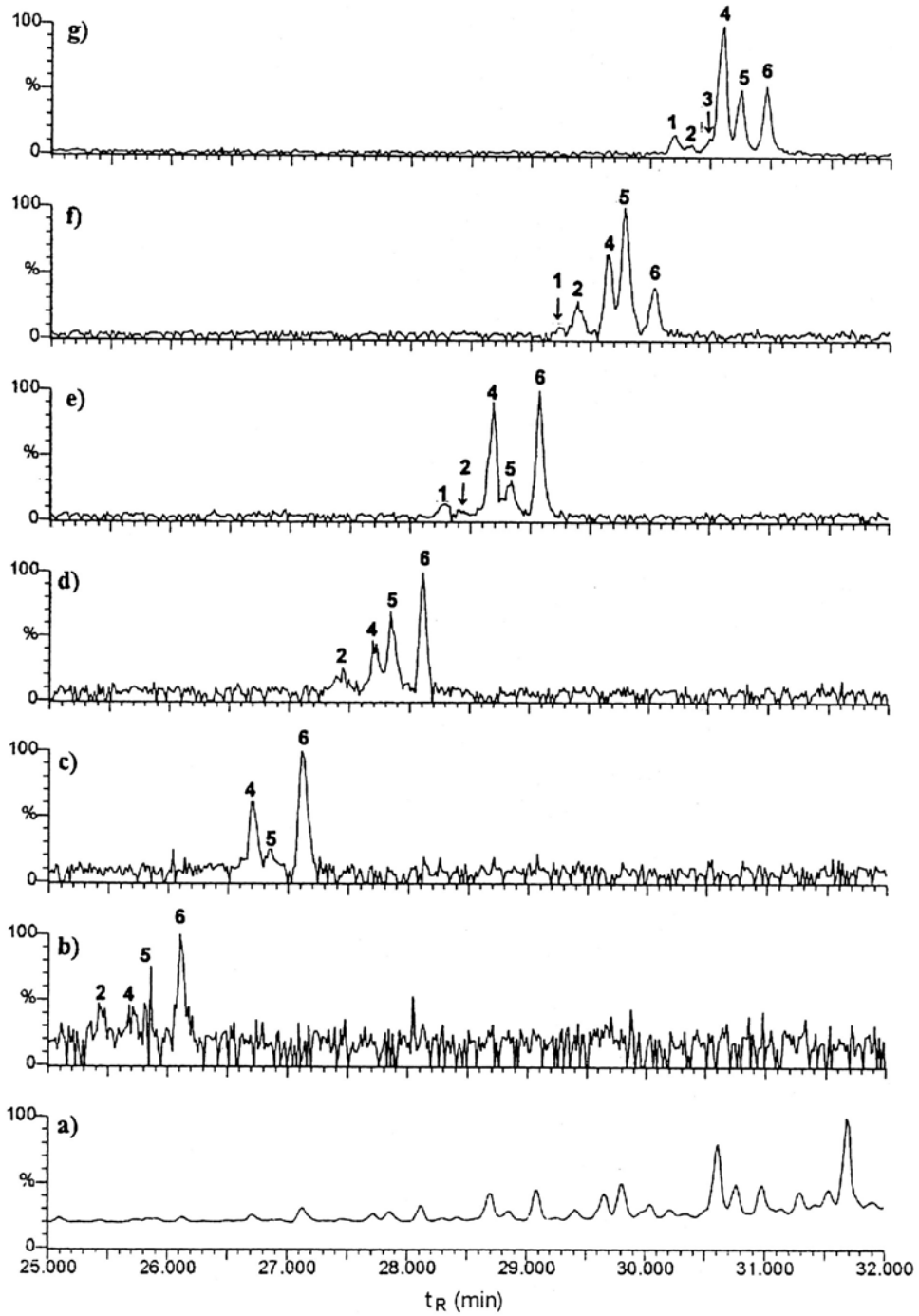


Fig. 3. GC–MS chromatogram of the aliphatic esters in the range of $N=37–42$ (M 550–620): (a) Total ion current (TIC), (b) m/z 550 ($N=37$), (c) m/z 564 ($N=38$), (d) m/z 578 ($N=39$), (e) m/z 592 ($N=40$), (f) m/z 606 ($N=41$), and (g) m/z 620 ($N=42$). Peaks 1–6 are described in Table 1. GC–MS conditions are described in the Experimental section.

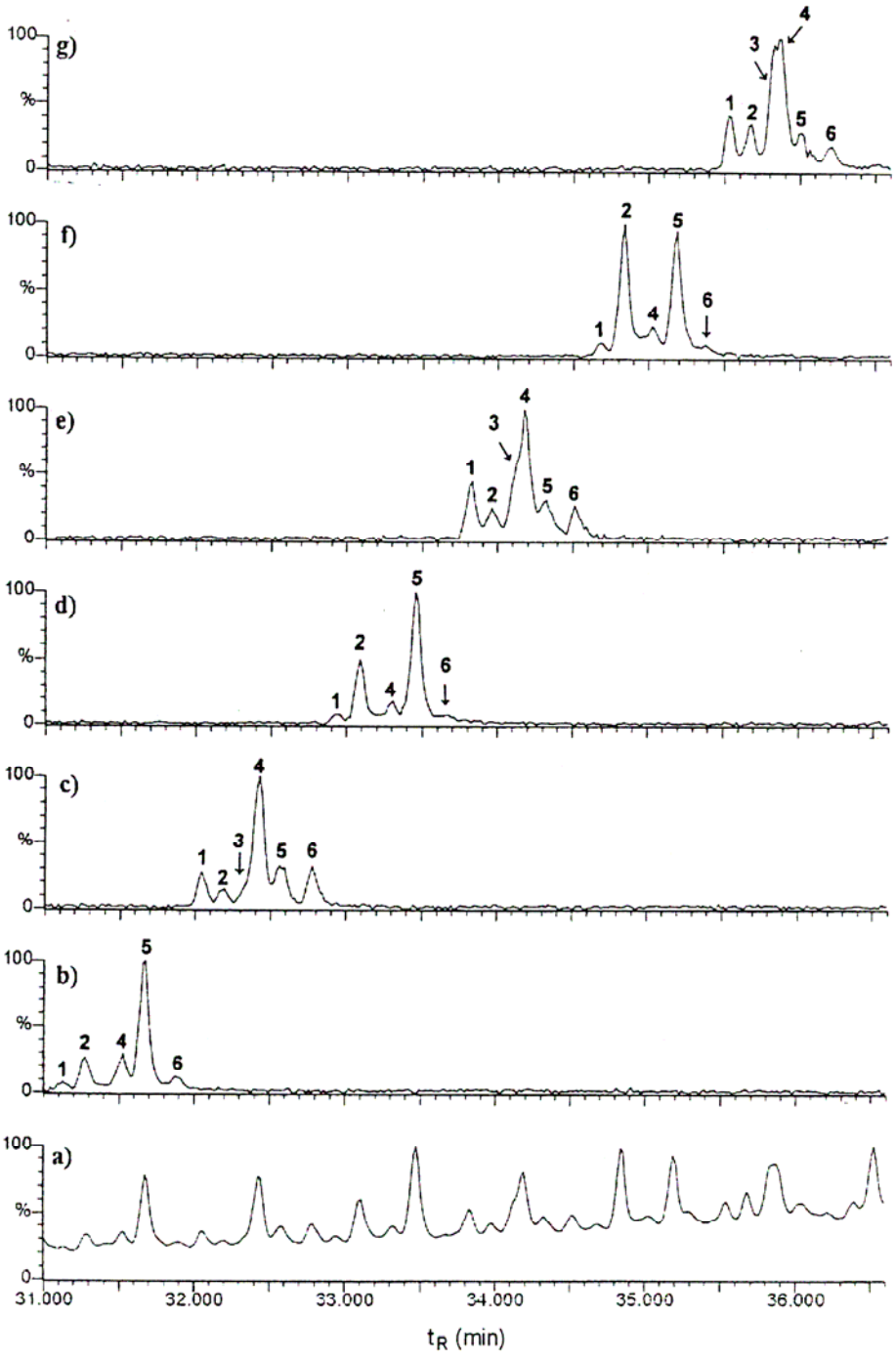


Fig. 4. GC–MS chromatogram of the aliphatic esters in the range of $N=43–48$ (M 634–704): (a) TIC, (b) m/z 634 ($N=43$), (c) m/z 648 ($N=44$), (d) m/z 662 ($N=45$), (e) m/z 676 ($N=46$), (f) m/z 690 ($N=47$), and (g) m/z 704 ($N=48$). Peaks 1–6 are described in Table 1. GC–MS conditions as in the Experimental section.

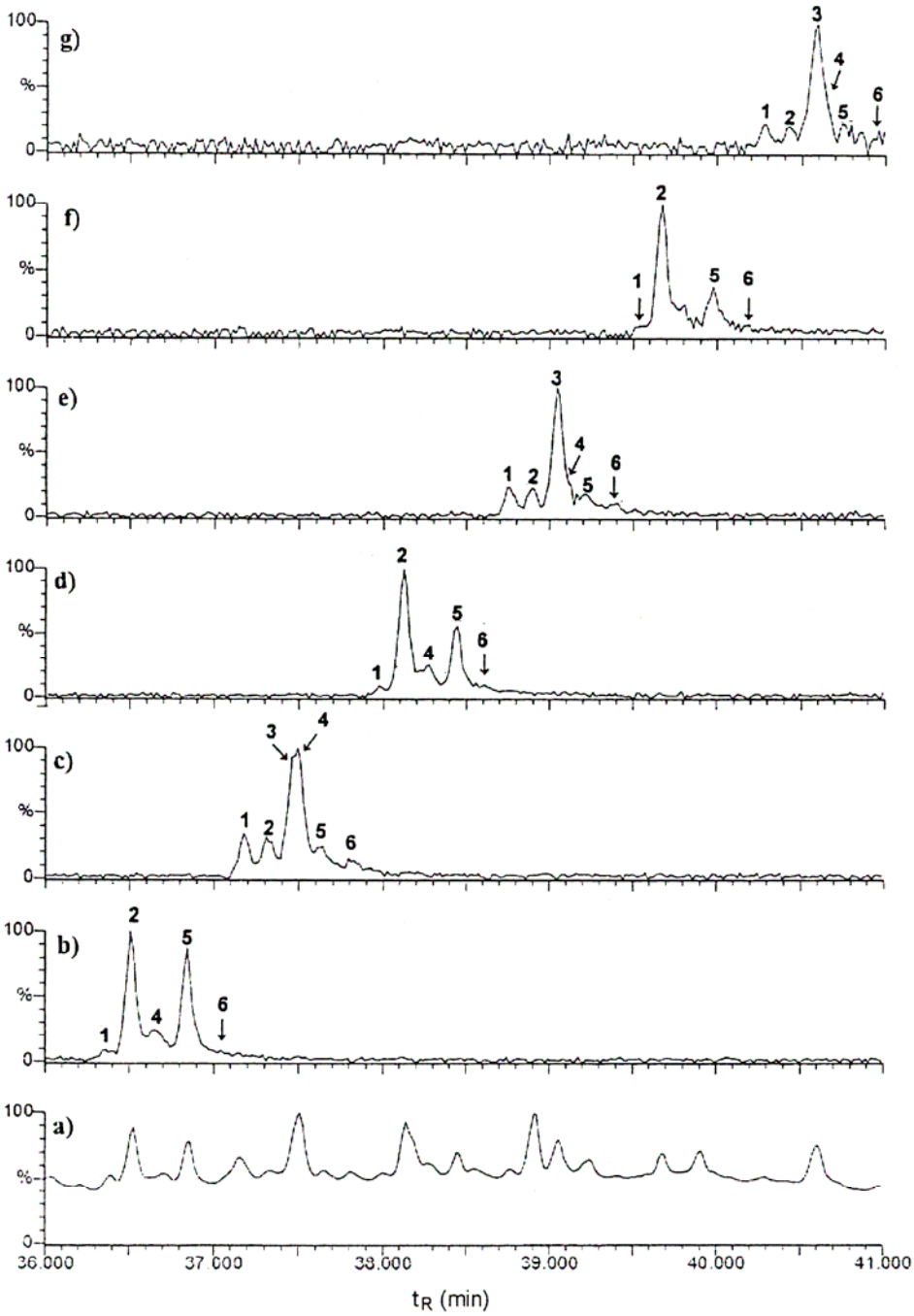


Fig. 5. GC–MS chromatogram of the aliphatic esters in the range of $N=49–54$ (M 718–788): (a) TIC, (b) m/z 718 ($N=49$), (c) m/z 732 ($N=50$), (d) m/z 746 ($N=51$), (e) m/z 760 ($N=52$), (f) m/z 774 ($N=53$), (g) m/z 788 ($N=54$). Peaks 1–6 are described in Table 1. GC–MS conditions as in the Experimental section.

chain structure, and vice versa) relative to the former.

The simultaneous registration of the ion with m/z 327, base peak of the compound 47 (21:26), with m/z 397, base peak for the compound 47 (26:21), in the region of $M^+ = 690$ ($N = 47$), gives two superimposed profiles, as shown in Fig. 6. This observation is consistent with the hypothesis that the effect of chain branching (*iso* or *anteiso*) on the retention time is the same for both positions, i.e., the acid or alcohol moieties. As a consequence, from Eqs. (1)–(4), the following equivalent pairs of structures have the same retention time:

$$[a_1{:}i_2]\,(\text{from Eq. (1)}) = [i_1{:}a_2]\,(\text{from Eq. (2)}) = [a{:}i] \quad (5)$$

$$[n_1{:}i_2]\,(\text{Eq. (1)}) = [i_1{:}n_2]\,(\text{Eq. (2)}) = [i{:}n] \quad (6)$$

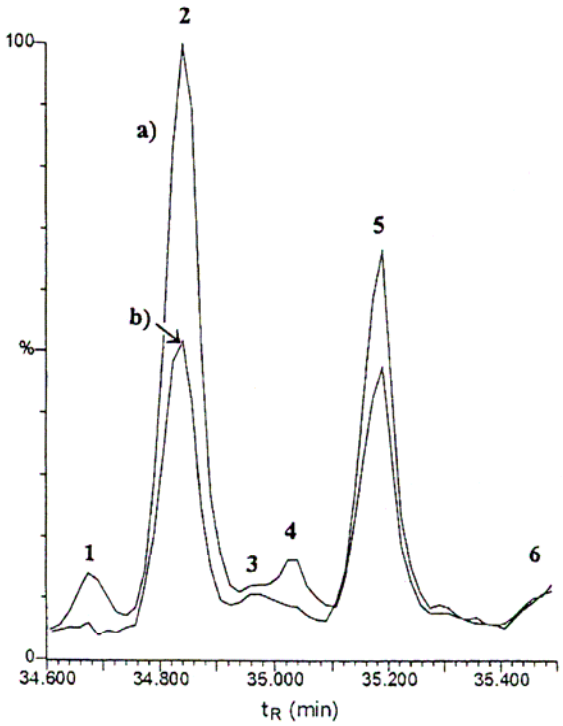


Fig. 6. Mass chromatogram of the base peaks for symmetrical compounds: (a) m/z 327, compound 47 (21:26); (b) m/z 397, compound 47 (26:21). Peaks 1–6 are described in Table 1. GC–MS conditions as in the Experimental section.

$$[a_1{:}n_2]\,(\text{Eq. (1)}) = [n_1{:}a_2]\,(\text{Eq. (2)}) = [a{:}n] \quad (7)$$

$$[a_1{:}n_2]\,(\text{Eq. (3)}) = [n_1{:}a_2]\,(\text{Eq. (3)}) = [a{:}n] \quad (8)$$

$$[i_1{:}n_2]\,(\text{Eq. (4)}) = [n_1{:}i_2]\,(\text{Eq. (4)}) = [i{:}n] \quad (9)$$

Taking into account that the latter terms from Eqs. (1)–(4) correspond to the same structure $[n{:}n]$, Eqs. (1–9) show that for an ester of [O] type four structures are resolved chromatographically, and for an ester of [E] type, five structures are resolved by GC, as can be seen from Eqs. (10) and (11):

$$[\text{O}] = [i{:}a] + [i{:}n] + [a{:}n] + [n{:}n] \quad (10)$$

$$[\text{E}] = [i{:}i] + [a{:}a] + [i{:}n] + [a{:}n] + [n{:}n] \quad (11)$$

There are six distinct ester structures: $[i{:}n]$, $[a{:}n]$ and $[n{:}n]$ common for both types of esters [O] and [E], $[i{:}i]$ and $[a{:}a]$ specific only for [E] esters and $[i{:}a]$ specific only for [O] esters.

