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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

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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-

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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].

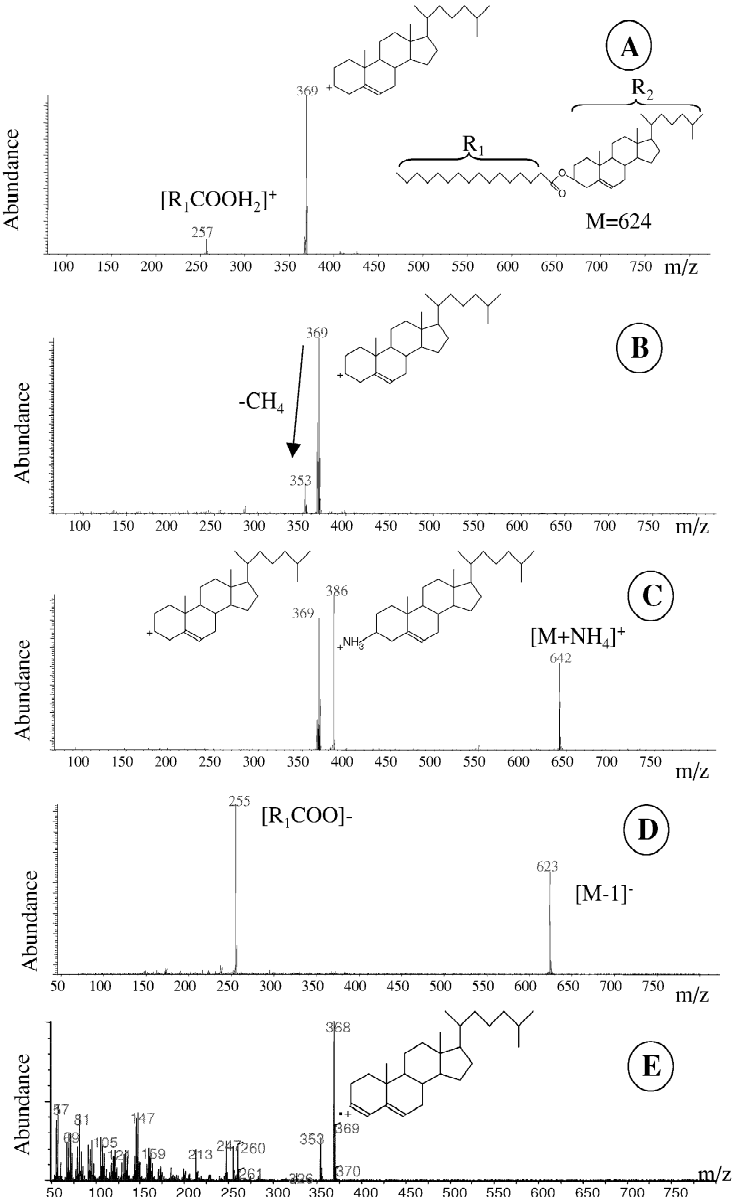

Fig. 1. Spectrum of cholesteryl palmitate using different ionisation modes: (A) isobutane PCI; (B) methane PCI; (C) ammonia PCI; (D) ammonia NCI; and (E) EI.

