ISOLATION OF CERAMIDES FROM WOOL BY SUPERCRITICAL EXTRACTION PROCESSES

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Abstract

Ceramides play a major role in structuring and maintaining the water permeability barrier function of the skin, and are widely used in different products for skin care and treatment. Internal wool lipids (IWL), which are rich in cholesterol, free fatty acids, cholesteryl sulphate and mainly ceramides, were extracted using organic solvents. The repairing effect of these lipid extracts structured as liposomes have been demonstrated when applied onto the skin.

This work was focused on the isolation of IWL extracts enriched in ceramides by supercritical fluid extraction with \( \text{CO}_2 \) to minimize the environmental impact of the organic solvent extraction process. Selected extraction variables (type and percentage of modifier, pressure, temperature and \( \text{CO}_2 \) volume) were optimized at laboratory and pilot plant levels. The lipidic composition of the different collected extracts was quantitatively analysed. Physical and chemical evaluations of extracted wool fibre were carried out to determine its feasibility for textile purposes.

Our results showed that higher amounts of ceramides were obtained using 10\% of methanol or ethanol as modifiers. It was deduced that 60\(^\circ\)C was enough to achieve a high amount of ceramides when little pressure (160 atm) and high \( \text{CO}_2 \) volume (5-7 cell volumes) were applied. Moreover, the temperature and the percentage of the modifier could be reduced to 40\(^\circ\)C and to 5\% when methanol was used as modifier, obtaining slightly smaller amounts of ceramides. Although the different extraction conditions slightly modified some properties of the fibres, these differences were insignificant.

1. Introduction

Wool is a natural fibre mainly made up of protein. It contains external lipids (lanolin) and a small amount of internal lipids (1.5\%), which could arouse considerable interest given their high proportion of ceramides. Internal wool lipids (IWL) are rich in cholesterol, free fatty acids, cholesteryl sulphate and ceramides, and resemble those from membranes of other keratinic tissues such as human hair or stratum corneum from skin [1-5].

Ceramides, which are derivatives of sphingosine or phytosphingosine in amide linkage with nonhydroxy, \( \alpha \)-hydroxy and \( \omega \)-hydroxy acids, are the main component of the intercellular lipids in stratum corneum [6, 7]. These lipids play an important role in the barrier function of the skin, avoiding penetration of external agents and controlling the transepidermal water loss in order to maintain the physiological skin water content [8-10].
IWL contain an equilibrated mixture of ceramides with a composition and molar proportions similar to the ones present in skin [11]. These IWL have been shown to be capable of forming liposomes with a stable bilayer structure [12-14]. Moreover, IWL liposomes have been reported to improve the properties of human hair and skin when applied on them. Therefore, they seem suitable for incorporation into pharmaceutical or cosmetics formulations in the treatment and care of skin [15] and human hair [16].

In order to obtain IWL extracts with large amounts of ceramides, organic solvent extraction has been optimized at laboratory [11] and pilot plant level [17, 18]. The extraction of wool with this methodology confirmed the presence of ceramides at about 0.2% on wool weight. However, a large amount of organic solvent is needed to extract the fibre, resulting in adverse environmental consequences. Supercritical fluid extraction (SFE) is an attractive alternative to traditional extraction techniques in order to avoid or minimize the use of organic solvents.

SFE is therefore a much more appropriate methodology to obtain IWL extracts rich in ceramides. SFE is an important tool to extract thermolabile analytes or complex mixtures such as ceramides from wool under mild experimental conditions especially low temperatures and short extraction time. Supercritical fluids have been applied in the extraction of wool wax in different ways. Diverse studies have focused their attention on the extraction of wool wax from raw wool with supercritical carbon dioxide (SCCO₂) [19-21] as well as on the solubility of wool wax in SCCO₂ [22]. SCCO₂ has also been used to extract unrefined wool grease [23] and to extract wool wax from wool scour wastes [24].

In a preliminary study [25], IWL from wool fibres were obtained using SFE with CO₂ at laboratory level. The type of modifier and selected extraction variables were studied by using experimental design approaches. The highest amounts of IWL and in particular ceramides were obtained using 10% of methanol or ethanol as modifiers, at 60 ºC and at a different pressure and CO₂ volume.

In this work, the best conditions to obtain IWL by SFE with CO₂ at laboratory level were tested. Moreover, an experimental design was performed to reduce the percentage of modifier and the temperature when methanol was used as modifier. The best experimental conditions achieved at laboratory level were performed at pilot plant level to scale up for industrial use. Physical and chemical evaluations of extracted wool at pilot plant level were also carried out to determine its feasibility for textile purposes.