Fig. 7 shows the chromatographic position of all isomeric peaks for the compounds with $M^+ = 676$ ($N = 46$). The ion of structure $[i{:}a]$ (characteristic for the odd esters) is present probably because the *a* compounds are of minor contribution in either the even acid or the alcohol moieties; or because there are small quantities of *i* compounds in the odd acid or the alcohol moiety.

Specifying the retention behaviour using the terminology of the equivalent chain length (ECL) is very useful for compound identification [19,20]. This parameter is independent of operating conditions. The ECL values for *normal* esters [of $(n{:}n)$ type] are integers equal to their carbon atom number $[(\text{ECL})_N^{(n:n)} = N]$. The ECL for branched esters is less than for the corresponding *normal* homologues. The ECL value for an isomer $(x{:}y)$ containing $(N+1)$ carbons can be written as:

$$(\text{ECL})_{N+1}^{(x:y)} = (\text{ECL})_N^{(n:n)} + (\text{FCL})^{(x:y)} = N + (\text{FCL})^{(x:y)} \quad (12)$$

where $(\text{FCL})^{(x:y)}$ is the fractional chain length (fraction of the carbon atom number attributed to the methyl branch) [19]. FCL is a fractional number in the range of 0.00–1.00 and is independent of N. For the $(n{:}n)$ isomers, the FCL is 1.00. Similarly, taking

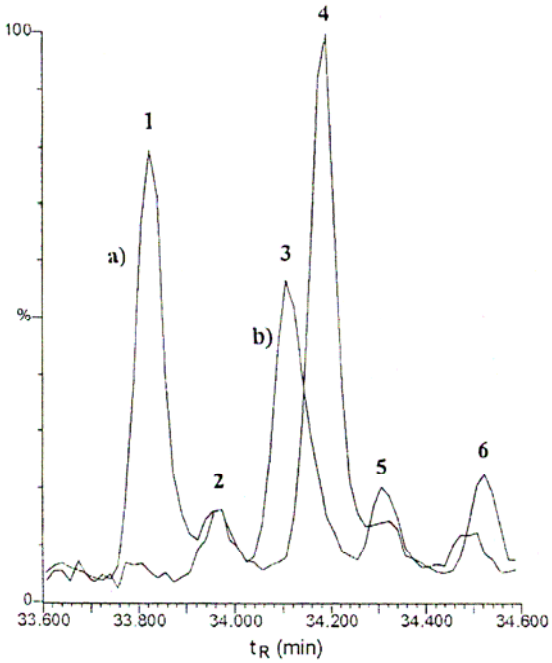


Fig. 7. Isomeric position peaks for the esters of $M^{+\cdot}=676$ ($N=46$); (a) m/z [base peak of compound 46 (20:26)]; (b) m/z 383 (base peak of compound 46 (25:21)]. Peaks 1–6 are described in Table 1. GC–MS conditions as in the Experimental section.

as a reference the *normal* compound with N carbon atoms, the FCL parameters for a *normal* compound containing $N+1$ carbon atoms is 1.00, but it is a fractional number (in the range of 0.00–1.00) for the others.

Table 1 shows the experimental FCL values (as average values obtained for the $N=44$ and 46 isomers). The resolution obtained on the separation of the six isomeric structures is better than 1 except for the separation of the peaks 3 and 4 ([a:a] and [i:n]), for which resolution is in the range of 0.5–1.0 (see Fig. 7).

Table 1
Fractional chain length (FCL) for ester isomers as the mean value obtained for $N=44$ and 46 compounds

Peak No.	Structure	FCL	Ester type	
1	*i*:*i*	0.19	E	
2	*i*:*a*	0.36		O
3	*a*:*a*	0.51	E	
4	*i*:*n*	0.61	E	O
5	*a*:*n*	0.76	E	O
6	*n*:*n*	1.00	E	O

i, *a* and *n* denote the *iso*, *anteiso* and *normal* structures, respectively, of the acid and alcohol moieties.

As can be seen only from the chromatographic retention parameters, it is not possible to see if the branched structure is on the acid or the alcohol moiety. The complete structural elucidation can be obtained by using chromatographic data in conjunction with mass spectral information. As previously stated, the base ion gives the carbon number of the acid (k), and the molecular ion M^+ gives the total number of carbon atoms N, thus the carbon atom number of the alcohol moiety (m) can be deduced. If k and m are known, the branched structure of the acid and alcohol moiety also can be found: i and n for [O] and a and n for [E] types.

3.2.2. Effect of the relative size of the acid and alcohol chain (k:m)

A shift of the peaks containing the same N and the same structure but different values for k and m (carbon atom distribution on the acid and alcohol chain, respectively) was observed (the "chain shift"). The shift is in the direction of the lower retention time when the k value is increasing. The position of the peaks m/z 313 (20:26), m/z 285 (18:28) and m/z 257 (16:30) for compounds with $k+m=46$ ($M=676$) are shown in Fig. 8. All these compounds have the same isomer structure (the acid and alcohol moieties are of even type). The shift effect $\Delta t/\Delta k$ is in the order of 0.006 min. This means that the retention time is changed by 0.006 min when the chain length is changed by one carbon atom (the sum $k+m$ is unchanged). The chain shift leads to an increase of peak width (decrease of the resolution when Δk is large) when the isomer number is increasing.

On the basis of Table 1 and mass spectral information, the structural distribution of the wool wax aliphatic esters in the molecular mass range 550–788 ($N=37$–54) was obtained. The results are shown in Table 2.

3.3. Isomeric ester distribution

Using isomer abundance as a criterion for both

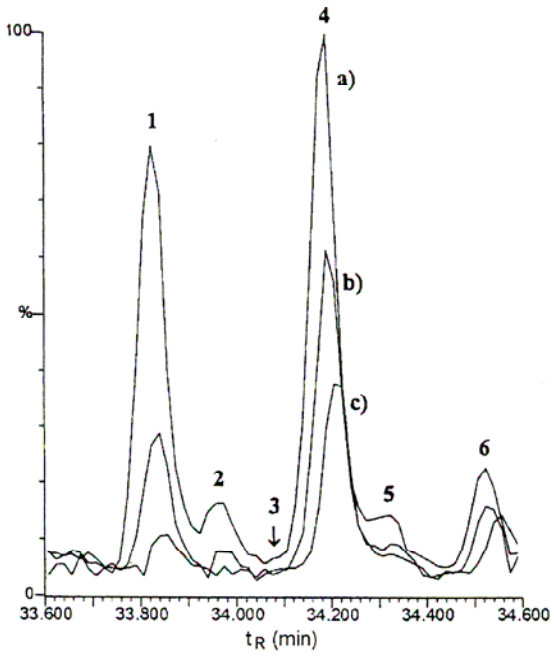


Fig. 8. Peak shift by the effect of the carbon atom distribution in the acid and alcohol moiety (*k*:*m*): (a) *m*/*z* 313, compound 46 (20:26); (b) *m*/*z* 285, 46 (18:28); (c) *m*/*z* 257, 46 (16:30). Peaks 1–6 are described in Table 1. GC–MS conditions as in the Experimental section.

types of esters, the three regions of the chromatogram can be seen.

3.3.1. Isomer distribution for odd esters ([O] type esters)

(a_1) The $N=37–39$ range (the unbranched linear chain region): for the esters with $N=37$ or 39, the major isomeric abundance corresponds to the [*n*:*n*] structure, being peak 6 the base peak. The structures [*n*:*i*] (peak 4, Fig. 3) and [*n*:*a*] (peak 5, Fig. 3) also have important contributions.

The mass spectrometric analysis of the individual esters shows that all compounds of higher abundance have the acid chain of type *n*. The contribution of type [*a*:*n*] and [*i*:*n*] was not detected (only [*n*:*a*] and [*n*:*i*]). This observation is consistent with the fact that for $N=37$ and 39, in the biosynthetic process preferentially combines acids and alcohols which produce esters with minimally branched chains (only one branched chain, on either the acid or the alcohol moiety). The combinations of [*i*:*i*] and [*a*:*a*] type (two branched chains, acid and alcohol moiety) are present only in very small abundances (under 8%). The dominant contribution in this region is given by the compounds 37 (15:22) and 39 (15:24).

(a_2) The $N=41–45$ range (the single branched chain region): in this *N* range, the contribution of the [*n*:*a*] type isomers (peak 5) is dominant. The main contribution is given by the compounds 41 (16:25), 43 (16:27) and 45 (18:27). In every situation, the acid chain is of *normal* type. The increase of the relative abundance of [*i*:*a*] isomers (peak 2, Figs 3 and 4) with an *N* increase should be noted (23% in the compounds of $N=41$ to 62% in the compounds of $N=45$). Peak 6 is decreasing from 49% ($N=41$) to 6.7% ($N=45$).

(a_3) The $N=47–53$ range (the two branched chain region): in the range of $N=47–53$, the chromatogram profile is dominated by the structure [*i*:*a*] (peak 2, Figs. 4 and 5), corresponding to a two branched chain ester structure (branched acid and alcohol moiety). The structure [*n*:*a*], peak 5, has also an important contribution. The relative intensity of peak 5 decreases with increasing *N* (82% for $N=47$ to 29% for $N=53$). The main contribution to the base peak is given by the compounds 47 (20:27), 49 (22,27), 51 (24,27) and 53 (26,27).

The characteristic configuration of the [O] type esters is the change of the isomeric structures with the increase of *N*, from [*n*:*n*] (unbranched structure, 100% for $N=37$) to [*i*:*a*] (two branched structure, 100% for $N=53$).

3.3.2. Isomeric distribution of even esters ([E] type esters)

(b_1) The $N=38–40$ range (the unbranched linear chain region): the main contribution to the ester peaks with $N=38$ or 40 consists of structures [*n*:*n*] (peak 6, Fig. 3) and [*n*:*i*] (peak 4, Fig. 3). Peak 6 is of 100% abundance and peak 4 of 48% and 82%, respectively. The main contribution is given by compounds 38 (14:24) and 40 (16:24). The preference for the minimally-branched compounds is a characteristic of this region.

(b_2) The $N=42–50$ range (the single branched chain region): the base peak is peak 4 with [*n*:*i*] structure. The contribution of the structures [*n*:*n*] (peak 6, Figs. 3 and 4) decreases from 58% ($N=42$)

Table 2
The ester isomeric distribution (% from the highest peak) in the range of $N=37–54$; $N=$total carbon atom; $k=$the carbon atom number on acid moiety; $\Delta k=k$ range; $c=$concentration (%, v/v); *i*, *a*, and *n* denote the *iso*, *anteiso* and *normal* structures, respectively, of the acid and alcohol moieties

N (*M*)	*c* (%)	Isomer distribution						
		Δk	*i*:*i* (1)	*i*:*a* (2)	*a*:*a* (3)	*i*:*n* (4)	*a*:*n* (5)	*n*:*n* (6)
37 (550)	0.47	14–18	0.0	8.0	0.0	35.2	67.6	100
38 (564)	1.35	14–18	0.0	0.0	5.5	46.7	8.9	100
39 (578)	2.21	14–19	0.0	11.9	0.0	42.8	52.4	100
40 (592)	3.95	14–20	2.2	0.0	4.4	82.2	33.3	100
41 (606)	4.78	14–20	7.0	23.2	0.0	72.1	100	48.8
42 (620)	6.21	14–22	17.8	4.4	11.1	100	48.9	57.8
43 (634)	7.29	14–20	11.4	36.4	0.0	29.5	100	13.6
44 (648)	7.87	14–26	26.6	13.3	13.5	100	31.1	30.4
45 (662)	9.41	15–27	13.3	62.2	0.0	15.5	100	6.7
46 (676)	9.02	16–27	44.4	26.7	62.2	100	28.9	22.2
47 (690)	10.0	14–29	11.4	100	0.0	31.8	81.8	4.5
48 (704)	8.21	16–28	40.0	24.4	71.1	100	24.4	15.5
49 (718)	7.56	16–29	6.7	100	0.0	22.2	80.0	2.2
50 (732)	6.41	18–30	37.8	26.7	88.8	100	15.5	6.7
51 (746)	6.08	18–31	2.5	100	0.0	17.8	51.1	2.2
52 (760)	4.23	21–30	20.0	17.8	100	48.9	11.1	6.7
53 (774)	2.91	20–33	3.3	100	0.0	4.4	28.9	0.0
54 (788)	2.02	23–33	15.5	13.3	100	33.3	11.1	3.3

to 15% ($N=50$). The isomers of major contributions are 42 (16:26), 44 (18:26), 46 (20:26), 48 (22:26) and 50 (24:26). The increase in the relative abundance of the structures [*a*:*a*] (peak 3, Figs. 3 and 4) is to be noted. The abundance of these isomers increases from 11% ($N=42$) to 89% ($N=50$).

(b_3) The $N=52–54$ range (the two branched chain region): for the $N=52$ and $N=54$ esters, the major contribution is of the isomers with [*a*:*a*] structure, peak 3 (100%), having both moieties branched (acid and alcohol). The base contribution is given by the compounds 52 (25:27) and 54 (27:27). The abundance of the structures [*i*:*n*] (peak 4, Fig. 5) decreases from 49% ($N=52$) to 33% ($N=54$). The contribution of the structures [*i*:*i*] (peak 1) is small, in the range 15–20%.

4. Conclusions

The most abundant aliphatic esters, obtained from wool wax by SFE, are in the carbon number range from 37 to 54. The corresponding carbon atom number of the acid moiety is from 14 to 33 and for the alcohol moiety from 19 to 33.

The isomeric structure for every *N*, resulting from the *n*, *i* and *a* structures of the acid and alcohol moieties, can be determined from the chromatographic profile and mass spectral information. When *N* increases from 37 to 54, the major isomeric contribution changes from the [*n*:*n*] structure (peak 6) to [*a*:*n*] (peak 5) and [*i*:*a*] (peak 2) structures, for the odd type esters. For the even type esters, the increase of *N* changes the major isomeric contribution from [*n*:*n*] (peak 6) to [*i*:*n*] (peak 4) and [*a*:*a*] (peak 3) structures.

The carbon number in the acid moiety, for the major contributing compounds, is under 50% [as $(k\times100)/(k+m)$]. It increases from 40.5 to 50% when *N* increases from 37 to 54.

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Complete characterisation of lanolin steryl esters by sub-ambient pressure gas chromatography–mass spectrometry in the electron impact and chemical ionisation modes

Eric Jover[a], Zaharie Moldovan[b], Josep Maria Bayona[a,*]

[a]*Environmental Chemistry Department I.I.Q.A.B.-C.S.I.C., Jordi Girona 18-26, E-08034 Barcelona, Spain*

[b]*National Institute for Research and Development for Isotopic and Molecular Technologies, P.O. Box 700, 3400 Cluj-Napoca 5, Romania*

Abstract

Steryl esters occurring in lanolin have been characterised by sub-ambient pressure gas chromatography coupled to mass spectrometry. Electron impact and chemical ionisation modes with different reagent gases have been evaluated in order to carry out unambiguous peak identification. Steryl esters with different sterol (i.e. cholesterol, lanosterol and dihydrolanosterol) and acid moieties either according to carbon number (i.e C_{10}–C_{23}) or isomeric forms (i.e. normal, iso and anteiso) have been identified. Identification of the sterol and acid moieties has been carried out by means of the mass spectral information obtained in the electron impact, chemical ionisation mode either in the positive or negative modes using methane, isobutane and ammonia as reagent gases. Isomeric identification has been achieved by chromatographic retention parameters (i.e. entire-chain length and fractional-chain length) and by the free fatty acid profile also present in lanolin.

Keywords: Sub-ambient pressure gas chromatography–mass spectrometry; Mass spectrometry; Lanolin steryl esters

1. Introduction

Lanolin is the wool grease secreted by the sheep sebaceous glands. This wool grease is a complex mixture of high-molecular mass lipidic compounds including fatty acids and alcohols, sterols, hydroxy acids, diols, aliphatic and steryl esters [1,2]. The high complexity of lanolin is highlighted by the composition of the monoester family estimated in ca. 10^4 individual components [3].

Lanolin is widely used in cosmetic and pharmaceutical formulations for its surfactant properties [2,4]. It also represents the world first source of sterols such as cholesterol and lanosterol. The study of minor lipids has shown also interest in order to assess the quality and authenticity of cosmetic and pharmaceutical products and also the steryl esters have already been used to proof the authenticity of eatable oil [5].

