Chromatographic Characterization of Internal Polar Lipids from Wool

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ABSTRACT: Wool internal polar lipids were isolated and separated into different fractions based on polarity. Qualitative and quantitative analyses of the different fractions were performed by thin-layer chromatography and thin-layer chromatography coupled to flame-ionization detection, respectively. Cholesterol esters, free fatty acids, sterols, ceramides, glycosylceramides, and cholesterol sulfate were the main components, with ceramides being in the highest proportion. The fatty acid composition of ceramides and glycosylceramides was determined by gas chromatography/mass spectrometry. As for other keratinized tissues, long-chain fatty acids predominated in comparison to either free fatty acids or phospholipid-linked fatty acids; in both cases, stearic and lignoceric acids were the most abundant fatty acids, and a low amount of 18-methyleicosanoic acid was found. This work opens new avenues in the study of lipid rearrangement in more complex and realistic vesicle structures than conventional liposomes.

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KEY WORDS: Ceramides, GC/MS, polar lipids, TLC/FID, wool.

Wool fiber is histologically constituted of cuticle and cortical cells held together by the cell membrane complex (CMC), which forms the only continuous phase in the keratin fiber. The influence of this minor component, which accounts for only 6% of total fiber weight, on the physical, chemical, and mechanical properties of wool fiber is well known (1).

It is possible to distinguish the different CMC regions according to their dyeability. There are two resistant membranes, two unstained layers called the β-layers, and a dark stained central layer, the δ-layer. Chemically, the CMC is mainly made up of proteins, except the β-layers, which are constituted of lipids and are assumed to form a bilayer structure.

Such membrane structures have been interpreted in terms of the fluid-mosaic model, proposed by Singer and Nicolson (2), in which globular proteins are buried in a phospholipid bilayer.

All qualitative analyses of internal wool lipids (1,3) are in agreement regarding the presence of free fatty acids, triglycerides, cholesterol, and cholesterol ester, although their proportions differ. However, a lack of phospholipids was observed (4,5) as in other keratinized tissues (6). It has been reported that phospholipids tend to disappear from the intercellular regions during keratinization (7).

The presence of other polar lipids, such as glycolipids (8,9), ceramides, and cholesterol sulfate (10), has been detected in wool fiber and, in some cases, quantitated by densitometric tracing of the corresponding thin-layer chromatographic separation. Qualitative and quantitative studies of these compounds are important to establish their disposition as a bilayer, because similar lipid compositions found in other keratinized tissues have been shown to develop bilayer structures (11).

Thin-layer chromatography coupled to a flame-ionization detector (TLC/FID) was used in the present work. This technique has considerably enhanced the sensitivity of TLC and allows quantitation of separated materials (12). In fact, it was used earlier to quantitate the different wool lipid extracts (13,14). The chemical structure of polar lipids was also studied to compare them with those from other keratinized tissues and to investigate their distribution in the bilayer. The different sphingolipid-linked fatty acids were isolated and then analyzed by TLC and gas chromatography/mass spectrometry (GC/MS).

The knowledge of the internal lipid composition of the fiber is fundamental to determine the lipid rearrangement in the internal membranes of the CMC, which are known to play an important role in the processing of chemicals into the fiber.

EXPERIMENTAL PROCEDURES

Materials. Raw Australian Merino wool was used for these analyses. Prior to internal-lipid extraction, the wool was dried for two days in a desiccator over NaOH and P_2O_5 and subsequently cleaned to remove the surface lipids and contaminants by rinsing with dichloromethane three times at room temperature and with water three times at room temperature. After being dried again for two days in the desiccator, the wool was Soxhlet-extracted with dichloromethane and equilibrated in a conditioned room (20°C, 60% relative humidity).

The chemicals were of analytical grade and the standards used were supplied by Sigma Chemical Co. (St. Louis, MO)

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for ceramides type III, galactoceramides type II, and cholesterol-sulfate, and by Fluka (Buchs, Switzerland) for cholesteryl-palmitate, palmitic acid, and cholesterol. Silica gel 60 (230–400 mesh) and high-performance thin-layer chromatography (HPTLC) plates (silica gel 60), both from Merck (Darmstadt, Germany), were used for chromatography.