2. Materials and methods

Extraction procedures

Raw Merino wool from Spain was supplied by SAIPEL (Terrassa, Spain). Wool samples were industrially cleaned with sodium carbonate and non-ionic polyoxyethylene surfactant to remove lanolin before being mechanically shaken and rinsed with water to eliminate vegetable matter and dust. Finally, the wool was heat dried at 40ºC for 2h.
SFE at laboratory level was performed using a Lab Scale supercritical fluid extraction system, (Superx-Prepmaster), equipped with a cell of extraction of 50 mL of capacity. This cell was filled with 10 g of wool in each experiment. Ethanol and methanol were used as modifiers, and delivered to the system at 10, 7.5 and 5% concentration with respect to the CO$_2$ flow. The experiments were performed at different temperatures (40, 50 and 60 °C), pressures (160, 200 and 250 atm) and CO$_2$ volumes (200, 250, 300 and 350 mL). The extracts were collected through a fixed restrictor system directly in an empty and weighted screw-capped vial (20 mL) hermetically sealed with a septum and with an outlet to vent the decompressed CO$_2$. The extracts were concentrated to dryness under a gentle stream of nitrogen and quantified gravimetrically.

At pilot plant level, a CSFF (Iberfluid Instruments / ICP-CSIC, Spain) was used to carry out different experiments. A cell of extraction of 375 mL of capacity was filled with 50 g of wool in each experiment. Ethanol and methanol were employed as modifiers, and delivered to the system at 10, 7.5 and 5% concentration with respect to the CO$_2$ flow. The experiments were performed at different temperatures (40 and 60 °C) and pressures (160 and 200 atm). The CO$_2$ volumes used per extraction was 4, 5 and 7 cell volumes. The depressurization process until ambient conditions was carried out in three separators, where the extracts were collected. The extracts were concentrated to dryness using a Savant SpeedVacPlus SC210A (Thermoquest) and quantified gravimetrically.

IWL extracts obtained at laboratory and pilot plant levels were stored in chloroform/methanol (2/1, v/v) at -20ºC until their analysis. Wool was heat dried at 40 ºC for 2h to evaluate its physical and chemical properties.

**Lipid analysis**

The quantitative analysis of the samples was performed by thin layer chromatography coupled to an automated ionization detector (TLC-FID) Iatroscan MK-5 analyzer (Iatron, Tokyo, Japan). This technique enables a rapid separation and precise quantification of different lipid classes without sample pretreatment [26]; in fact, this method has been used to study the composition of different lipidic extracts [4, 27-29].

Samples were applied on Silica gel S-III Chromarods using a SES (Nieder-Olm, Germany) 3202/15-01 sample spotter. The determination of the composition was made using an optimised TLC-FID protocol to analyse lipid content [11]. Before performing a total scan, the rods were developed to a distance of 2.5 cm with chloroform / methanol / water (57:12:0.6), after this to 8 cm with hexane / diethyl ether / formic acid (50:20:0.3) and finally to 10 cm with hexane / benzene (35:35).

**Wool fibre evaluation**

Physical and chemical wool modifications before and after the extraction process were determined by the evaluation of several parameters such as bulk,
resilience, whiteness index, yellowness index, fibre length and alkaline solubility.

Wool bulk is a measure of the core volume of an assembly of fibres which is defined as the specific volume in cm³/g under a compression stress of 1 kPa after a defined preparation history and two previous cycles of compression and recovery at 3 kPa. The procedure to measure core bulk using an autobulkometer is described at a standard test method developed for use in a laboratory [30]. Using the same procedure but including some modifications, bulk (the specific volume of the fibres under 1kPa) and the resilience (the springiness of the fibre mass from 3kPa to 1 kPa) of wool were measured using a MT-LQ dynamometer under the following conditions: Duplicated wool samples of approximately 10 g previously opened and conditioned in standard conditions were placed in a glass measuring cylinder of 500 cm³ with internal diameter of 60 mm. The sample was subjected to a two compression cycles at a rate of 1cm³/s up to 3 kPa. When this stress was reached the sample was held under this pressure for 30 seconds. Then the stress was removed and the sample was able to come back to its original volume for 30 seconds. At the end of the second compression cycle, the specific volume of the compressed sample at 3kPa V3 in cm³/g was recorded. Then, the sample was able to return to its original volume for 30 seconds and a third compression cycle at the same rate up to 1kPa was performed. The specific volume at this stress was the bulk of the sample V1 in cm³/g. The resilience, measured as the recovered volume from 3 kPa to 1 kPa given by the difference V1-V3 in cm³/g was recorded.