Lanolin has been usually characterised following the ester bond cleavage of the aliphatic and steryl esters by hydrolysis [6–8]. That approach gave useful information about its composition but not about of the original structure of the ester mixture. Fatty acids and alcohols are independently analysed and they represent the sum of the originally free and esterified compounds. In order to avoid this problem, intact lanolin must be analysed without hydrolysis. Recent improvements in high-temperature gas chro-

*Corresponding author. Fax: +34-93-204-5904.
E-mail address: jbtqam@cid.csic.es (J.M. Bayona).

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matography (HTGC) combined with the extended use of mass spectrometry techniques are the analytical techniques of choice for such determination. In this regard, free fatty acids (FFA) by GC [9] and aliphatic esters by HTGC [10] have been successfully analysed from lanolin.

Analysis of the original steryl esters of lanolin has not been reported yet. So this work will address this lack of knowledge. However, intact steryl esters have already been carried out in other matrices by gas chromatography–mass spectrometry (GC–MS) in the electron impact (EI-MS) and positive ion chemical ionisation (PCI/MS) using different reagent gases [11]. Often these analyses involve difficult methodologies with several chromatographic steps. In cocoa butter, they have been characterised by on-line LC–GC–FID and PCI-MS confirmation using ammonia as reagent gas [12]. Other steryl esters have been also analyzed in their intact form (i.e. amyrin and lupeol esters) from aspen wood by off-line argentation-silica gel chromatography GC–FID and GC–EI-MS [13]. Ergosteryl, egost-8-enyl, zymosteryl, cholestadienyl, methylergostadienyl esters have been identified in yeast by thin layer chromatography (TLC) in combination with off-line HPLC–GC–FID [14]. In this kind of complex analysis tend to complicate chromatographic steps as shown in the analysis of ergostatetraenyl, ergosteryl, ergostadienyl, methylergostenyl, cholestadienyl and zymosteryl esters in algae and yeast using on-line NPLC–RPLC UV detection and GC-CI–MS using ammonia as reagent gas [15].

Special attention has been paid to cholesteryl esters due to their importance in cholesterol metabolism, transport and storage in mammals [16]. However, cholesteryl esters need to be characterised by CI/MS because in the EI/MS mode their mass spectra give only information about the sterol moiety with a base peak at m/z 368 but without information about the acid moiety or molecular ion. Early work related to the blood serum lipid characterisation has been carried out in 1975 by off-line TLC–GC–FID and its confirmation carried out by direct probe introduction mass spectrometer working in the EI or PCI MS using different reagent gases [17]. Cholesteryl esters with a saturated or unsaturated acid moiety occurring in human plasma have been identified by GC–EI-MS and GC–NCI-MS both with a magnetic sector instrument using hydrogen, ammonia, methane and isobutane as reagents gases [18]. Cholesteryl, methylcholestadienyl, cholestadienyl, ethylcholestenyl esters were characterised in marine particulate matter by GC–EI-MS and GC–PCI-MS using methane as reagent gas [19]. Finally, a complete study on cholesteryl, stigmasteryl, sitosteryl and campesteryl esters from human plasma, barley seedlings, palm oil and rape seed oil have been published using off-line TLC, HPLC, GC–EI-MS and GC–NCI-MS on a magnetic sector with ammonia as reagent gas [20]. Nevertheless, steryl esters are high-molecular mass compounds with high-boiling point, which difficult their GC analysis. In order to circumvent this limitation, HTGC has been used [21] but thermal labile components can be degraded during the GC conditions. The aim of the work was to evaluate the suitability of fast gas chromatography using sub-ambient pressure conditions to allow a lower elution temperatures and faster analysis speed [22,23], combined with MS in the EI, PCI and NCI ionisation modes for the characterisation of intact steryl esters occurring in lanolin. Analysis of intact lanosteryl and dihydrolanosteryl esters is carried out for the first time. Also compared to described techniques, the methodology presented in this work is much easier and permitted in one injection, the complete identification of three steryl ester families including their isomeric characterisation.

2. Experimental

2.1. Standards and reagents

Cholesteryl palmitate (97% purity) was provided by Aldrich, (Steinheim, Germany). Isooctane for trace analysis, HPLC grade ethyl acetate and cyclohexane and *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were obtained from Merck (Darmstadt, Germany). Lanolin was Corona Lanolin, a refined wool wax from Croda (Snaith Goole, UK). Helium 99.9995% from Air Liquide (France) was used as carrier gas. Reagent gases for ionisation were electronic grade ammonia, 99.9995% methane and 99.95% iso-butane all from Air Liquide (France).

2.2. Sample preparation

About 100 mg of lanolin were weighed and dissolved in 5 ml of ethyl acetate–cyclohexane 1:1. Then the sample was filtered through a 0.45 μm nylon membrane filter (Lida, Kenosha, WI, USA). Then 10 μl of the solution were placed in a 2 ml conic vial, then 10 μl of BSTFA was added. The closed vial was maintained at 70 °C over 1 h and then evaporated to dryness under gentle nitrogen stream. Iso-octane (50 μl) was added into the vial to reconstitute the sample and analysed before 48 h to avoid the hydrolysis of the TMS group. Main polar constituents of lanolin as free fatty acids, hydroxy acids, diols can interfer in the analysis. Silylation permits chromatographic and detection system to more easily eliminate these polar compounds.

2.3. Instrumental analysis

A sub-ambient pressure CP Sil 8 CB/MS capillary column (5% diphenyl-dimethylpolysiloxane) of 10 m×0.53 mm I.D. and 0.25 μm of film thickness fitted to a deactivated restrictor of 50 cm length and 0.1 mm of internal diameter at the injection port was obtained from Chrompack (Middelburg, The Netherlands). One microliter of sample was injected in the splitless mode at 320 °C activating the injector purge at 90 s from injection. Initial column temperature was held at 90 °C for 1 min, and then programmed at 10 °C min^{-1} to 320 °C keeping the final temperature for 20 min (44 min each run). Chromatographic analysis was performed in the constant flow mode at 1.2 ml min^{-1}.

2.3.1. Chemical ionisation mode

A GC 6890A from Agilent Technologies (Palo Alto, CA, USA), coupled to an MS 5973N was used. Quadrupole was held at 150 °C and transfer line at 280 °C because band broadening was not observed at these temperatures. In order to optimise the sensitivity in the positive and negative ion ionisation modes with the different reagent gases, a standard mixture containing 30 ppm of an aliphatic ester ($C_{15}COOC_{22}$) and a cholesteryl ester ($C_{15}COOCholesterol$) were injected at different pressures (from $8.5*10^{-5}$ to $14.8*10^{-5}$ Torr) and temperatures (from 180 to 250 °C). Optimal conditions were $12.8*10^{-5}$ Torr and 200 °C for ammonia PCI, $14.8*10^{-5}$ Torr and 230 °C for ammonia NCI, $8.5*10^{-5}$ Torr and 200 °C for isobutane PCI and $10.8*10^{-5}$ Torr and 200 °C for methane PCI.

2.3.2. Electron impact mode

A GC from Fisons (Manchester, UK), GC 8060 coupled to an MS detector MD 800 was used. Transfer line and ion source were held at 280 and 230 °C, respectively. Other chromatographic conditions were identical to those reported in the CI section.

3. Results and discussion

3.1. Mass spectrometry optimisation

The characterisation of the steryl esters occurring in lanolin due to the high complexity can be only carried out by a high resolution technique such as GC–MS in combination with different ionisation modes including both CI and EI, which provide either molecular mass or structural information. For these reasons, we have evaluated the CI-MS either by PCI or NCI using different reagents gases such as ammonia, isobutane and methane. The proposed chromatographic technique compromises speed of analysis and resolution. The 0.53 mm internal diameter is necessary to achieve sub-ambient pressure conditions, along the column but in any case a better resolution than conventional LC due to the higher efficiency is obtained.

3.1.1. Isobutane

According to the electron capture mechanism prevailing in the NCI mode, steryl esters gave very poor sensitivity in all the conditions evaluated when isobutane was used as reagent gas [24]. Consequently, it was disregarded in the NCI mode. However, in the PCI mode the sterol moiety was clearly identified with a base peak corresponding to $[R_2]^+$ (i.e. m/z 369 for cholesteryl, 409 for lanosteryl and 411 for dihydrolanosteryl). Also the acid moiety can be detected with an abundant ion $[R_1COOH_2]^+$ but the low abundance of the molecular ion $[M+1]^+$, which does not scale up in the figures. It was not useful for the molecular ion assignment because in

real samples with noisy background, it was very often impossible to detect this ion (Figs. 1 and 2). Accordingly, the molecular ion can be deduced by the following expression:

$$M = [R_1COOH_2] + [R_2] - 2$$

Formation of $[R_1COOH_2]^+$ and $[R_2]^+$ was already reported for aliphatic esters and could be easily correlated to steryl esters (Fig. 3), first reaction channel corresponding to an alkene elimination and the second one corresponding to the carboxylic acid elimination after ester protonation [25].

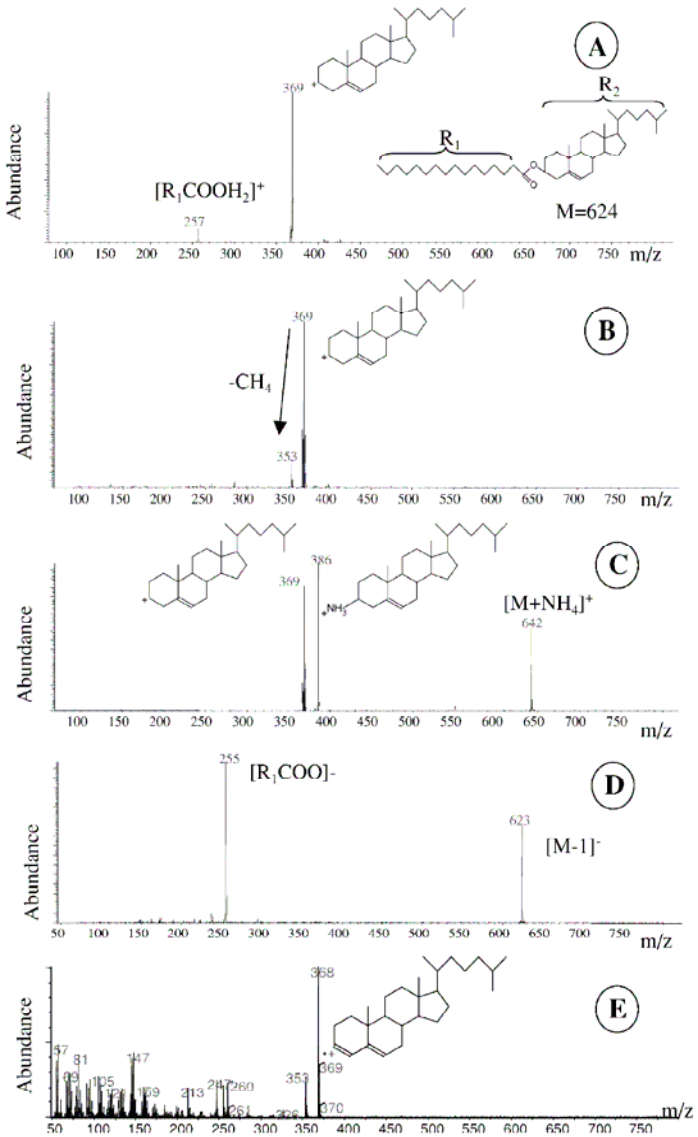

Fig. 1. Spectrum of cholesteryl palmitate using different ionisation modes: (A) isobutane PCI; (B) methane PCI; (C) ammonia PCI; (D) ammonia NCI; and (E) EI.

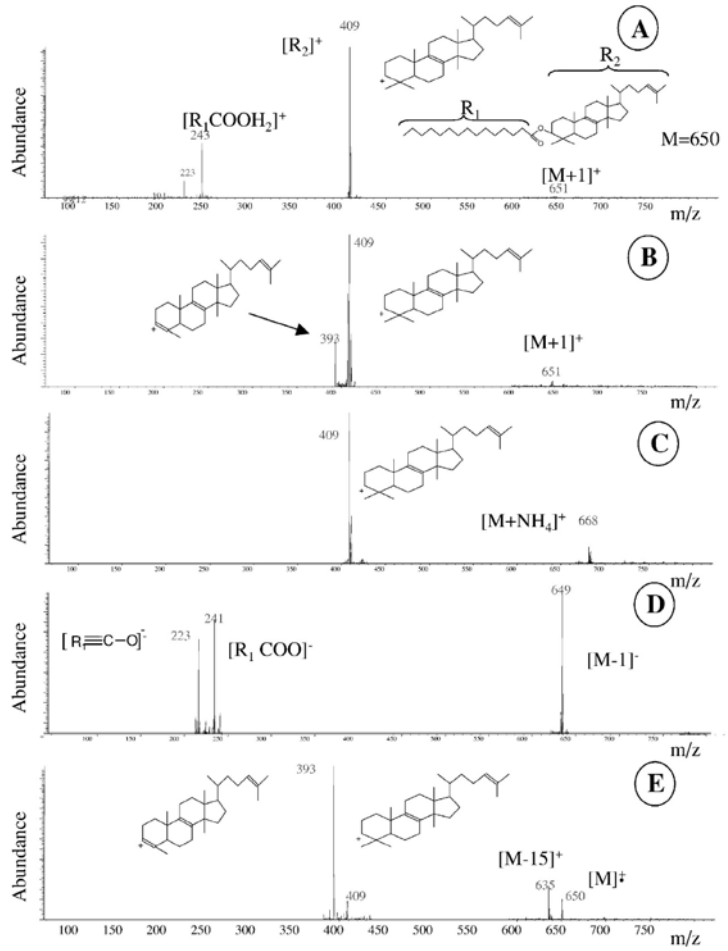

Fig. 2. Spectrum of lanosteryl pentadecanoate using different ionisation modes: (A) isobutane PCI; (B) methane PCI; (C) ammonia PCI; (D) ammonia NCI; and (E) EI.

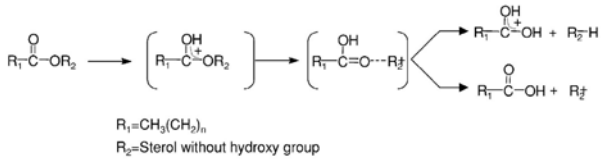

Fig. 3. Mechanism of ionisation of steryl ester in PCI.

3.1.2. Methane

As observed in the NCI mode with isobutane, a poor response was obtained and no further attempts were carried out to optimise it. In the PCI mode, methane behaves similarly that isobutane but different ionisation patterns could be observed due to methane higher proton affinity, increasing proton transfer exothermicity and therefore fragmentation. Also, no ion indicating the acid moiety could be detected in methane PCI (Figs. 1B and 2B). For lanosteryl and dihydrolanosteryl esters, in addition to $[R_2]^+$, we found $[R_2-16]^+$ corresponding to a methyl loss followed by a double bond formation giving an ion at m/z 393 for lanosteryl esters (Fig. 2B) and 395 for dihydrolanosteryl esters.

3.1.3. Ammonia

The best results in terms of structural identification for the entire range of target analytes were obtained with ammonia both in the PCI and NCI modes. In the NCI $[M-1]^-$ was one of the most abundant ions, which is very useful for the identification purposes (Figs. 1D and 2D). Also a strong ion corresponding to the carboxylate formation $[R_1COO]^-$ can be detected. For lanosteryl and dihydrolanosteryl esters, a second ion from the acid moiety, which corresponds to $[R_1COO^- - H_2O]^-$ was detected as reported previously for aliphatic esters [25]. Thus the sterol moiety can be deduced according to the following equation:

$$R_2 = [M-1] - [R_1COO] + 1$$

Using PCI, an adduct of all target compounds was obtained with the ammonium ion $[M+NH_4]^+$ (Figs. 1C and 2C). Conversely to PCI, NCI formed an ion characteristic of sterol moiety $[R_2]^+$ and for cholesteryl esters only $[R_2+NH_3]^+$ corresponding to sterol molecular mass was observed. For lanosteryl and dihydrolanosteryl esters no secondary ion corresponding to sterol moiety could be found. Acid moiety can be deduced from the following equation:

$$R_1COO = [M+NH_4] - [R_2] - 18$$

3.1.4. Electron impact

EI is the most widely used ionisation technique for lipid characterisation. However, it is useful as screening but not always for identification purposes. On the one hand, EI of lanosteryl and dihydrolanosteryl esters gave enough structural information to carry out the complete analysis (Fig. 2E) but on the other hand, cholesteryl esters gave all the same fragments corresponding to the sterol moiety (Fig. 1E). Therefore, the acid moiety or molecular ions could not be detected. As a consequence, no identification was possible using only EI-MS but a very abundant ion characteristic of the cholesterol moiety was found at m/z 368 corresponding to a water elimination with formation of a double bond in position 3. Also for all the studied compounds, the best sensitivity was obtained in the EI-MS.