Methods. The internal lipids were Soxhlet-extracted from cleaned wool (4 g) with chloroform/methanol azeotrope (250 mL, 79:21, vol/vol) for 5 h (15).

The lipid extract was separated into classes of compounds. The dry extract (10–15 mg) was redissolved in chloroform/methanol (2:1, vol/vol), separated into three different polarity fractions by silica-gel column chromatography (8 cm × 0.5 cm i.d.), and eluted sequentially by 10 mL of chloroform, 10 mL of acetone, and 10 mL of methanol (16).

Each fraction was qualitatively analyzed by thin-layer chromatography with two different solvent systems: (A) hexane/diethyl ether/acetic acid (80:20:1, vol/vol/vol), and (B) chloroform/methanol/water (65:25:4, vol/vol/vol). The spots were detected with iodine vapor and perchloric acid 40%, both for lipids in general, and specifically with α-naphthol for sugar derivatives (17), ferric chloride for sterol derivatives (18), ninhydrin for primary amines (19), Cl_2/toluidine for compounds able to form chloramines (20), Cl_2/KI-starch for sphingolipids (21), and by Vaskovsky's method for phospholipids (22).

Quantitative analysis was performed with the Iatroscan TH-10 TLC/FID analyzer (Iatron Lab. Inc., Tokyo, Japan) (13). The dry fractions, obtained from the silica column, were redissolved in chloroform/methanol (2:1, vol/vol) (20–30 mg/mL). Samples were spotted with a sample spotter SES 3202/IS-01 (SES GmbH, Nieder-Olm, Germany) on silica gel SIII Chromarods (1 μL) and developed with solvent system i [*n*-hexane/ethyl ether/formic acid (50:20:0.3, vol/vol/vol)] to separate the nonpolar lipids. After a partial scan to quantitate and eliminate the apolar lipids, the redevelopment of Chromarods with system ii [chloroform/methanol/ammonia (58:10:2.5, vol/vol/vol)] twice for 7 cm, leads the separation and quantitation of the polar lipids (23). The same procedure was applied to the standards of cholesteryl-palmitate, palmitic acid, cholesterol (Chol), ceramides (CA), glycosylceramides (GCer), and cholesterol sulfate (Chol-S) to determine their response factors.

The fractions, eluted with acetone and methanol from the silicic acid column, were subjected to preparative TLC to isolate each polar lipid. These fractions (15 mg) were developed on preparative TLC sheets with solvent B, and the spots were visualized in an iodine atmosphere. Each component, as well as the standards, were scraped from the silica, extracted with chloroform/methanol (2:1, vol/vol), and dried under an N_2 stream.

The isolated polar lipids and standards were hydrolyzed under reflux in aqueous methanolic hydrochloric acid (1 N HCl; 8.7 M water) for 18 h (24). The methyl esters of the nonpolar moieties were extracted with hexane, dried under an N_2 stream, redissolved in *iso*-octane, and analyzed by GC/MS (Konik 3000 HRGC; Sant Cugat, Spain; and VG TS-250; Fisons, Manchester, United Kingdom).

The GC/MS analysis was performed under the following conditions: injection, splitless (0.7); column, Ultra 1 fused silica Hewlett Packard (100% dimethylpolysiloxan, 12 m × 0.2 mm i.d.); column program, 70°C, 2 min: 20°C/min to 150°C; 5 min: 8°C/min to 300°C; 5 min; ionization, 70 eV/EI; multiplier, 3200; resolution, 500.

RESULTS AND DISCUSSION

Internal wool lipids were obtained from cleaned raw Merino wool by extraction in a soxhlet with chloroform/methanol azeotrope. The yield of lipids obtained by this method was 0.80% on wool weight. Although internal wool lipids are believed to account for higher percentages [1.2% (5) to 1.5% (1)], an even lower percentage (0.45%) has been obtained by other authors with the same extracting method (13).