Whiteness index (Berger 59) and yellowness index (ASTM D1925) of non-extracted and extracted wool fibres were measured by using a spectrophotometer Color-Eye 3000 (Macbeth, U.S.A.) with D65 illuminant and 10⁰ observer.

Fibre length in mm of non-extracted and extracted wool fibres were evaluated following the corresponding guidelines (IWTO-17-85) by the Almeter Al-100 with 0.5 g to 2.5 g of samples corresponding to approximately 20,000 to 120,000 fibres.

Alkaline solubility of extracted wool fibres was evaluated by triplicate following a normalised methodology (UNE-40-204.72) [31].

3. Results and discussion

Lipid extraction and analysis

Raw Spanish Merino wool was extracted at laboratory and pilot plant levels with SFE in order to obtain the lipid extract rich in ceramides. Merino wool from Spain was used because its internal lipid composition resembles that found in the stratum corneum of skin [11].

In a preliminary study [25], IWL from wool fibres were obtained with the SFE methodology at laboratory level using 10% of methanol and ethanol as modifiers. The lower temperature (60 ºC) and pressure (160 atm) assayed were
enough to obtain larger amounts of ceramides. The volume of CO\textsubscript{2} necessary was 250 mL when 10\% of methanol was used and 350 mL with 10\% of ethanol. In this work, these theoretical conditions derived from Box-Behnken 3\textsuperscript{2} experimental design studies were verified in assays 1 and 2. The lipidic extracts obtained were analysed by TLC-FID and the results are shown in Table I.

**Table I.** Total lipids and ceramides extracted with SFE at laboratory level. The results are expressed as percentages on the total wool weight extracted (o.w.w.).

<table>
<thead>
<tr>
<th>Assay</th>
<th>SFE conditions</th>
<th>Total extract (% o.w.w.)</th>
<th>Ceramides (% o.w.w.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T (ºC)</td>
<td>P (atm)</td>
<td>V\textsubscript{CO\textsubscript{2}} (mL)</td>
</tr>
<tr>
<td>1</td>
<td>60</td>
<td>160</td>
<td>350 (7 Vc)</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>160</td>
<td>250 (5 Vc)</td>
</tr>
</tbody>
</table>

Vc: cell volume

Since 10\% of methanol seem to be the best modifier to obtain larger amounts of lipids and in particular ceramides, another experimental design (Taguchi) was performed by SFE at laboratory level to reduce the temperature and percentage of this modifier. The variables and their range used for Taguchi experimental design were: temperatures (40, 50 and 60 ºC), percentages of methanol (5, 7.5 and 10\%), pressures (160, 200 and 250 atm) and CO\textsubscript{2} volumes (200, 250 and 300 mL). Nine different experiments at Taguchi experimental conditions were performed and the lipid composition was determined by TLC-FID. The results are detailed in Table II.

**Table II.** Analytical results of the extracts obtained from the 9 SFE experiments following the Taguchi experimental design using methanol as modifier. The results are expressed as percentages on the total wool weight extracted (o.w.w.).

<table>
<thead>
<tr>
<th>Assay</th>
<th>SFE conditions</th>
<th>Total extract (% o.w.w.)</th>
<th>Ceramides (% o.w.w.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T (ºC)</td>
<td>P (atm)</td>
<td>V\textsubscript{CO\textsubscript{2}} (mL)</td>
</tr>
<tr>
<td>3</td>
<td>40</td>
<td>160</td>
<td>200 (4 Vc)</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
<td>200</td>
<td>250 (5 Vc)</td>
</tr>
<tr>
<td>5</td>
<td>40</td>
<td>250</td>
<td>300 (6 Vc)</td>
</tr>
<tr>
<td>6</td>
<td>50</td>
<td>160</td>
<td>250 (5 Vc)</td>
</tr>
<tr>
<td>7</td>
<td>50</td>
<td>200</td>
<td>300 (6 Vc)</td>
</tr>
<tr>
<td>8</td>
<td>50</td>
<td>250</td>
<td>200 (4 Vc)</td>
</tr>
<tr>
<td>9</td>
<td>60</td>
<td>160</td>
<td>300 (6 Vc)</td>
</tr>
<tr>
<td>10</td>
<td>60</td>
<td>200</td>
<td>200 (4 Vc)</td>
</tr>
<tr>
<td>11</td>
<td>60</td>
<td>250</td>
<td>250 (5 Vc)</td>
</tr>
</tbody>
</table>