Therefore, CI-MS is necessary to confirm the steryl ester identification of cholesteryl derivatives, lanosteryl and dihydrolanosteryl esters can be quantified using EI-MS, for cholesteryl esters coelution in C_{17} acidic fragment range was observed and in this case quantification was carried out by PCI using ammonia. In order to gain structural information in the EI-MS, the electron impact ionisation energy was reduced from 70 to 20 eV but a remarkable loss in sensitivity was detected and, therefore not useful for quantitation purposes.

Characteristic ions of the different studied families in the tested ionisation modes are summarised in Table 1. In this table also appear ions used for quantification.

3.2. Homologous acid patterns

The different families of steryl esters have been charactarised using the ions shown in Table 1. At this point, the complexity of lanolin was evident as shown in Fig. 4 where both the total ion current (TIC) from steryl esters and the mass fragmentograms corresponding to diagnostic ions appeared crowded. As expected, very similar distribution patterns for lanosteryl and dihydrolanosteryl esters were obtained because these families are biosyntheticaly related. Carbon number of the acid moieties ranged from 10 to 23 with a maximum abundance, by adding peak areas of the different isomeric forms, for palmitic acid (C_{16}) (Fig. 5). The distribution patterns were monomodal, single maximum gaussian distribution, apart from dihydrolanosteryl esters, which was bimodal (two maxima distribution) with a minor maximum at C_{14}. For cholesteryl esters, the

Table 1
Summary of the characteristic ions used for identification in the different ionisation modes. Value between parentheses represents relative abundance of the ion. Underlined ions are used for quantification

	Cholesteryl esters m/z			Lanosteryl esters m/z			Dihydrolanosteryl esters m/z		
	Intact molecule	Acid moiety	Sterol moiety	Intact molecule	Acid moiety	Sterol moiety	Intact molecule	Acid moiety	Sterol moiety
NH_3 PCI	$[M+18]^+$ (54)	–	386(100), 369(88)	$[M+18]^+$ (20)	–	409(100)	$[M+18]^+$ (23)	–	411(100)
NH_3 NCI	$[M-1]^-$ (57)	$[R_1COO]^-$ (100)	–	$[M-1]^-$ (100)	$[R_1COO]^-$ (64), $[R_1COO\text{-}H_2O]^-$ (46)	–	$[M-1]^-$ (100)	$[R_1COO]^-$ (64), $[R_1COO\text{-}H_2O]^-$ (46)	–
CH_4 PCI	$[M+1]^+$ (<1)	–	369(100), 353(17)	$[M+1]^+$ (<1)	–	409(100), 393(33)	$[M+1]^+$ (<1)	–	411(100), 395(38)
C_4H_{10} PCI	$[M+1]^+$ (<1)	$[R_1COOH_2]^-$ (10)	369(100)	$[M+1]^+$ (<1)	$[R_1COOH_2]^+$ (33)	409(100)	$[M+1]^+$ (<1)	$[R_1COOH_2]^+$ (33)	411(100)
EI	–	–	368(100), 353(15)	$[M]^{\cdot+}$ (4), $[M-15]$ (14)	–	393(100), 409(15)	$[M]^{\cdot+}$ (4), $[M-15]$ (14)	–	395(100), 411(13)

distribution was different with a major abundance of the longer acid carbon number. In this case acid carbon number ranged from 11 to 23 with a prominent maximum at 17 caused by a coelution. In order to avoid these coelutions considering that in the electron impact mode several cholesteryl derivatives gave the m/z 368 fragment, PCI-MS fragmentograms of pseudo-molecular ion using ammonia were used for quantification, also in this mode acid fragment information was available confirming in this way compound identity. Therefore, obtained pattern had a maximum at 19 acid being atypical but no other coelution was found. The shape of this distribution was monomodal. Therefore for choles-

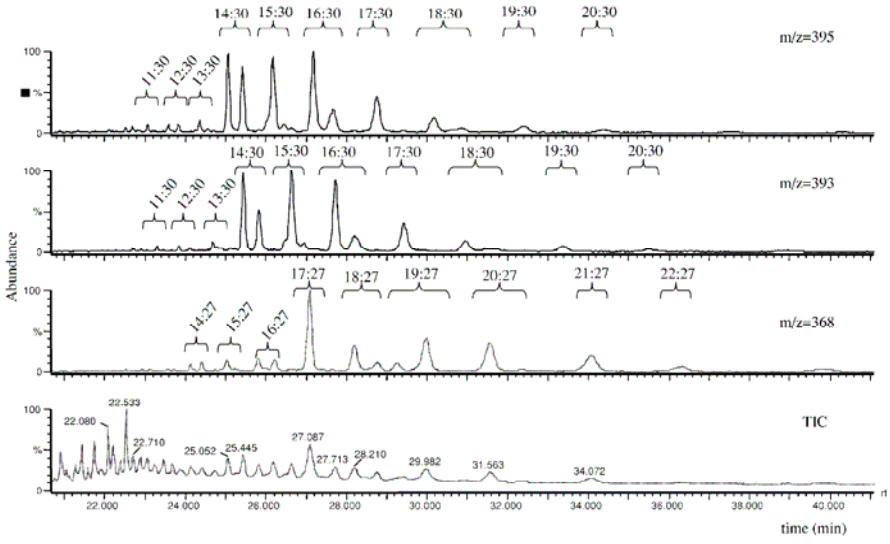

Fig. 4. Fragmentograms of a characteristic lanolin sample showing the total ion current (TIC) and steryl ester characteristic ion at m/z 395 for dihydrolanosteryl esters, m/z 393 for lanosteryl esters and m/z 368 for cholesteryl esters. The composition of acid and alcohol moieties forming the steryl esters is indicated on each peak apex.

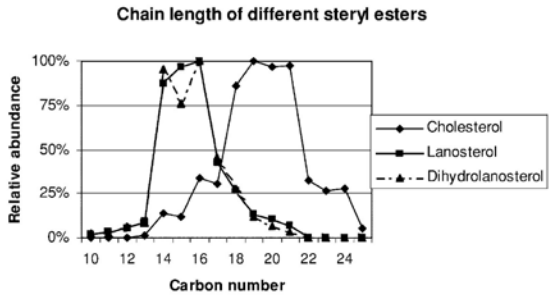

Fig. 5. Relative abundance of steryl esters against acid chain length.

teryl esters, odd acid chains are predominant instead of even for lanosteryl and dihydrolanosteryl esters as it should be expected. Fig. 5 shows the main differences in the steryl ester distributions.

3.3. Isomeric identification of steryl esters

In lanolin free fatty acids (FFA) occur in three different isomeric forms namely, normal, iso and anteiso [2]. Iso corresponds to (ω-1)-monomethyl-substituted FFA and anteiso to (ω-2)-monomethyl-substituted FFA. Therefore, for each acid carbon number, three different isomers can be found. These FFA come from the hydrolysis of aliphatic and steryl esters secreted by the sebaceous glands [26]. Accordingly, it is expected the same isomeric distribution for the FA forming the steryl esters.

Since no significant differences could be observed between different isomers of a same steryl ester by mass fragmentography, the identification of the different isomers has been carried out by means of chromatographic parameters such as equivalent-chain length (ECL) and fractional-chain length (FCL) using an earlier reported equation [27]. It has already been applied to the isomeric characterisation of FFA and free fatty alcohols in lanolin [9]. It is asumed a similar influence of branched FA chain for steryl esters than for the trimethylsilylesters of FFA. This assumption was confirmed by the experimental results when possible because steryl esters with a normal acid of the same carbon number than the studied compound but also an steryl ester with a normal acid of one carbon less than studied compound was needed for calculation. Normal acids were sometimes present at very small amounts, which made the ECL calculations difficult. Nevertheless, we identified when it was possible, the isomeric steryl ester composition by means of ECL with these calculated points and confirmed that similar isomeric pattern is found between FFA and FA in steryl esters. Therefore, when ECL calculation was not possible, we identified FA isomers assuming that FFA isomer pattern was respected.

In Table 2, FCL of the different isomers of free fatty acids and steryl esters are listed. Remarkably high correlation was observed between the different values, so these chromatographic parameters have shown to be suitable for steryl ester isomer identification.

Total isomeric distribution for cholesteryl esters is 44% iso, 46% anteiso, and 10% normal; for lanosteryl esters 42% iso, 38% anteiso, and 20% normal. For dihydrolanosteryl esters are 42% iso, 35% anteiso and 23% normal. Finally for FFAs are 27% iso, 30% anteiso, and 43% normal. For odd acid chain carbon numbers, anteiso is the most abundant representing from 89% to 98% for the three families. Small differences between compounds appeared for minor isomers, normal for lanosteryl and dihydrolanosteryl esters representing around 6% and iso 4% but for cholesteryl esters, normal represents only 2% and iso even less. For even carbon acid chain only slight differences were observed between the different families of esters, iso and normal isomers were the most abundant; iso representing around 70% of the total amount and 30% the normal isomer. The difference between iso and normal tended to increase for longer chain acids raising from 50% compared to lower molecular mass esters to the detection of only iso for the bigger ones.

As shown in Section 3.2, we are able to characterise the steryl esters by means of the acid chain length and by means of ECL and FCL, thus the complete

Table 2
Comparison between FCL of steryl esters and free fatty acids, between brackets appears number of points used

	Iso	Anteiso
Free fatty acids	0.619–0.656 (23)	0.717–0.762 (23)
Cholesteryl esters	0.595–0.608 (5)	0.734–0.783 (5)
Lanosteryl esters	0.580–0.610 (4)	0.727–0.738 (3)
Dihydrolanosteryl esters	0.582–0.619 (5)	0.707–0.739 (3)

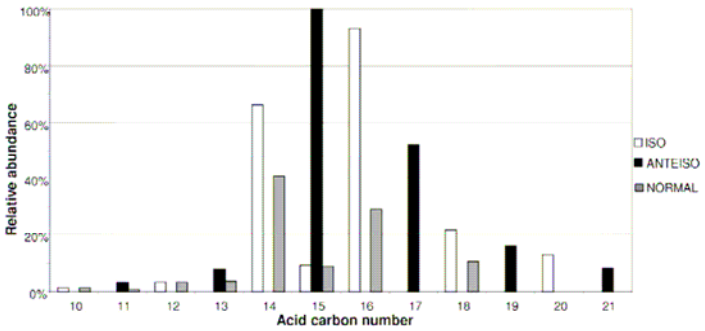

Fig. 6. Lanosteryl esters pattern against acid chain carbon number presenting the isomeric distribution.

analysis with individual compound identification has been obtained, results for lanosteryl esters are shown in Fig. 6.

4. Conclusion

Intact steryl esters from lanolin have been analysed for the first time, permitting to fill the lack of knowledge in the exact composition of lanolin. Also for the first time, lanosteryl and dihydrolanosteryl esters have been individually identified.

One of the main interests of this paper was to develop an analytical methodology, which permitted by means of sub-ambient pressure GC–MS fragmentography and chromatographic parameters to totally determine the 64 compounds belonging to three different lipid classes. Those compounds have been reported as difficult to analyse due to their high-molecular mass and to poor information given by EI-MS. Also the isomers normal, iso and anteiso have been identified for each family by means of chromatographic retention data. A comparison between different ionisation techniques has been carried out in order to optimise target compound identification. EI-MS has shown to be the most sensitive but providing poor structural information for cholesteryl esters not permitting to identify the acid moiety or molecular ion. On the other hand, CI-MS offered the best results in terms of structural information obtained in the PCI with ammonia as reagent gas. CI-MS was selected to carry out compound identification but for quantification EI-MS, more sensitive, was preferred.

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3.2 Caracterització de contaminants orgànics

En aquest apartat s'ha desenvolupat una metodologia analítica per a la determinació de diferents contaminants orgànics. La tècnica proposada està basada en un sistema cromatogràfic monodimensional acoblat a una detecció dual amb un detector de captura d'electrons i un detector específic de nitrogen i fòsfor (GC-ECD/NPD). Com ja s'ha vist en la introducció, aquest tipus de tècniques permeten d'ampliar l'interval de compostos que es poden analitzar. En el cas que ens pertoca, aquesta tècnica s'ha aplicat a la determinació de compostos organoclorats (lindà, ...), plaguicides organofosforats (diazinon, ...), piretroids (cypermethrin), un tiocarbamat (molinat) i compostos triazínics (atrazina, ...) en lanolina i en mostres d'aigua de l'estuari del riu Ebre.

En aquests casos, on es treballa amb matrius complexes, la correcta identificació dels compostos d'interès es fa difícil i és necessari emprar alguna tècnica de confirmació. En el primer treball s'ha utilitzat la GC-NCIMS i amb amoníac de gas reactiu com a tècnica de confirmació, en canvi en el cas del segon treball s'ha simplificat el procés utilitzant algunes de les propietats de la detecció dual GC-ECD/NPD i més específicament el quocient de les respostes entre els dos detectors.

3.2.1 Aplicació de la cromatografia dual a la determinació de plaguicides en lanolina

L'article d'aquest apartat, *"Trace level determination of organochlorine, organophosphorus and pyrethroid pesticides in lanolin using gel permeation chromatography followed by dual gas chromatography and gas chromatography-negative chemical ionization mass spectrometric confirmation."*, presenta la determinació de plaguicides en mostres de lanolina emprant la tècnica de la cromatografia dual.

L'interval de propietats fisicoquímiques dels plaguicides d'interès i dels compostos que conformen la matriu se superposen dificultant així la purificació que es fa com etapa prèvia a la seva determinació, en aquest cas s'utilitza la cromatografia semi-preparativa d'exclusió molecular (GPC). Com es mostra a la Figura 6, els coeficients de partició octanol/aigua (Log P) es solapen per les diferents famílies de compostos. A més cal destacar, que com s'ha vist en la secció 3.1 la lanolina conforma una matriu d'una elevada complexitat. Alguns compostos com els hidroxiàcids o els diols mostren valors de Log P que poden arribar a ser inferiors a 4 i en contrapartida els èsters alifàtics de cadena llarga poden arribar a valors de Log P superiors a 20.

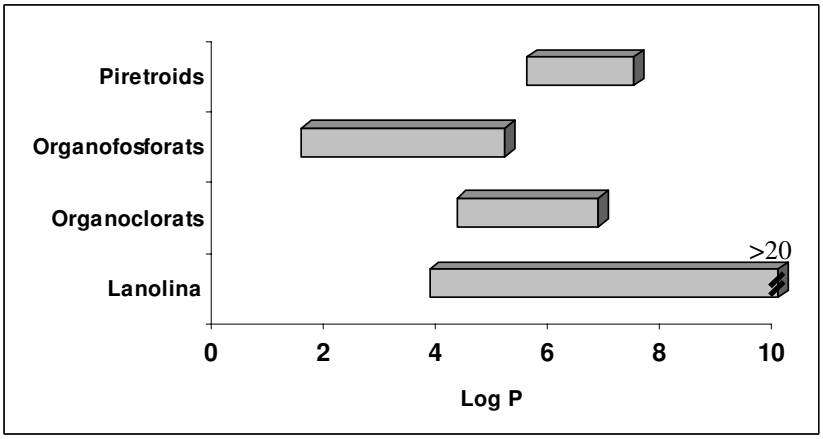

Figura 6. Distribució dels Log P, per a les diferents famílies de plaguicides estudiades i pels compostos que conformen la matriu. Els valors de Log P han estat estimats segons la teoria dels fragments [Syracuse Research Corporation, 2006].

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Trace level determination of organochlorine, organophosphorus and pyrethroid pesticides in lanolin using gel permeation chromatography followed by dual gas chromatography and gas chromatography–negative chemical ionization mass spectrometric confirmation ☆

Eric Jover, Josep Maria Bayona*

I.I.Q.A.B.–C.S.I.C., Environmental Chemistry Department, Jordi Girona 18–26, E-08034 Barcelona, Spain

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Abstract

A methodology for multi-class pesticide determination at trace level in lanolin is presented. Gel permeation chromatography on a Bio-Beads SX-3 column followed by a dual GC chromatographic determination has been developed. The effluent of the analytical column (50% diphenyl–methyl- or 14% cyanopropyl–phenylpolysiloxane) was split into an electron-capture and a nitrogen–phosphorus detection system. The chromatographic system was optimised for 28 pesticides commonly used to control sheep pests and corresponding to organochlorine, organophosphorus and pyretroid classes. Identification has been carried out by gas chromatography coupled to negative chemical ionization mass spectrometry. Recoveries ranged from 72 to 94% and the detection limits from 20 to 97 ng/g depending on the pesticide class, the RSDs were below 10%. Finally, the developed analytical methodology has been successfully applied to the determination of pesticides in several lanolin samples.