To chemically characterize the different compounds, the total lipid extract was separated according to polarity into three fractions by absorption chromatography with silica gel and eluting with chloroform, acetone, and methanol. These fractions were dried under a nitrogen stream and quantitated gravimetrically. The percentages calculated for the total lipid extract were 43.0% for the chloroform fraction, 41.6% for the acetone fraction, and 15.4% for the methanol fraction. According to the literature (16), the chloroform fraction is believed to contain nonpolar lipids, the acetone fraction glycolipids, and the methanol fraction phospholipids.

Development of the chloroform fraction with solvent system A (Table 1) detected the presence of five nonpolar compounds and one polar residue. Comparison with standards allowed us to identify them as sterol esters, triglycerides, free fatty acids, diglycerides, and sterols. Development of this chloroform fraction with solvent system B, use of the specific detector for sphingolipids, Cl_2/KI-starch, and comparison with standards showed that the polar residue was mainly composed of ceramides.

Fractions eluted with acetone and methanol were developed with solvent system B (Table 1). Comparing the colorimetric behavior and the retention time of the compounds with standards, their chemical nature was established as follows. Spots 6 and 9 present the characteristic properties of sphingolipids. Both of them give a blue color with Cl_2/KI-starch (specific stain for these compounds) and yellow with toluidine (amido group), but only 9 shows the presence of a glycosyl group (purple with α-naphthol). Comparison with standards allowed us to identify spots 6 and 9 as ceramide and glycosylceramide, respectively, the latter being in agreement with previous studies (8,9). Spots 7 and 8 give a blue color with sterol stain, red with perchloric acid, and blue with α-naphthol, which seems to indicate that they contain a glycosyl and a sterol group. Both have retention times that are similar. Spots 10 and 11, like cholesterol-sulfate standard, give a blue color with α-naphthol and with sterol. Because spot 10 has exactly the same retention time as the cholesterol-sulfate

TABLE 1
Thin-Layer Chromatography Analysis of Lipids from the Chloroform, Acetone, and Methanol Fractions and the Retention Factors (R_f) with Solvent Systems[a]

Spot number	Chloroform fraction	Acetone fraction	Methanol fraction	Solvent system A (R_f)	Solvent system B (R_f)	Compound
1	+[b]	–[c]	–	0.62	—	Sterol esters
2	+	–	–	0.40	—	Triglycerides
3	+	–	–	0.28	—	Free fatty acids
4	+	–	–	0.15	—	Diglycerides
5	+	–	–	0.11	—	Sterols
6	+	+	+	0.04	0.80	Ceramides
7	–	+	–	–	0.73	
8	–	+	–	–	0.66	
9	–	+	–	–	0.58	Glycosylceramides
10	–	+	+	–	0.37	Sterol sulfate
11	–	+	+	–	0.30	
12	–	–	+	–	0.20	
13	–	–	+	–	0.12	

[a](A) hexane/diethyl ether/acetic acid (80:20:1, vol/vol/vol) and (B) chloroform/methanol/water (65:25:4, vol/vol/vol). [b]Detected. [c]Not detected.

standard, it may be assumed that 11 could be a derivative. Other compounds, 12 and 13, with a lower retention time, were found in the methanol fraction. It seems that they are amino acids or protein derivatives because they are detected with ninhydrin. The Vaskovsky stain does not detect any spots, which corroborates the absence of phospholipids in internal wool lipids.

Application of the TLC/FID technique to these fractions permitted us to quantitatively determine their composition. The applied method consists of multiple development of Chromarods with solvent systems described in the experimental section with a partial scan to solve the nonpolar lipids, and after redevelopment, a total scan for the polar lipids. The same procedure was applied to the standard compounds with the following response factors: cholesterol ester (CE), $0.40.10^{-3}$; free fatty acids (FFA), $0.35.10^{-3}$, Chol, $0.24.10^{-3}$; CA, $0.30.10^{-3}$, GCer, $0.42.10^{-3}$; and Chol-S $0.92.10^{-3}$. The percentages of the total lipid extract were obtained from the amount of each compound after multiplying each area by the corresponding response factor (Table 2).