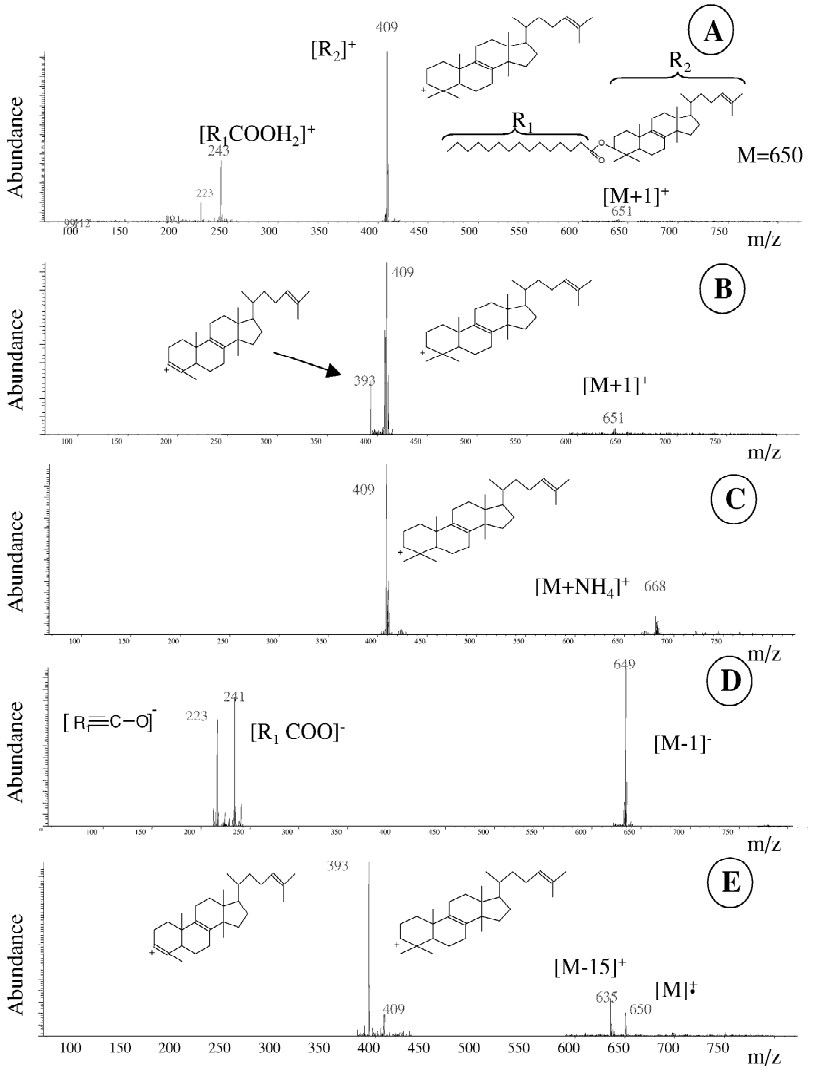

Fig. 2. Spectrum of lanosteryl pentadecanoate using different ionisation modes: (A) isobutane PCI; (B) methane PCI; (C) ammonia PCI; (D) ammonia NCI; and (E) EI.

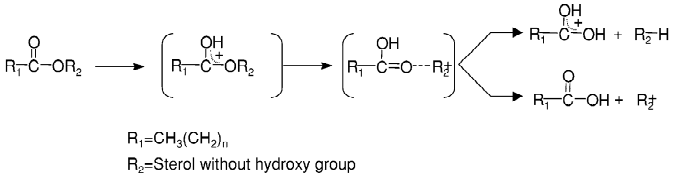

Fig. 3. Mechanism of ionisation of steryl ester in PCI.

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-

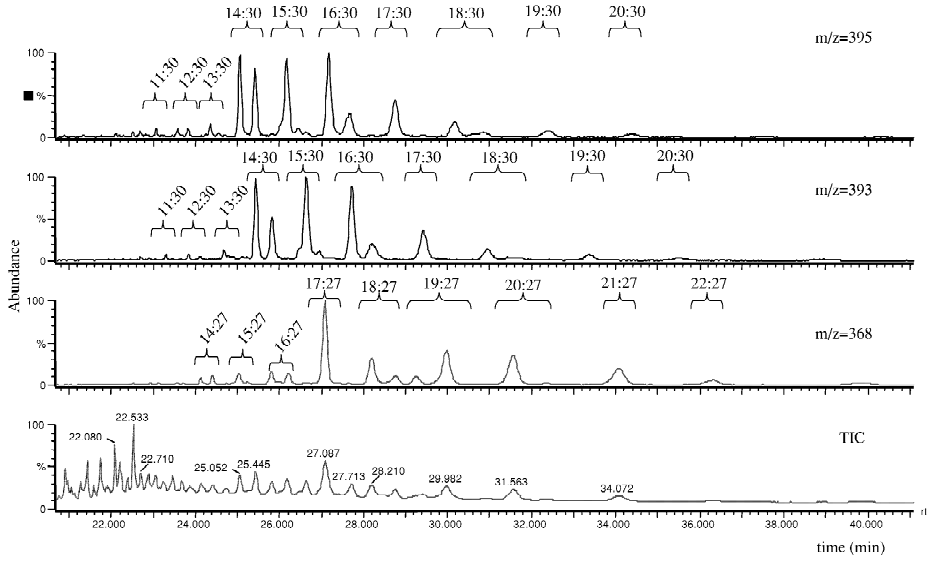

Fig. 4. Fragmentograms of a characteristic lanolin sample showing the total ion current (TIC) and steryl ester characteristic ion at m/z 395 for dihydrolanosteryl esters, m/z 393 for lanosteryl esters and m/z 368 for cholesteryl esters. The composition of acid and alcohol moieties forming the steryl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

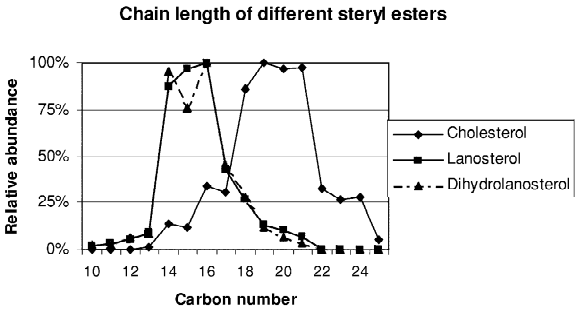

Fig. 5. Relative abundance of steryl esters against acid chain length.

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)

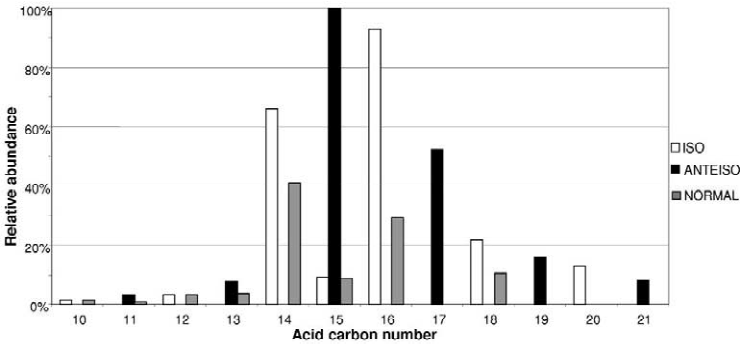

Fig. 6. Lanosteryl esters pattern against acid chain carbon number presenting the isomeric distribution.

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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