Keywords: Waxes; Organochlorine compounds; Organophosphorus compounds; Pyrethroids; Pesticides; Lanolin

1. Introduction

To control the sheep pests, insecticides such as organochlorine and organophosphorus compounds and pyrethroids are applied. Since most of these pesticides are lipophilic, they tend to accumulate into lipids, so a main part of them can be found in lanolin, the wool wax of sheep. Also, indirect input of pesticides in lanolin appears to come from the ingested grass [1]. Despite the fact that organochlorine pesticides are commonly forbidden, they are still in use in several countries.

Lanolin is a complex mixture of fatty acids and alcohols, diols, hydroxy-acids, sterols, esters, sterol esters and diesters. This blend forming the wool wax has similar properties to the human skin wax; so

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*Corresponding author. Tel.: +34-93-4006-119; fax: +34-93-2045-904.

E-mail address: jbtqam@cid.csic.es (J. Maria Bayona).

lanolin and its by-products are widely used in many applications such as baby care, cosmetics and pharmaceuticals. Furthermore, it is suspected that lanolin can vehiculate pesticide transport through the human skin. Direct ingestion by infants is possible after treatment of the mother nipple [2]. For these reasons, pesticide occurrence in lanolin has to be closely controlled. Pesticide content, according to the 1990 European Pharmacopoeia, cannot exceed 50 ng/g for individual organochlorine pesticides, 500 ng/g for other individual pesticides and 1 μg/g as total.

Pesticides occurring in lanolin have already been analysed by gas chromatography (GC) following clean up by gel permeation chromatography (GPC) [3–6]. Several detection systems such as electron-capture detection (ECD) [3–8], flame photometric detection (FPD) [3,7,8], atomic emission detection (AED) [7], thermionic specific detection (TSD) [4] and mass spectrometry (MS) [3,7] have been used for this purpose. However, due to the wide range of pesticides occurring in lanolin, the use of several detectors with different selectivity to target different analytes appears to be the most promising approach.

In this work, we present a fast methodology based on a dual GC system with ECD and nitrogen–phosphorus detection (NPD). The use of this system has already been described for pesticide analyses [9,10] but it has not been used yet for trace level determination of pesticides in lanolin. The dual detection system offers advantages over mono-elemental detection systems, such as higher sensitivity than AED for nitrogen and higher selectivity than GC–MS in the election impact ionization (EI) mode. Moreover, GC–negative chemical ionization (NCI) MS has been selected for confirmation due to its high selectivity and sensitivity for most of the target pesticides [11]. Special attention has been paid to pyrethroids since they are increasingly replacing the organophosphorus pesticides. Furthermore, they show poor response in both NPD and ECD. Therefore, detection has been optimised for these specific compounds.

The main goal of this work is to show the suitability of a single-step clean up by GPC followed by a dual GC determination of pesticides occurring in lanolin. GPC has been demonstrated to be the best option as lanolin clean-up method, but the detection system needs an improvement to permit a faster analysis.

2. Experimental

2.1. Standards and reagents

Ethyl acetate was obtained from Merck (Darmstadt, Germany). HPLC-grade cyclohexane was purchased from Panreac (Barcelona, Spain). Analytical-grade allethrin, cypermethrin, chlorpyriphos-methyl, deltamethrin, bromophos-methyl, fenthion, ethion, coumaphos, chlorfenvinphos, permethrin, hexachlorbenzene, 4,4′-DDT, 4,4′-DDD and 4,4′-DDE were obtained from Dr. Ehrenstorfer (Augsburg, Germany). Quantitative grade endosulfan, pirimiphos-ethyl, diazinon and malathion were from Riedel-de Häen (Seelze, Germany). Qualitative grade lindane, allethrin, heptachlor, aldrin, dieldrin and endrin were obtained from PolyScience (Niles, IL, USA). Lanolin 1 was a refined wool wax, Lanolin 4 a technical product, they were purchased from a local brand. Lanolins 2 and 3 are wool wax extracts obtained by supercritical fluid extraction (SFE) in our own laboratory.

2.2. Sample preparation

Standard stock solutions of 2000 μg/g have been prepared with ethyl acetate. All solutions were stored at 4 °C. Lanolin samples (1 g) were dissolved in a known volume of GPC mobile phase (5 ml). Hexachlorobenzene (HCB) (0.4 μg) dissolved in ethyl acetate, was used as surrogate and spiked into the solution that was filtered through a 0.45 μm nylon membrane (Lida, Konosha, WI, USA). Samples were injected into a high-performance liquid chromatographic system (HPLC) system equipped with a Rheodyne high-pressure injection valve (Rhonert Park, CA, USA) and a 100 μl loop. The HPLC system used was from Shimadzu (Kyoto, Japan). The system consisted of two LC-10AT pumps, an SPD-10AV UV detector, an SCL-10A controller and Class-VP software. A 450 mm×10 mm I.D. column packed with Bio Beads SX-3, 200–400 mesh (Bio-Rad, Hercules, CA, USA) was used. Ethyl acetate–cyclohexane (1:1) with a 2 ml/min flow-rate was used as mobile phase for GPC. To carry out the pesticide determination, each lanolin sample was injected four times, each of them corresponding to ca. 20 mg of lanolin on column.

Two different fractions, high-molecular-mass

compounds (t_R<8 min) and low-molecular-mass (t_R>8 min) were collected. The latter that may contain pesticides, was rotaevaporated to a volume of ca. 2 ml. Then the samples were placed into tared conic vials before evaporation under a gentle nitrogen current to ca. 100 μl, then the vials were weighed to know the exact lanolin concentration before injection into the dual GC system.

2.3. Instrumental analysis

2.3.1. Dual GC system

For the GC analysis, a chromatograph MFC 500 from Carlo Erba (Milan, Italy) coupled to NPD 800 and ECD 800 detectors from Fisons was used. Helium from Abello Linde (Barcelona, Spain) was used as carrier gas at 190 kPa, and nitrogen as ECD make-up gas at 80 kPa from Abello Linde. In NPD, the additional gas was hydrogen at 50 kPa and air at 30 kPa and as make-up gas, helium at 70 kPa. Several capillary columns were tested: 5% phenyl–methylpolysiloxane (META TX-5, Teknokroma, Sant Cugat, Spain; 30 m×0.25 mm I.D., 0.25 μm), 50% phenyl–methylpolysiloxane (DB17, J&W, Folsom, CA, USA; 30 m×0.25 mm I.D., 0.25 μm) and a 14% cyanopropyl–phenyl–methylpolysiloxane (DB1701, J&W; 30 m×0.25 mm I.D., 0.25 μm). The best results in terms of resolution were achieved with the latter two columns. Oven temperature was programmed from 70 °C (1 min), at 10 °C/min to 220 °C (0 min), and then at 5 °C/min to 280 °C (18 min). Injector and detector temperatures were held at 280 °C except the ECD 800 body that was maintained at 310 °C. A vitreous fused-silica outlet splitter purchased from SGE (Ringwood, Australia) is used to split the capillary column effluent to the two detectors. An AS 200 autosampler from CTC Analytics (Zwingen, Switzerland) was used.

2.3.2. GC–NCI-MS system

A GC system from Agilent Technologies 6890A (Geneva, Switzerland) was used, coupled to an MS detector 5973N. Ammonia was chosen as ionization gas and its pressure was optimised to $1.6 \cdot 10^{-4}$ Pa in the ion source that was held at 175 °C. Transfer line and the quadrupole temperatures were maintained at 280 and 150 °C, respectively. Helium at a constant flow-rate of 1 ml/min was used as carrier gas.

2.4. Calibration curve and quantification

The calibration curves did not have to be corrected by the internal standard because a strong linear correlation has been found without correction ($0.996<R^2<0.999$). In order to minimise detector variability, samples and calibration standards were injected in a randomised order.

3. Results and discussion

3.1. GPC fractionation

The cut-off time has been selected by injecting lanolin samples and pure pesticide solutions into the GPC system. Different pesticide classes have been tested, and lindane eluted first. Therefore, the choice of cut-off time depends on the trade off between lindane recovery and clean-up efficiency. In Fig. 1, two GPC chromatograms of a lanolin sample (Lanolin 1) and lindane are shown where the cut-off time of 8 min is indicated. To evaluate the efficiency of clean-up, the UV response of all lipidic compounds was considered to be equivalent. With the chosen cut-off time, a lindane recovery of 85% was obtained by injecting a lindane stock solution into the GPC system. The clean-up efficiency evaluated by injection of a lanolin sample into the GPC system was 79%.

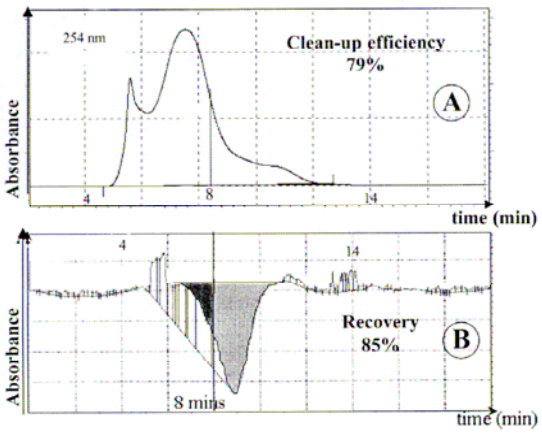

Fig. 1. GPC chromatograms of a (A) lanolin sample (20 mg) and (B) lindane (17 mg) injected on column.

3.2. Optimisation of GC–ECD–NPD

In order to optimise the chromatographic conditions, a mixture of 23 pesticides has been analysed by dual detection GC. The oven temperature program has been optimised to obtain a satisfactory separation for all target compounds. Three different pesticide classes are present in the mixture: nine organophosphorus pesticides (diazinon, chlorpyrifos-methyl, malathion, pirimiphos-ethyl, bromophos-methyl, fenthion, chlorfenviphos, ethion, and coumaphos); 10 organochlorine pesticides (heptachlor, lindane, aldrin, dieldrin, endrin, endrin ketone, endosulfan, 4,4′-DDT, 4,4′-DDD and 4,4′-DDE); and four pyrethroid pesticides (allethrin, permethrin, cypermethrin, and deltamethrin). A 14% cyanopropyl–phenyl–methylpolysiloxane (DB1701) also recommended for pesticide analysis has been evaluated but poor separation was observed between three organophosphorus pesticides (malathion, pirimiphos-ethyl and bromophos-methyl) with an α value of 1.004 between pirimiphos-ethyl and malathion, and 1.003 between malathion and bromophos-methyl. The ECD and NPD chromatograms of the mixture are shown in Fig. 2.

As confirmation method, GC–NCI-MS has been used. The mixture has been injected into the GC–NCI-MS system in full-scan mode in order to define the characteristic ions that will be used later to confirm the presence of pesticides in real samples by selected ion monitoring (SIM). A total ion current trace of the pesticide mixture by GC–NCI-MS is shown in Fig. 3 and Table 1 presents relevant data for pesticide identification.

3.3. Real lanolin and wool wax sample analysis

Recovery was calculated using hexachlorobenzene as surrogate. Recoveries from 72 to 94% were obtained (n=4) and are shown in Table 2. Lanolin represents a very complex mixture with a wide range of molecular masses and polarities. For this reason, the ECD chromatogram contains a lot of peaks that makes identification and integration difficult. How-

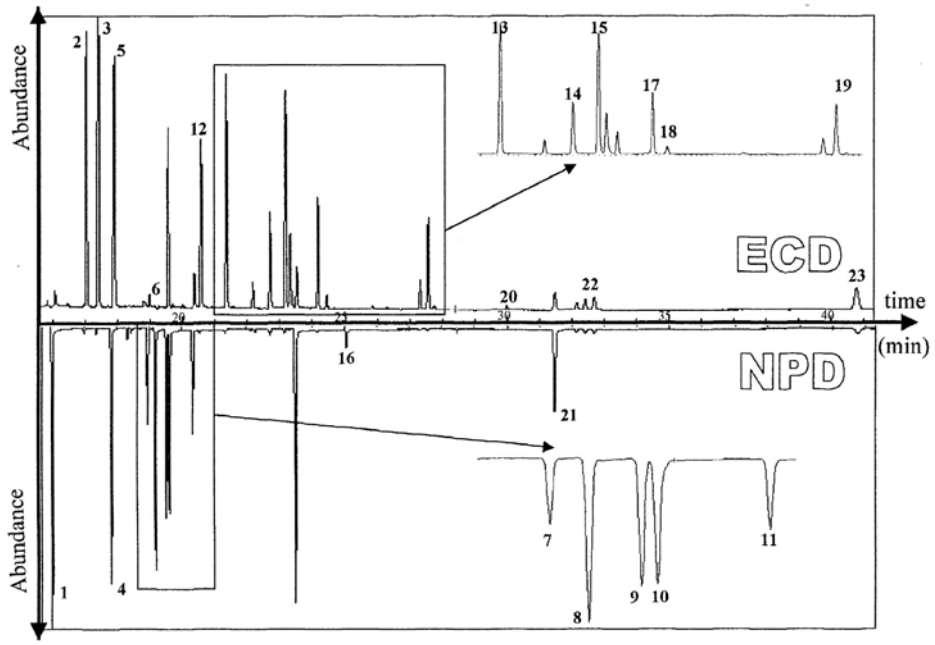

Fig. 2. Gas chromatogram of a 23-component pesticide mixture detected by ECD and NPD at concentrations ranging from 500 to 1500 μg/ml.

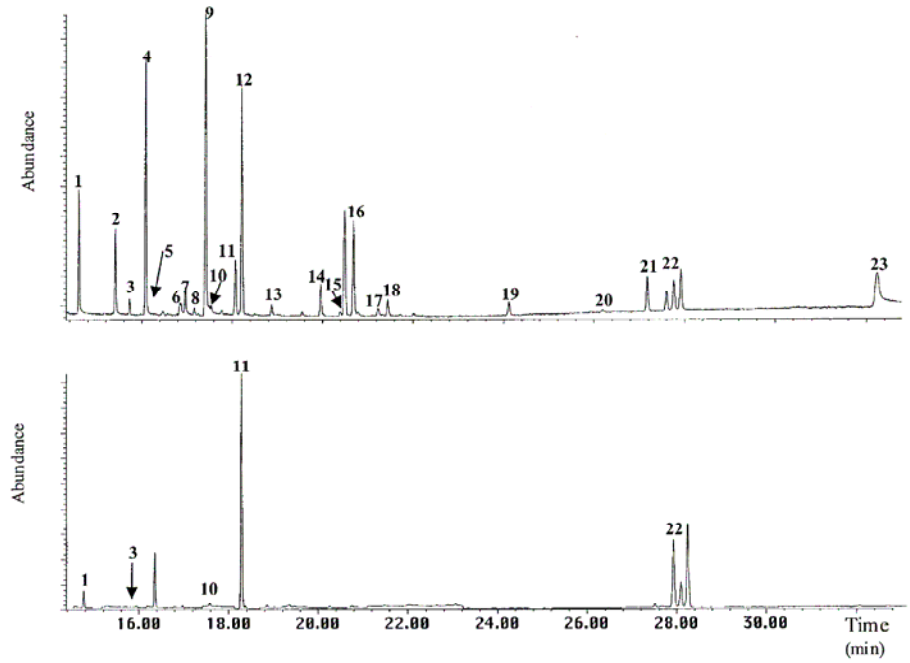

Fig. 3. Two total ion current traces obtained by NCI-MS using NH_3 at $1.2 \cdot 10^{-4}$ Pa. (A) Standard mixture of 23 pesticides with concentrations between 500 and 1500 μg/ml in the full scan mode and, (B) a real lanolin sample detected in the SIM mode.

ever, the NPD chromatogram is cleaner due to a higher detector selectivity (Fig. 4).

First of all, a qualitative study has been carried out looking for the presence of target compounds. Several of them have been detected (diazinon, chlorfenviphos, fenthion, bromophos-methyl, lindane, 4,4′-DDE and cypermethrin). Peak identification has been confirmed by GC–NCI-MS. The technique proved to be excellent, allowing high sensitivity and additional information for compound identification.