Analysis of the chloroform fraction developed with solvent system i shows the presence of nonpolar lipids, together with a polar residue in which not only sphingolipids but also a small amount of Chol-S was found after scanning the redeveloped Chromarods with solvent system ii. Other compounds are made up of diglycerides and triglycerides. The absence of nonpolar lipids in the acetone and methanol fractions was also confirmed by this technique. Only sphingolipids and Chol-S were found, apart from other more polar nonidentified peaks, which were identified as amino acid derivatives by TLC. The presence of sphingolipids in the three fractions is the most significant and accounted for more than 40%.

TABLE 2
Amounts of the Different Compounds in the Chloroform, Acetone, and Methanol Fractions

	(% of total lipid extract)					
Fraction	CE	FFA	Chol	CA + GCer[a]	Chol-S	Others
Chloroform	2.9	13.9	11.1	12.6	0.3	2.2
Acetone	—[b]	—	—	20.1	10.2	11.3
Methanol	—	—	—	8.8	2.3	4.3

[a]Because the total separation of both compounds was not possible, they were quantitated together by applying the response factor of the ceramides (CA) that are the most abundant. CE, cholesterol ester; FFA, free fatty acid; Chol, cholesterol; GCer, glycosylceramides; Chol-S, cholesterol sulfate.
[b]Not found.

The acetone and methanol fractions and the standards, such as CA type III, galactoceramides type II, Chol, and Chol-S, were developed on preparative TLC sheets with chloroform/methanol/water (65:25:4, vol/vol/vol) to determine the chemical composition of the polar compounds.

The spots, detected by iodine vapor, were scraped from the silica, eluted with chloroform/methanol (2:1, vol/vol) and dried under a stream of N_2. Compounds corresponding to spots 6 and 9 and the standards were removed individually from the TLC plates, whereas pairs 7 and 8 and 10 and 11 were removed together, owing to their similar retention times.

Each isolated material, once extracted with chloroform/methanol (2:1, vol/vol), was hydrolyzed under reflux for 18 h with aqueous MeOH · HCl. The reaction mixture was extracted with hexane to remove the methylated free fatty acids (FFA-OMe) and the other nonpolar constituents, such as cholesterol. These extracts were dried under a stream of N_2, redissolved in *iso*-octane, and analyzed by GC/MS.

GC/MS chromatograms from CA and galactoceramide standards show only the presence of FFA-OMe, and the Chol-S standard shows dehydrated Chol, Chol-OMe and Chol.

The total ion current chromatograms of samples from spots 6 and 9, which correspond, respectively, to CA and GCer, are similar and are composed of the FFA-OMe listed

TABLE 3
Composition of FFA-OMe Obtained from Ceramides of Spot 6, Glycosylceramides of Spot 9, and Literature Values

	Ceramides		Glycosylceramides		
Component	Spot 6	Ref. 30[a]	Spot 9	Ref. 9[b]	Ref. 30[a]
C12:0	—[c]	—	—	—	4.0
C14:0	—	2.5	—	—	5.4
C16:0	3.4	12.6	6.8	9.5	17.2
C17:0br[d]	trace[e]	—	trace	—	—
C17:0	0.3	—	trace	—	—
C18:1	2.2	9.9	2.3	—	—
C18:0	28.4	22.5	40.5	32.9	31.8
C19:0br	0.6	—	trace	—	—
C19:0	0.4	—	trace	—	—
C20:0	8.5	6.9	8.3	6.0	3.9
C21:0br	1.6	38.0	0.7	—	24.5
C21:0	0.6	—	0.7	—	—
C22:1	—	—	—	7.4	—
C22:0	11.2	5.4	0.8	11.9	4.4
C23:0br	1.5	—	trace	—	—
C23:0	1.4	—	trace	—	—
C24:1	11.3	—	—	—	—
C24:0	24.5	8.0	36.9	15.2	9.0
C25:0br	1.3	—	trace	—	—
C25:0	0.5	—	trace	—	—
C26:0	2.1	—	3.0	—	—
Others	—	—	—	17.1	—

[a]Lipids obtained by enzymatic digestion of wool.
[b]Lipids obtained by extraction with organic solvents.
[c]Not found.
[d]Ante-iso methyl-branched.
[e]Trace amounts accounting for less than 0.1%.

in Table 3. Both samples are mainly composed of linear fatty acids, such as palmitic acid (C16:0), oleic acid (C18:1), stearic acid (C18:0), arachidonic acid (C20:0), behenic acid (C22:0), and lignoceric acid (C24:0). The main difference is the presence of nervonic acid (C24:1) in spot 6.

It is important to emphasize the presence of the odd-chain fatty acids, even though they only account for 8.2% in the ceramides (spot 6) and 1.4% in the GCers (spot 9). Identification of these compounds was confirmed by the mass spectrum, including the molecular ions at *m/e* 284, 312, 340, 368, and 396 for the C17:0, C19:0, C21:0, C23:0, and C25:0 for both linear and branched fatty acid methyl ester derivatives. The fatty acid methyl esters all have an M – 29 (loss of CH_3-CH_2 ethyl) more prominent than M – 31 (loss of CHO_3O acylium ion) and this implies the presence of ante-iso methyl branching (25).

Significant amounts of 18-methyleicosanoic acid, the C21:0 ante-iso methyl branching, have been previously reported to be covalently bound to the wool surface through ester or thioester linkages (26). Wertz and Downing confirmed the presence of this fatty acid in human hair (25) and other mammalian hairs (27), thus supporting the hypothesis of Evans *et al.* (26) that it is attached to the cell surface through ester or thioester linkages. 18-methyleicosanoic acid has also been found in keratinized tissues of a broader range of animals (28). Recent results support a model of the epicuticle membrane of keratin fibers in which 18-methyleicosanoic acid is acylated to the protein as thioester and do not support the hypothesis of a ceramide-fatty acid linkage (29).

Although 18-methyleicosanoic acid is the odd-chain fatty acid present in the largest proportion, it only accounts for 1.6% in ceramides and 0.7% in the glycosylceramides. Our results therefore confirm that the large amount of C21:0 found, when wool is alkali-treated, could not come from the CA.

Table 3 shows the percentages of FFA-OMe in spots 6 and 9, compared also with other results already published (9,30). According to the results obtained, C18:0 and C24:0 are the main components present in spot 6. These do not agree completely with other published results (30), where a higher amount of branched C21:0, identified as 18-methyleicosanoic, was found. Apart from the possible importance of the different method of extraction, the different sample preparation must also be taken into account. In the present work, only ceramides present in the acetone and methanol fractions have been analyzed, although the TLC/FID study demonstrated the presence of such compounds in the chloroform fraction, which could have a different fatty acid composition.

Spot 9 is mainly composed of C18:0 and C24:0, which are also the main components in spot 6, although not in such a high proportion. The smaller proportion of unsaturated FFA in this sample should also be borne in mind. Discrepancies with other authors (30) may also be observed, mainly in the branched C21:0 content. Again, there are differences in sample preparation because the sample analyzed by Körner and Rouette (30) does not only contain GCers but also the rest of the polar lipids. On the other hand, our results are in accordance with those obtained by Nogués *et al.* (9), who also used solvent extraction to remove the lipids from wool.

In a recent report, Körner *et al.* (31) also demonstrated that the high amount of 18-methyleicosanoic acid, believed to be linked to sphingolipids, comes mainly from the hydrophobic protein extracted by enzymatic-reductive treatment with papain/dithioerythritol of the CMC. Therefore, it may be concluded that, even though a quantitative extraction of lipids could not be achieved with chloroform/methanol azeotrope extraction, a smaller proportion of lipoproteins may be present and a more accurate analysis of sphingolipids could be achieved.