Pesticides present at higher concentrations (i.e., diazinon, chlorfenviphos, lindane and cypermethrin) have been quantified. Limits of quantitation (LOQs) are not limited by the detectors themselves but by the matrix background. Also, the splitter ratio can be modified to reach the configuration limit of a single detector, if needed. Even with the 1:1 splitter ratio used, limits of detection (LODs) of 76 ng/g were obtained for diazinon, 30 ng/g for chlorfenvinphos, 21 ng/g for lindane and 97 ng/g for cypermethrin. The LOQs of these compounds were suitable, with no important differences for any of the pesticide classes, so they can be considered representative if no coelution is observed. We cannot exclude possible coelutions because major compositional differences can be observed between samples depending on the origin of lanolin. Diazinon and chlorfenviphos have been determined using NPD, cypermethrin and lindane by ECD. Limits of detection were evaluated using three times the area of the background matrix response in the neighborhood of the retention time of the target peak and 10 times the area ratios to obtain limits of quantification (Table 3). RSDs for the different compounds are shown in Table 2. Levels above the allowed limits were observed for three samples out of the four analysed (Fig. 5). So, it is evident that this kind of methodology is suitable for routine analysis.

4. Conclusion

The developed methodology is suitable for the determination of pesticides in real lanolin samples.

Table 1
Summary of data for the identification of 29 pesticides obtained by GC–NCI-MS

Name	Number	Pesticide class	Confirmation ions	k_{DB17}	k_{DB1701}	Detection method
Dichlorvos		Organophosphorus	125, 205, 170	8.45	–	NPD
Diazinon	1	Organophosphorus	169, 303, 275	15.83	15.09	NPD
Heptachlor	2	Organochlorine	266, 300, 237	16.85	15.92	ECD
Lindane	3	Organochlorine	255, 145	17.27	17.92	ECD
Parathion-methyl		Organophosphorus	263, 154, 141	17.99	17.67	NPD
Pirimicarb		Carbamate	237, 193, 166	17.64	–	NPD
Chlorpyrifos-methyl	4	Organophosphorus	141, 212, 95	17.72	16.52	NPD
Aldrin	5	Organochlorine	237, 330, 214	17.75	16.54	ECD
Allethrin	6	Pyrethroid	167, 134, 301	18.49	18.67	ECD
Malathion	7	Organophosphorus	157, 172, 125	18.86	18.11	NPD
Pirimiphos-ethyl	8	Organophosphorus	169, 332, 304	19.13	18.03	NPD
Bromophos-methyl	9	Organophosphorus	257, 81, 141	19.47	18.16	NPD
Fenthion	10	Organophosphorus	277, 263, 247	19.57	23.88	NPD
Chlorfenvinphos	11	Organophosphorus	153, 288, 322	20.32	19.29	NPD
Endosulfan	12	Organochlorine	406, 242, 372	20.54	19.06	ECD
4,4′-DDE	13	Organochlorine	281, 246, 315	21.37	19.69	ECD
Dieldrin	14	Organochlorine	237, 346, 380	22.76	20.73	ECD
4,4′-DDD	15	Organochlorine	248, 283, 319	23.28	22.08	ECD
Ethion	16	Organophosphorus	185, 355, 153	23.63	22.26	NPD
4,4′-DDT	17	Organochlorine	281, 249, 260	24.34	22.61	ECD
Carbophenotion		Organophosphorus	185, 143, 153	24.47	–	NPD
Endrin	18	Organochlorine	272, 380, 308	24.61	23.59	ECD
Endrin ketone	19	Organochlorine	308, 272, 346	27.82	25.95	ECD
Permethrin	20	Pyrethroid	207, 354, 390	30.39	27.94	ECD
Azinphos-ethyl		Organophosphorus	185, 316, 133	31.79	–	NPD
Coumaphos	21	Organophosphorus	225, 362, 191	31.93	31.00	NPD
Cypermethrin	22	Pyrethroid	207, 171, 379	32.62	30.82	ECD
Deltamethrin	23	Pyrethroid	81, 297, 217	41.45	36.84	ECD

The dual chromatographic system supplies more information in less time and helps us to improve identification by comparing the response of a compound by two different detectors. Sample clean-up is the most important step of analysis for this kind of complex mixture. GPC eliminates 79% of the lipidic compounds but matrix peaks still appear in the ECD chromatogram. Therefore, an additional clean-up is necessary for most real samples if organochlorine pesticides are targeted. However, the developed

Table 2
HCB recoveries for the different samples and RSDs for the different compounds analysed

		Recovery (%)		RSD (%, n=4)
Lanolin 1	Replicate 1	83.30	HCB	6.50
Lanolin 1	Replicate 2	72.40	Diazinon	12.80
Lanolin 1	Replicate 3	87.10	Chlorfenvinphos	15.00
Lanolin 1	Replicate 4	93.50	Cypermethrin	8.50
Lanolin 2	SFE wool extract	83.4	Lindane	12.10
Lanolin 3	SFE wool extract	79.40		
Lanolin 4	Technical lanolin	82.50		

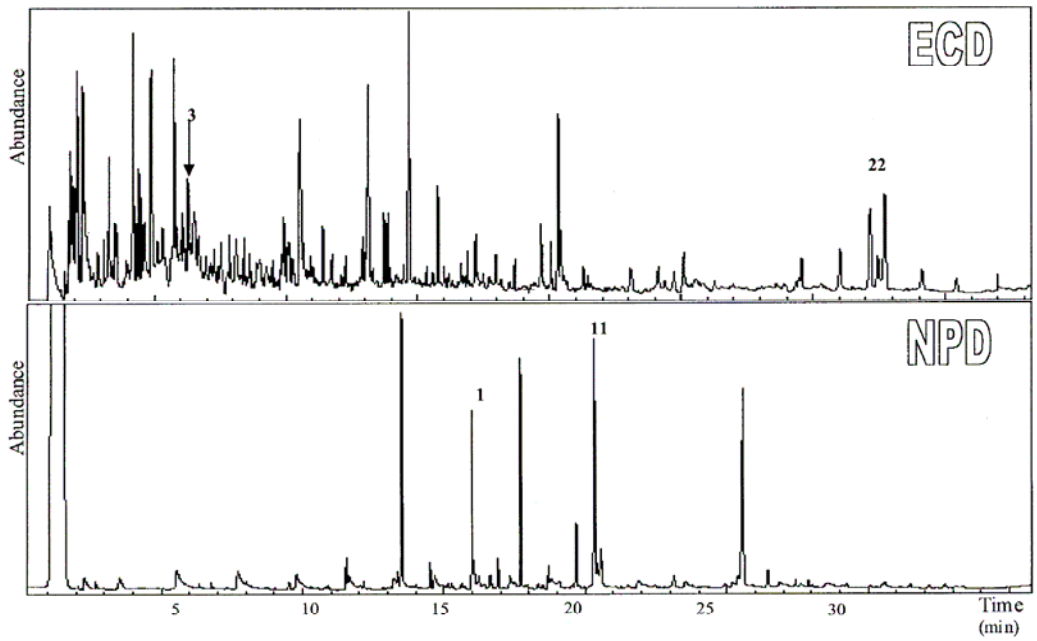

Fig. 4. Gas chromatogram of a real lanolin (1.1 mg on column) sample after GPC detected by ECD and NPD.

analytical methodology is successful for organophosphorus and pyrethroid pesticide classes that are used most nowadays.

Acknowledgements

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Table 3
LODs and LOQs for the target compounds evaluated in real lanolin samples (1.1 mg on column) by GC–ECD–NPD

	Test compound	LOD (ng/g)	LOQ (ng/g)
Organochlorine	Lindane	21	57
Organophosphorus	Diazinon	76	253
	Chlorfenvinphos	30	117
Pyrethroid	Cypermethrin	97	331

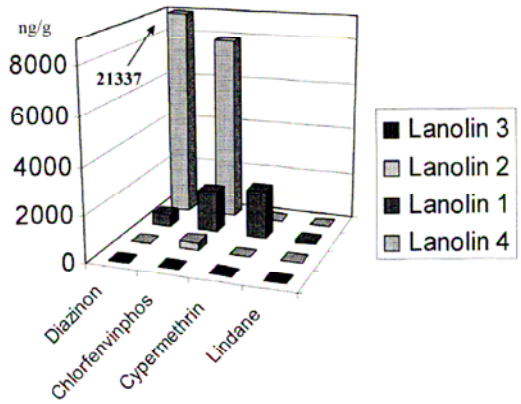

Fig. 5. Quantification of the main pesticides in four lanolin samples by GC–ECD–NPD.

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3.2.2 Utilització de la cromatografia dual per a la identificació de contaminants orgànics

A l'article, *"Development and application of a dual detection system: Electron capture and nitrogen phosphorus detectors coupled to gas chromatography. Application to polar pesticide determination in aqueous matrices"*, es presenta una metodologia per la que la tècnica de la cromatografia dual es podria utilitzar per a confirmar la identificació dels anàlits d'interès. D'aquesta manera, es podria evitar les metodologies de confirmació clàssiques que consisteixen a emprar dues columnes cromatogràfiques de polaritat diferent o, una tècnica d'espectrometria de masses. Encara que en el treball presentat es considerin tan sols plaguicides en mostres d'aigua, aquesta metodologia de treball es pot aplicar per a qualsevol anàlit i en qualsevol matriu mentres que l'anàlit presenti una mínima resposta en els dos detectors emprats i la matriu no provoqui coelucions amb els compostos d'interès. En aquest article s'ha emprat un sistema de cromatografia dual GC-ECD/NPD al ser el sistema més utilitzat per la determinació de contaminants orgànics.

To be submitted to CHROMATOGRAPHIA

DEVELOPMENT AND APPLICATION OF A DUAL DETECTION SYSTEM: ELECTRON CAPTURE DETECTOR AND NITROGEN PHOSPHORUS COUPLED TO GAS CHROMATOGRAPHY. APPLICATION TO POLAR PESTICIDE DETERMINATION IN AQUEOUS MATRICES

Eric Jover, Anna I. Gómez and Josep Maria Bayona

Abstract

Dual chromatography has already been used in order to analyse pesticides and organic contaminants in several matrices. Moreover, several studies report the potential use of this technique for compound identification using the detector response ratio (DRR) between the two detectors. In this study, DRR was defined as depending on the analyte concentration. This ratio was applied to compounds showing both positive and negative responses fitting to linear or logarithmic curves. DRR use has been evaluated for several pesticide chemical classes in aqueous matrices (organochlorine, organophosphorus, triazine and thiocarbamate) using an electron capture and a nitrogen phosphorus detector. A confirmation limit (LOC) was defined as the minimum concentration required to identify a compound using the DRR and the obtained results were confirmed by using a mass spectrometric method.

1. Introduction

An increasing number of active compounds are used as pesticides in agriculture and also in household applications. Moreover, apart of these new compounds, residues from banned products are still present in the environment. Due to their toxic effect on human health and on the ecosystems, those products need to be controlled in the food chain and in the different environmental compartments.

For these reasons, a lot of work is being done in regulating pesticides and controling them by different governemental agencies like European Union, Environmental Protection Agency (EPA) or the Food and Drug Administration (FDA). In this way, several analytical methods have been developped in order to analyse these compounds in different matrices.

Detectors such as electron capture detector (ECD) [1] for the organochlorine compounds and nitrogen phosphorus detector (NPD) [2] for the organophosphorus and triazinic compounds are widely used. Dual detection consists in splitting the effluent of the chromatographic column into two different detectors. Dual detection methodologies have already been applied in order to extend the regulated pesticide families for example by using a thermoionic detector (TSD) coupled with a flame photometric detector (FPD) for the characterisation of organophosphorus pesticides [3] and ECD:FPD for organophosphorus and organochlorine pesticides in plants [4]. Nevertheless, the prefered dual detection system for the analysis of a wide range of drugs and pesticides is the conjunction of ECD with NPD which has been applied for benzodiazepines [5,6] and imidazopyridines [5] in plasma; carbaryl and organophosphorus pesticides in water [7,8], sediment and biota [8]; 110 pesticides in water samples [9]; organophosphorus pesticides in fruit [10]; pyrethroids, organophosphorus and organochlorine pesticides in lanolin [11]; organochlorine and organophosphorus pesticides in water, sediment and benthic organisms [12]; ethylenethiourea in food commodities [13] and organochlorine, organophosphorus and nitrogenated pesticides in water [14]. All these applications use a parallel configuration, but a serial one can also be considered with the ECD, being a non destructive detector, prior to the NPD [15].

In order to confirm the identification of a chemical compound in gas chromatography, it is necessary to use mass spectreometry or to compare their retention time with an authentic standard in two different polarity chromatographic columns. The first option is still expensive for routine analysis and the second one time consuming.

Dual detection coupled to GC is a rapid, effective and low-cost identification avoiding further confirmation. This hypothesis is based on the fact that an analyte can be detected in two independent detectors and its response at each detector is a function of sample concentration as well as some molecular property of the analyte [16]. Therefore, on a dual detection system, a detector response ratio (DRR) can be used as a compound confirmation tool. In this way, ECD and flame ionisation detector (FID) have been used to identify volatile sulphides in garlic [17] and the ECD:NPD DRRs have been applied for the identification of organophosphorus pesticides [18] and to their characterization in fruit [19].

In this work, the DRR relationship with sample concentration has been calculated for different pesticide families such as organochlorine, organophosphorus, pyrethroid, triazinic compounds and even for a specific thiocarbamate with a negative response in the ECD. A proposed relationship between DRR and concentration has been tested on real surface water samples for compound identification which has been confirmed by GC-MS working in the electron impact mode.

2.Experimental

2.1-Standards and reagents

Ethyl acetate, methanol and hexane analytical grade were obtained from Merck (Darmstadt, Germany). Lindane, atrazine, simazine, diazinon and molinate quantitative grade were from Dr. Ehrenstorfer (Augsburg, Germany). Stock solutions of the different pesticides have been prepared (2000 μg/g). Fresh calibration standards were prepared from these solutions monthly.

2.2-Sample preparation

Surface water samples (2 L) from Ebre river (Spain) were filtered through GF/F filters (0.7 m). Thereafter they were preconcentrated by using solid-phase extraction (SPE) on a 100 mg polymeric phase Strata^{TM} from Phenomenex (Torrance, CA, USA). Cartridges were eluted with 10 mL of ethyl acetate-hexane (1:1) and rotaevaporated to roughly 1 mL. Extracts were fractionated on a 3 g alumina column 3% deactivated (w/w). Fraction I was eluted with 5.5 mL of hexane and contained some organochlorine compounds (OCls); fraction II with 6 mL hexane-ethyl acetate (9:1) contained the rest of OCls and finally, fraction III was eluted with 12 mL of ethyl acetate and consisted of the most polar compounds including organophosphorus an triazinic pesticides. Samples

were rotaevaporated to 1 mL and then evaporated under gentle nitrogen current at roughly 100 µL and then 10 µL of bromophos-methyl (6 µg/g) were added as internal standard. Bromophos-methyl was chosen as it showed a good response in both ECD and NPD.

2.3-Instrumental analysis

Dual GC system consisted on a chromatograph MFC 500 from Carlo Erba (Milan, Italy) coupled to NPD 800 and ECD 800 detectors from Fisons (Manchester, UK) and has been already described in an earlier publication [11]. A 14%cyanopropyl-polydimethylsiloxane (DB-1701) capillary column obtained from JW (Folsom, CA, USA) was used. Oven temperature was programmed from 70ºC (1 min), at 15º/min to 200ºC (0 min) and then at 4º/min to 280ºC (18 min). A vitreous fused silica outlet 1:1 splitter from SGE (Ringwood, Australia) was connected at the end of the chromatographic column to split the effluent into the two detectors. The capillary silica tubing used to connect the splitter to the detectors was of the same length in order to avoid differences between the two detectors retention times.

As confirmation method, GC-MS working in the electron impact mode was carried out using a Trace GC-MS 2000 system from Thermo Finnigan (Manchester, UK) with Xcalibur software-based data acquisition.

3. Results and discussion

3.1-DRR model description

DRR has been defined in other publications [10, 18], in our work DRR was slightly redifined and was considered as shown in eq. 1

$$DRR_x = \frac{\frac{A_{xd1}}{A_{I.S.d1}}}{\frac{A_{xd2}}{A_{I.S.d2}}} \qquad \textit{eq. 1}$$

being DRR_x, the DRR for the component x; A_{xd1} area obtained for x in detector1; A_{xd2} area obtained for x in detector 2; $A_{I.S.d1}$ area obtained for the internal standard (bromophos-methyl) in detector 1 and $A_{I.S.d2}$ the area obtained for the internal standard in the detector 2. As detector response is dependent on analyte concentration, DRR was considered as a function of concentration. Using a dual detection system in every single

analysis two responses were obtained. Therefore, when an external calibration was carried out, two different calibration plots were obtained, one for each detector. Working in the linearity interval of the two detectors, calibration plots could be experssed as eqs. 2 and 3.

$$\frac{A_{xd1}}{A_{I.s.d1}} = a_1 \frac{C_x}{C_{I.S.}} + b_1 \qquad \textit{eq. 2}$$

$$\frac{A_{xd2}}{A_{I.s.d2}} = a_2 \frac{C_x}{C_{I.S.}} + b_2 \qquad \textit{eq. 3}$$

where a_1 is the slope and b_1 the ordinate in the origin of detector *1* calibration plot; a_2 the slope and b_2 the ordinate in the origin of detector 2 calibration plot and C_x the concentration of the analyte *x*. Therefore if eq. *1* is expressed as a function of the analyte concentration it becomes eq. 4.

$$DRR_x = \frac{a_1 \frac{C_x}{C_{I.S.}} + b_1}{a_2 \frac{C_x}{C_{I.S.}} + b_2} \qquad \textit{eq. 4}$$

An example of the different calibration plots and of the $DRR_x(C_x)$ function are presented in Figure 1. It has to be highlighted that DRR_x is dependent of the used detectors and cannot be extrapolated from one instrument to another one. Also other experimental conditions such as split ratio, make-up pressure, gas purity and detector temperature can modify this value.