These results were also compared with the fatty acid analyses from sphingolipids of other keratinized tissues. CAs and GCers consist of heterogeneous lipid classes that include four possible combinations of sphingosine or phytosphingosine as the long-chain base constituent with nonhydroxy or hydroxy fatty acids (α or ω) in amide linkages (32). Sphingolipids of human and pig stratum corneum are also mainly composed of long-chain fatty acids (C_{22}–C_{24}), which predominate with respect to either neutral lipid or phospholipid-linked fatty acids (33). Even though the present work only provides the analyses of fatty acids liberated from the mixture of CAs and GCers, it is necessary to mention the absence of hydroxy acids for the two fractions studied. This complete lack of hydroxy acids in the wool cell membrane lipids is in agreement with the results of Körner *et al.* (31).

Ion current chromatograms of compounds from the isolated spot pairs 7 and 8 and 10 and 11 were also performed. Cholesterol derivatives were detected, which agrees with the qualitative analyses performed by TLC.

Methyl ester derivatives of fatty acids have also been detected in the pair of compounds of spots 7 and 8; mainly oxidized compounds, tetrahydrofuran pentanoic acid derivatives with a main fragment ion of *m/z* 185, and also small amounts of C16:1, C16:0, C18:1, C18:0, C20:0, and C24:1. Minor sterol derivatives are also found, mainly desmosterol derivatives with large *m/z* 366, representing loss of water from desmosterol, *m/z* 384.

The pairs composed of spots 10 and 11 from the acetone and methanol fractions have in common a major presence of dehydrated cholesterol, *m/z* 368, and a minor presence of methyl cholesterol, *m/z* 400, which are also found in the Chol-S standard, owing to their formation under methylation conditions. There are small amounts of C16:0 and C18:0 fatty acids in the acetone fraction and other sterol derivatives, which could correspond to dehydrated desmosterol, *m/z* 366, and hydroxycholesterol, *m/z* 402, in the methanol fraction.

Although the presence of free desmosterol on wool has been demonstrated (15,34), there is only one previous study that refers to the presence of different sterols [cholesterol, hydroxy-desmosterol, and 19-norcholesta-1,3,5(10)-triene-6-one] in the wool polar lipid fraction (31). Our analyses show not only the presence of cholesterol sulfate but also the presence of different polar compounds that are sterol derivatives; in spots 6 and 7, mainly desmosterol derivatives are found, and in spots 10 and 11, mainly cholesterol but also desmosterol and hydroxycholesterol derivatives are detected.

In summary, internal wool lipids have been isolated and separated into different polarity fractions to facilitate their qualitative and quantitative analyses. The TLC/FID technique has been optimized by developing new solvent systems to separate and quantify wool lipids. Cholesterol esters, FFA, Chol, CA, GCers, and Chol-S are the main components, the CAs being the family of compounds obtained in the largest proportion. The chemical composition of the polar compounds were determined by GC/MS after being suitably hydrolyzed and derivatized. The fatty acid composition of CAs and GCers was determined, stearic and lignoceric acids being the most abundant, and 18-methyleicosanoic acid being present in a low proportion in both materials.

As mentioned in the introduction, the major lipids present in the cell membrane complex of wool (that is, sterols, fatty acids, CAs) are quite unlike those normally found in viable cells (that is, phospholipids) but similar to those found in membranes of keratinized stratum corneum of skin. However, there are major differences between the membrane structures of stratum corneum cells and those present in the CMC of wool. In the former, multiple lipid bilayer structures are found (32), whereas in the CMC of wool only two lipid bilayers are present (the β-layers). The functions of these membranes would also be quite different; for example, stratum corneum cells are constantly sloughed off the skin surface, whereas the same separation and loss of adhesion between cells in the keratin wool fiber do not occur.

Despite significant advances in recent years in the characterization of the lipid components of the CMC, little progress has been made in improving our understanding of the structure and arrangement of these and other components within the CMC. It has been reported (11,35) that the lipids of the CMC and stratum corneum are capable of forming liposomes, even without phospholipids. Taking into account our previous experience on the permeability of different vesicles (36–38), some work has been carried out (Coderch, Maza, Lopez, Bosch, and Parra, in preparation) on structural, physicochemical characterization and permeability of liposomes with internal lipids of wool, which could help us to understand the morphology as well as the particular behavior of this keratinized tissue.

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