Quantification

In order to quantify, only the response of one of the detectors will be used. The chosen detector will be the one with a higher sensitivity (steeper slope) and a largest linearity interval. Analyte concentration will be directly calculated from eq. 2 or eq. 3 and will be called experimental concentration ($C_{x\text{-}exp}$). Also from the detector used for quantification will be calculated the detection limit (LOD_x) and the quantification limit (LOQ_x) for the analyte x.

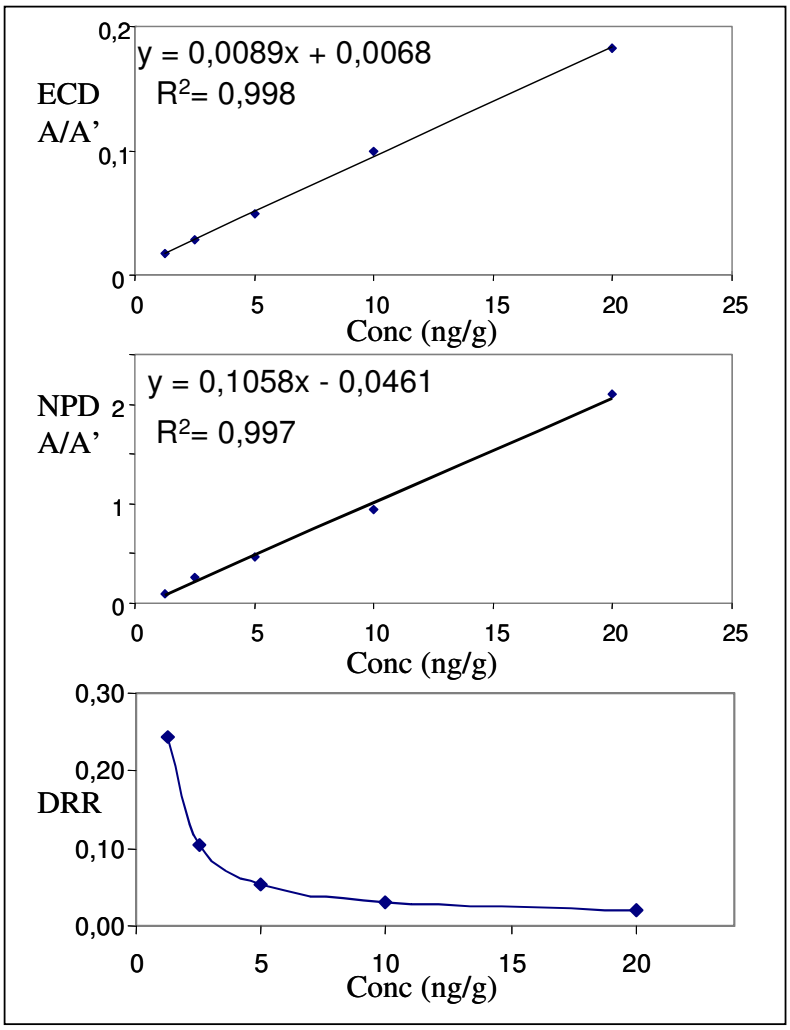

Figure 1. Diazinon ECD and NPD calibration plots; and the $DRR_{Diazinon}$ as a function of diazinon concentration.

Identification Confirmation

In order to confirm analyte identification, experimental DRR ($DRR_{x\text{-}exp}$) for the sample was compared with DRR_x obtained by replacing C_x by the $C_{x\text{-}exp}$ calculated with the most sensitive detector in *eq. 4*. If the $DRR_{x\text{-}exp}$ is equal to DRR_x, considering its RSD, compound identity is then confirmed. If not, the experimentally observed compound has the same retention time (or partially coeluting) but is not the analyte x. In order to confirm analyte identity, that compound has to respond in the two detectors. Therefore a limit of confirmation (LOC_x) can be considered as being the detection limit (LOD) for the detector giving the poorest signal for the analyte x.

Electron capture detector tends to have a short linearity intervals giving a response with a general logarithmic shape. No logarithmic curve can be used as a calibration due to large errors induced by this shape; but in order to confirm compound identity, it is feasible. Therefore, *eq. 2* or *eq. 3* can be replaced by the logarithmic equation modifying in this way the final DRR_x equation *eq. 4* becoming *eq. 5*.

$$DRR_x = \frac{a_1 \ln\left(\frac{C_x}{C_{I.S.}}\right) + b_1}{a_2 \frac{C_x}{C_{I.S.}} + b_2} \qquad \textit{eq. 5}$$

3.2-Application of DRR to different pesticide families and to real samples

This methodology was used in order to identify and quantify pesticides in samples of suface water from Ebro river. The following pesticides of four different pesticide families were identified: lindane (organochlorine), diazinon (organophosphorus), molinate (thiocarbamate), atrazine and simazine (triazinics), and desethyl atrazine (triazinic metabolite). Also other compound families were tested but not found in real samples such as pyrethroids (permethrin, cypermethrin, allethrin and deltamethrin) and a methoxy-s-triazine (terbumeton).

Two of the investigated compounds, molinate and terbumeton, gave a negative response in the ECD. Even in these conditions, DRR_x was successfully applied. The only special requirement was the need to have a high detector offset value in order to avoid negative peak saturation.

In Table I, important parameters obtained for the calibration plots for some of the encountered analytes are presented. It should be pointed out that the molinate slope was negative for the ECD as its response was negative. For the two triazines, a logarithmic fitting has been used for the ECD response. LODs ranged from 0.05 to 0.35 pg, LOQs from 0.28 to 1.40 pg and finally LOCs ranged from 0.31 to 5.02 pg.

Table I. Summary of the main calibration parameters for the encountered compounds.

	Chemical class	NPD Calibration plot		NPD R^2	ECD Calibration plot		ECD R^2	Quantification detector	LOD(1)	LOQ(2)	LOC(3)
		a	*b*		*a*	*b*					
Lindane	Organochlorine	5×10^{-4}	3×10^{-4}	0.990	6.26×10^{-2}	7.69×10^{-2}	0.999	ECD	0.08	0.95	5.02
Diazinon	Organophosphorus	1.058×10^{-1}	-4.61×10^{-2}	0.998	8.9×10^{-3}	6.8×10^{-3}	0.991	NPD	0.05	0.28	0.31
Molinate	Thiocarbamate	4.7×10^{-3}	-5×10^{-4}	0.999	-3×10^{-4}	-1.0×10^{-3}	0.989	NPD	0.35	1.28	2.55
Atrazine	Triazine	9.0×10^{-3}	-9.9×10^{-3}	0.999	1.82×10^{-2}(4)	1.25×10^{-2}	0.990	NPD	0.32	0.99	2.19
Simazine	Triazine	1.50×10^{-2}	-1.08×10^{-2}	0.999	2.01×10^{-2}(4)	-1.23×10^{-2}	0.985	NPD	0.28	1.40	3.11

(1)Detection limit was considered as three times blank level expresses in pg; (2) Quantification limit was considered as the lower calibration point expressed in pg; and (3) LOC was the quantification limit of the poorest response detector. (4) Logarithmic equation (*eq. 5*)

Figure 2 showed the DRR_x curves for two different pesticides, diazinon and chlorpyryfos-methyl. In addition, the $DRR_{x\text{-}exp}$ has been ploted for the different analysed samples. As can be seen, the diazinon identity has been confirmed and the chlorpyryfos-methyl rejected despite the same retention time coeluting within the cluster. These hypothesis have been corroborated by GC-EIMS.

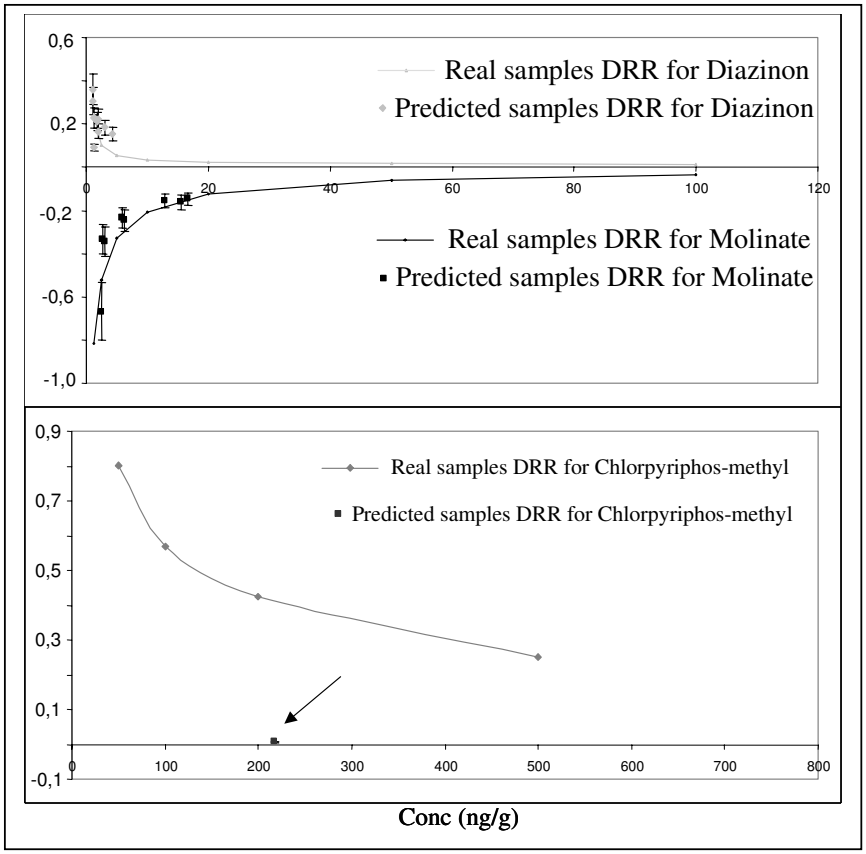

Figure 2. a) DRR_x plot for diazinon and $DRR_{x\text{-}exp}$ for the different real samples. **b)** DRR_x plot for chlorpyryfos-methyl and $DRR_{x\text{-}exp}$ for a real sample.

One of the problems of this technique is the possibility of a false negative induced by a chromatographic co-elution. In fact, if an interference is present in one of the detectors at the same retention time, it will change the $DRR_{x\text{-}exp}$ and it will be different to the DRR_x. In order to control this effect, the presence or absence of the studied analytes in all the samples was confirmed by GC-MS. And no false negative was encountered for the analysed samples but in several cases for minor compounds apparent partial co-elutions avoided the right calculation of DRR_x. Problems in DRR_x application will be proportional to the complexity of the studied matrix amd inversely proportional to the target analytes concentration. Therefore, an efficient sample

preparation and clean-up before the final determination are compulsory when dual detection is applied in real samples.

4. Conclusion

The GC-ECD/NPD has shown to be an efficient way to analyse multiclass pesticides such as organochlorine, organophosphorus, thiocarbamate and triazines. Furthermore, the use of DRR, which is a function of the analyte concentration, is helpful in order to confirm the compound identification. Therefore, the use of time consuming methods, such as GC-MS or the use of diferent polarity columns, being no more compulsory for trace level determination of pesticides in medium-to-low complexity matrixes.

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3.3 Discussió de resultats

Pel que fa els àcids i altres compostos lipídics polars, cal destacar que gràcies a la resolució cromatogràfica de la GC, la informació complementària que aporta la MS i al càlcul d'índexs de retenció (ECL/FCL) s'han pogut identificar més de 170 compostos individuals que corresponen a les famílies següents: FAs, FALs, diols, hidroxiàcids i VFAs. Les dues metodologies monodimensionals seleccionades (GC-EIMS i HSPME GC-NCIMS), que són complementàries, permeten determinar un interval molt ampli d'àcids grassos des de l'àcid acètic (C_2) al n-C_{33}. Si considerem la distribució d'aquests compostos, presenten un màxim corresponent a C_{16} i dos submàxims locals a C_{27} i C_2. L'àcid acètic prové de la β-oxidació d'àcids de cadena més llarga [Reactome, 2006]. Per a explicar l'origen dels dos altres màxims existeixen dues hipòtesis. La primera correspondria a l'existència de dues rutes enzimàtiques diferents, l'una realitzaria la síntesi de la distribució centrada a l'entorn del C_{16} i l'altre en faria l'extensió. Aquest tipus de funcionament ja s'ha observat per organismes terrestres [Kolattukudy, 1976]. La segona correspondria a una distribució que provindria dels FFAs i l'altre de la descomposició dels èsters. En el darrer cas hauríem de diferenciar entre la hidròlisi natural dels èsters que faria que en les mostres originals ja s'observés aquest perfil bimodal pels àcids de cadena llarga o que els resultats obtinguts tinguin un biaix experimental degut a l'etapa de saponificació que es realitza com a pretractament de la mostra. Les anàlisis realitzades posteriorment sense pretractament de la mostra portarien a descartar aquesta última hipòtesi.

En aquests treballs s'ha confirmat algunes de les particularitats de la lanolina que és la presència de sèries homòlogues pel que fa els FAs i els FALs sense una marcada diferència entre l'abundància dels compostos parells i senars. De la mateixa manera, tampoc s'ha aconseguit detectar la presència d'àcids grassos insaturats (PUFA). És interessant observar en el segon treball que pel que fa als VFAs, l'origen d'aquests compostos no és únic i que fins i tot en la lanolina comercial (purificada) s'observa un nivell elevat d'àcid isovalèric, característic de la degradació de proteïnes, que prové de la contaminació de la lanolina amb matèria fecal de les pròpies ovelles.

Pels hidroxiàcids i diols, s'han obtingut distribucions molt similars, on l'isòmer lineal és el més abundant. Aquestes similituds demostren un origen comú per aquestes dues famílies de compostos com podria ser la hidròlisi dels diesters presents a la lanolina. Tampoc no es pot descartar que una de les famílies provingui directament de l'altre per oxidació (de diol a hidroxiàcid) o bé per reducció (de hidroxiàcid a diol).

Encara que la tècnica utilitzada mostra una elevada resolució cromatogràfica, no es poden evitar completament les coelucions com es mostra a la Figura 3 de la (pàgina 32, [Moldovan *et al.*, 2002]). En aquests casos, és especialment necessària la utilització d'ions diagnòstic específics per a poder identificar correctament els compostos. Malgrat això, quan els compostos que coelueixen presenten estructures similars cal avaluar-ne l'especificitat. Per exemple, en les condicions experimentals avaluades, el FA C_{19} *iso* està coeluint amb el hidroxiàcid C_{17} normal i els dos compostos donen l'ió característic m/z 129 $[(CH_2)_2C(O)OSi(CH_3)_3]^+$. Així doncs, aquests compostos s'haurien de diferenciar utilitzant d'altres ions diagnòstic o bé emprant una altre fase estacionaria que presenti una selectivitat diferent.

Per a la determinació simultània de FAs, FALs, hidroxiàcids i diols, la sililació ha demostrat ser una tècnica adequada ja que encara que no sigui la més emprada per l'anàlisi de FAs (s'acostuma a emprar la metilació), si que permet, en una sola etapa, de derivatitzar les quatre famílies químiques estudiades. La sililació també permet obtenir ions diagnòstic intensos tant estructurals, que s'empren per a identificar la família a la que pertany el compost d'interès, com indicadors de la massa molecular (normalment $[M\text{-}CH_3]^+$). No obstant això, dues de les limitacions d'aquesta tècnica de derivatització, són la manca d'estabilitat dels compostos derivatitzats, ja que s'hidrolitzen ràpidament i la necessitat d'evaporar a sequedat per tal d'eliminar l'excés i els residus de l'agent derivatitzant abans de realitzar l'anàlisi. Aquest últim aspecte dificulta l'anàlisi de compostos de baixa massa molecular (volàtils) ja que es podrien perdre durant aquesta etapa d'evaporació. Però precisament, tal com s'ha esmentat en l'article que es refereix als VFAs [Jover et al., 2005], els compostos volàtils s'han determinat mitjançant HS-SPME i per tant la tècnica de sililació ha estat aplicada tan sols per la caracterització dels compostos semivolàtils.

És important, quan es treballa amb matrius tan complexes com la lanolina, de realitzar una estratègia de caracterització progressiva i seqüencial de la mostra. Així, es pot analitzar la lanolina seguint diferents etapes tot incrementant el nivell de detall. En una primera etapa, gràcies a ions amb informació estructural, podem identificar les diferents famílies químiques presents a la mostra. Després, mitjançant ions diagnòstic de la massa molecular del compost, s'identifica la massa molecular de cada component d'una mateixa família química. Finalment, s'identifica els diferents isòmers posicionals mitjançant els índexs de retenció cromatogràfics. Aquest tipus d'estratègia no tan sòls és d'aplicació en la caracterització de la lanolina sinó que també es podria utilitzar per a la

caracterització de qualsevol tipus de matriu lipídica complexa. Per a realitzar l'anàlisi quantitativa d'aquests compostos, degut a la gran quantitat de compostos individuals, és necessari considerar que el factor de resposta pels membres d'una mateixa família química, com per exemple els FAs, serà el mateix.

Pel que fa referència als èsters alifàtics, una primera conclusió evident seria que no podem esperar aconseguir separar completament els compostos individuals degut a l'extrema complexitat de la barreja (variació en la massa molecular, combinacions de diferents longituds de cadena dels fragments àcids i alcohols i diferents isòmers posicionals). El que aconseguim és tenir els compostos agrupats per *clusters* de la mateixa massa molecular. Dins d'aquests clusters, la separació cromatogràfica que s'obté, ve donada per la diferent combinació d'isòmers posicionals (utilització d'índexs de retenció). No obstant s'observa que no s'aconsegueix diferenciar entre la composició isomèrica de la branca alcohòlica de la de la branca àcida utilitzant aquests índexs. Així doncs, tindríem coelucions completes dels compostos d'estructura simètrica (Figura 7). Però el que no aconseguim amb la resolució cromatogràfica si que s'aconsegueix mitjançant l'espectrometria de masses.

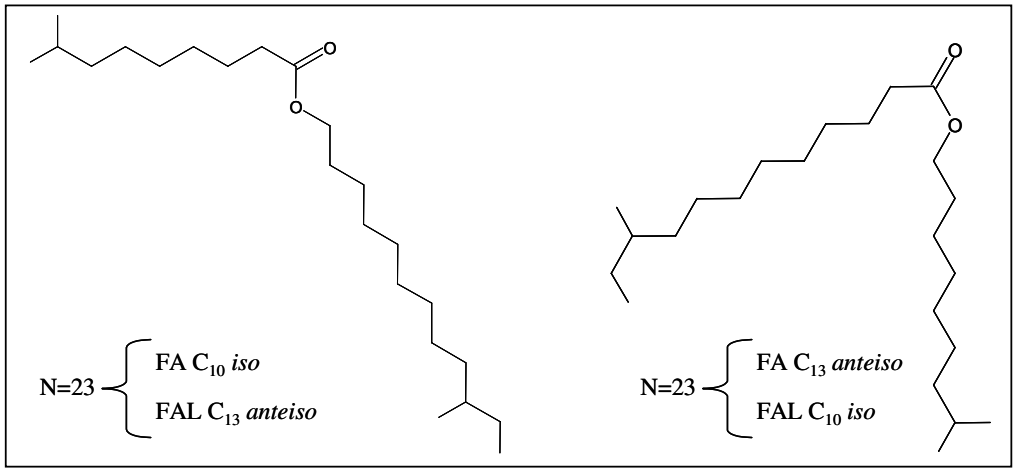

Figura 7. Exemple d'estructura química de compostos simètrics que coeluirien en el sistema de HTGC-MS.

Els dos compostos de la Figura 7 presenten un espectre de masses lleugerament diferent que ens permet identificar-los. Els dos tenen l'ió m/z 354 corresponent a la massa molecular de la substància, però en el cas FA C_{10} l'ió més abundant seria el m/z 173 i en canvi pel FA C_{13} seria m/z 215. Així doncs, l'espectrometria de masses i els

índexs de retenció són informacions complementàries que permeten caracteritzar a nivell molecular aquesta complexa barreja d'èsters alifàtics.

En els dos treballs corresponents a la determinació dels FAs i FALs [Moldovan *et al.*, 2002, Jover *et al.*, 2003] s'ha comentat que el seu origen podia ser la hidròlisi dels èsters sintetitzats a les glàndules sebàcies. És interessant comparar doncs l'estructura isomèrica dels èsters que s'ha determinat amb l'estructura que haurien de tenir si els FALs i FAs que hem analitzat fossin el resultat de la seva hidròlisi. En la Taula 2 s'observa aquesta comparativa, tot considerant que un èster de massa molecular X estaria tan sols format pel compost més abundant que s'ha trobat en el *cluster* corresponent a aquesta massa molecular. La coincidència de resultats és remarcable podent, així, confirmar la nostra hipòtesi. Solament en tres casos la forma isomèrica prevista no es correspon amb la determinada experimentalment i es poden explicar fàcilment per les aproximacions realitzades per a construir aquesta taula.

Taula 2. Comparació de l'estructura isomèrica dels èsters determinada experimentalment o gràcies a la caracterització isomèrica dels èsters i alcohols.

N èster[1]	**N FA[1]**	**FA[2]**	**N FAL[1]**	**FAL[2]**	**Prev.[3] Èster**	**Exp.[4] Èster**
37	15	n	22	n	**n:n**	**n:n**
38	14	n	24	n	**n:n**	**n:n**
39	15	n	24	n	**n:n**	**n:n**
40	16	n	24	n	**n:n**	**n:n**
41	16	n	25	a	**a:n**	**a:n**
42	16	n	26	i	**i:n**	**i:n**
43	16	n	27	a	**a:n**	**a:n**
44	18	n	26	i	**i:n**	**i:n**
45	18	n	27	a	**a:n**	**a:n**
46	20	i	26	i	**i:i**	**i:n**
47	20	i	27	a	**i:a**	**i:a**
48	22	i	26	i	**i:i**	**i:n**
49	22	i	27	a	**i:a**	**i:a**
50	24	i	26	i	**i:i**	**i:n**
51	24	i	27	a	**i:a**	**i:a**
52	25	a	27	a	**a:a**	**a:a**
53	26	i	27	a	**i:a**	**i:a**
54	27	a	27	a	**a:a**	**a:a**

[1]Número de carbonis dels fragment àcid o alcohòlic de l'èster més abundant amb aquest número de carbonis total.
[2]Estructura isomèrica més abundant del FA o FAL considerat [Moldovan *et al.*, 2002a].
[3]Estructura isomèrica de l'èster més abundant prevista considerant els isòmers més abundants trobats en la caracterització de FAs i FALs.
[4]Estructura isomèrica de l'èster més abundant determinada cromatograficament [Moldovan *et al.*, 2002b].

Els èsters esteroidals presents a la lanolina formen un grup menys complex de compostos que malgrat això, resulten més difícils d'analitzar. Formen una barreja menys complexa perquè tan sòls tenim tres variants del fragment alcohòlic (colesterol, lanosterol i dihidrolanosterol). Per altra banda, el fet ja comentat, de que són substàncies termolàbils, no ha permès d'analitzar tots els èsters conjuntament motivant la utilització d'una tècnica cromatogràfica específica (GC a pressió sub-ambient) per a la determinació d'aquest grup de compostos. Malgrat això, i degut a les seves característiques, emprar com a detector l'espectròmetre de masses amb ionització per impacte electrònic (EIMS) no ha estat suficient ja que pel que fa els èsters de colesterol tan sols s'obtenen els ions corresponents al fragment esteroidal. Així doncs, mitjançant GC-EIMS es pot identificar la família química del compost però no la longitud del fragment àcid o la seva massa molecular. Per això, es van desenvolupar tècniques d'ionització més suaus com la ionització química positiva (PCIMS) o la negativa (NCIMS) utilitzant diferents gasos reactius (amoníac, metà i isobutà). Aquestes tècniques d'ionització, al ser més suaus, eviten la fragmentació excessiva de les molècules làbils i en alguns casos permeten detectar l'ió molecular o el seu pseudo-ió molecular com quan es treballa en PCIMS amb amoníac ($[M+NH_4]^+$). De la mateixa manera que per les altres famílies de lípids presentades també s'han utilitzat índexs de retenció cromatogràfics per a identificar els diferents isòmers posicionals del fragment àcid. Com a la taula 2 pels èsters alifàtics, les abundàncies isomèriques dels èsters esteroidals també ens demostren que els FAs provenen de la hidròlisi dels èsters.

Tal com s'ha presentat en els articles d'aquesta secció, l'anàlisi a nivell molecular dels compostos lipídics que integren la lanolina, tot i la elevada complexitat i temps necessari per dur-la a terme, té nombrosos interessos ja que alguns compostos individuals, tenen propietats característiques. Així, a la introducció hem vist com alguns compostos tenen una especial toxicitat o al revés, aporten beneficis importants que fan que la seva anàlisi a nivell molecular sigui valuosa. De manera més genèrica, quan realitzem aquestes determinacions a nivell molecular generem una gran quantitat d'informació que ens pot permetre, com ho veurem a l'aplicació (secció 5.1), d'entendre millor el funcionament d'un ecosistema o com hem comentat en la secció 3.1.1, l'observació de la distribució dels àcids grassos individuals (amb 2 màxims locals) ens permet fer la hipòtesi de que la síntesi d'aquests compostos es fa seguint dues vies diferents. Així doncs, encara que laboriosa, l'anàlisi de lípids a nivell molecular és una important font d'informació. Potser la pregunta que ens haguéssim hagut de fer seria

més aviat si sempre és útil fer aquest tipus de caracteritzacions. La resposta en aquest cas és un no rotund. Hi han moltes aplicacions que no necessiten de tanta resolució en la caracterització, en molts casos les proporcions entre diferents famílies químiques ja són suficients. Per a citar un exemple proper, la Farmacopea Europea exigeix per a que una lanolina pugui ser comercialitzada que no tingui un contingut d'àcids grassos lliures superior a 0.56% [Ministerio de Consumo, 2002]. En aquests casos, en lloc de determinar la suma dels FAs, cal una tècnica separativa de menor resolució com pot ser la cromatografia de capa fina (TLC) i es quantifiquen tots els FAs, que apareixen com un sol pic cromatogràfic, conjuntament. De fet, la TLC acoblada a un FID ja ha estat utilitzada per a caracteritzar la lanolina i els lípids interns de la llana [Coderch *et al.*, 1996, Fonollosa *et al.*, 2000, Leaver *et al.*, 1988]. Així doncs, les dues tècniques són complementàries ja que aporten una informació diferent de la mostra. Per tant, no és estrany que s'hagin utilitzat conjuntament en la caracterització de diferents extractes de lanolina [Domínguez *et al.*, 2003]. Fins i tot la complementarietat d'aquestes dues tècniques separatives de diferent resolució cromatogràfica ha estat estudiada i considerada com a positiva [Jover *et al.*, 2006] en la caracterització d'un extracte de lanolina. En conclusió, per a poder escollir la metodologia analítica a emprar cal tenir present el nivell de resolució necessària per cada aplicació.

Pel que fa referència a la determinació de contaminants orgànics emprant tècniques monodimensionals, en aquest apartat s'ha desenvolupat tant els aspectes pràctics com els més teòrics de la cromatografia dual. A continuació resumirem els punts forts i punts febles d'aquesta tècnica. Una de les principals avantatges d'aquesta tècnica és la seva versatilitat, comparada amb la GC clàssica ja que permet determinar diferents famílies químiques de compostos simultàniament tot mantenint bons nivells de detecció. Un altre avantatge, que s'ha intentat de desenvolupar en el segon treball d'aquesta secció, és la possibilitat de simplificar el procés d'identificació d'un pic cromatogràfic. El que normalment s'havia de fer emprant un GC-MS o comparant temps de retenció en columnes de diferent polaritat, ara es pot fer directament amb el DRR que és un paràmetre basat en els quocients de resposta dels dos detectors. L'únic requisit previ és que els compostos d'interès han de presentar una resposta, encara que sigui negativa, en els detectors emprats. Aquest DRR per uns detectors donats dependrà de l'estructura fisicoquímica del compost i de la concentració en la que es troba present. En aquest sentit als generalment emprats LOD i LOQ, s'hi afegeix el límit de

confirmació (LOC) que correspon a la quantitat mínima d'anàlit que es necessita per a confirmar-ne la identificació emprant el sistema del DRR. Aquest tipus de muntatge pot ajudar a millorar la productivitat dels laboratoris d'anàlisi disminuint el temps necessari a la confirmació dels compostos identificats (generalment realitzada per GC-EIMS o GC-NCIMS).

Pel que fa els aspectes negatius, cal recordar que com per la majoria de processos cromatogràfics és necessari tenir un pretractament de la mostra que permeti tenir un extracte el més purificat possible. A més, és útil que aquest procés també serveixi d'etapa de preconcentració a fi de millorar la relació senyal soroll en el moment de la determinació final. Així doncs, l'efectivitat de la GC-ECD/NPD i de la cromatografia dual en general continua sent massa dependenta de la complexitat de l'extracte a analitzar. A més, si ho comparem amb una tècnica de GC clàssica amb la cromatografia dual hi ha l'agreujant que les coelucions amb d'altres compostos provenint de la matriu donaran peu a falsos negatius ja que els DRR determinats experimentalment no es correspondran amb els DRR teòrics calculats a partir de les rectes de calibració considerant aleshores el compost d'interès com a no present. Finalment, si es considera el continu desenvolupament de l'espectrometria de masses (quadrupols) com a tècnica estàndard destacant de manera especial les millores en aspectes relacionats a la robustesa, la velocitat, la sensibilitat i el cost de la tècnica cal pensar que la MS i especialment la EIMS amb quadrupol s'anirà imposant pel que fa a la detecció de la majoria de contaminants orgànics en GC.

3.4 Conclusions

En aquest treball s'han desenvolupat diferents tècniques cromatogràfiques monodimensionals per a la caracterització de lípids i contaminants orgànics. En el cas de la determinació dels lípids i degut a la seva complexitat s'han hagut de desenvolupar tècniques específiques per a les diferents famílies químiques que la composen. En aquest sentit s'ha desenvolupat les diferents metodologies següents:

- HS-SPME GC-NCIMS per a la determinació de VFAs.
- GC-EIMS per a la determinació de FFAs, FALs, hidroxiàcids i diols.
- HTGC-EIMS per a la determinació d'èsters alifàtics.
- GC a pressió sub-ambient acoblada a diferents tècniques d'espectrometria de masses per a la determinació d'èsters esteroidals.

Aquestes tècniques s'han aplicat a la caracterització de la lanolina que s'ha emprat com a matriu de referència. A part d'aconseguir la caracterització completa de les diferents famílies químiques, els resultats obtinguts han permès obtenir una valuosa informació dels mecanismes existents de formació dels diferents lípids. En aquest sentit és interessant de destacar els punts següents:

- Pel que fa els VFAs s'observa una barreja de compostos que provenen de tres orígens ben diferenciats. Trobem la distribució habitual dels àcids grassos de la lanolina que se superposa amb una abundant proporció d'àcid acètic que prové de la -oxidació dels FAs i a uns elevats nivells d'àcid iso-valèric, producte de degradació de les proteïnes que prové d'una contaminació fecal de la lanolina.
- La caracterització dels FAs així com la dels corresponents èsters tant alifàtics com esteroidals, ha permès determinar la seva procedència a partir de la hidròlisi dels èsters. Cal considerar que la distribució bimodal observada estaria causada per la intervenció de dues vies enzimàtiques diferents; la primera responsable de la síntesi *de novo* dels àcids grassos i la segona de la seva extensió.
- En aquest apartat també s'ha desenvolupat una metodologia monodimensional basada en la cromatografia dual per a la detecció de contaminants orgànics. En aquest sentit, s'han desenvolupat aspectes tant teòrics com aplicats de la cromatografia dual. A més s'ha aplicat per primer cop aquesta tècnica per a la detecció de plaguicides a la lanolina i s'ha aprofundit en el concepte del quocient entre la resposta dels detectors en matrius aquoses per a utilitzar-lo per a la confirmació de la identificació de contaminants orgànics.