Vc: cell volume

Very similar amounts of ceramides were obtained for all extraction treatments from 0.19 to 0.23\% on wool weight. They were always inferior to the amount extracted in assays 1 and 2. However, the mild extracted conditions must be considered. The best results obtained under the lower mild conditions in Taguchi experimental design were achieved in the assays 3 and 4, at 40 ºC using 5\% and 7.5\% of methanol under different conditions of pressure and CO\textsubscript{2} volume.
Therefore, the best experimental conditions achieved in assays 1, 2, 3 and 4 with SFE at laboratory level were also implemented at pilot plant level in order to ascertain whether similar amounts of ceramides could be achieved at both levels. The IWL extracts obtained were quantified by TLC-FID and the results are indicated in Table III.

**Table III.** Composition of extracted lipids with SFE at laboratory (LAB) and pilot plant (PP) level. The results are expressed as percentages on the total wool weight extracted (o.w.w.).

<table>
<thead>
<tr>
<th>Assay</th>
<th>SFE conditions</th>
<th>Total extract (% o.w.w.)</th>
<th>Ceramides (% o.w.w.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T (ºC)</td>
<td>P (atm)</td>
<td>VCO2</td>
</tr>
<tr>
<td>1</td>
<td>60</td>
<td>160</td>
<td>7 Vc</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>160</td>
<td>5 Vc</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
<td>200</td>
<td>5 Vc</td>
</tr>
<tr>
<td>3</td>
<td>40</td>
<td>160</td>
<td>4 Vc</td>
</tr>
</tbody>
</table>

Vc: cell volume

IWL content has been reported to be present in the wool fibres in the range of 1.2% o.w.w. [32] to 1.5% o.w.w. [33]. Accordingly, appropriate yields were obtained in the assays 1 and 2 obtained with SFE at laboratory and pilot plant level using 10% of modifier, in which lipids were extracted in about 1.2%. A comparison of the ceramide content analysed in these extracts shows that a higher amount is obtained using 10% of methanol than 10% of ethanol at both levels. Slightly lower amounts of lipids and ceramides were achieved in the assays 3 and 4 when the percentage of methanol was reduced, probably because of the lower temperature of the extraction procedure and the lower percentage of modifier used. In order to compare the results obtained at laboratory and pilot plant level from Table III, the percentages of ceramides are given in Figure 1.

![Figure 1](image-url)  

**Figure 1.** Amount of ceramides in each final extract. The results are expressed as percentages on the total wool weight extracted (o.w.w.).
It seems that slightly smaller amounts of ceramides are always obtained when the experiments were performed at pilot plant level. This could be due to the higher dead volume found in the pilot plant design. At both levels, the highest amounts of ceramides are obtained when 10% of methanol is used as modifier. At laboratory level, similar amounts of ceramides are achieved with 10% of ethanol or with 7.5% and 5% of methanol. However, at pilot plant level lower amounts of ceramides are obtained when 5% of methanol is used at 40°C.

Therefore, the similar results obtained at laboratory and pilot plant level show that the highest amounts of ceramides are achieved with SFE at 60 °C, 160 atm and 5 cell volumes of CO₂ using 10% of methanol.

After the extraction procedure, the evaluation of the wool extracted at pilot plant level under different experimental conditions was assessed to study the feasibility of the extracted wool for textile purposes.

**Wool Fibre Evaluation**

Several parameters such as bulk, resilience, whiteness degree, yellowness index, fibre length and alkaline solubility were evaluated on non-extracted and extracted wool fibres in order to determine the possible physical and chemical wool modifications due to lipid SFE at pilot plant level. The results obtained in the evaluation of these physical and chemical parameters are indicated in Table IV.

**Table IV.** Determination of physical and chemical parameters of non-extracted (N-E) and pilot plant extracted wool samples under different experimental conditions. The parameters determined were bulk, resilience, whiteness index (W.I.), yellowness index (Y.I.), fibre length and alkaline solubility (Alk.Sol.).

<table>
<thead>
<tr>
<th>Assay</th>
<th>SFE Conditions (°C / atm / Vc / %M)</th>
<th>Bulk (cm³/g)</th>
<th>Resilience (cm³/g)</th>
<th>W.I.</th>
<th>Y.I.</th>
<th>Length (mm)</th>
<th>Alk.Sol. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-E</td>
<td>-</td>
<td>20.41</td>
<td>2.96</td>
<td>8.71</td>
<td>13.23</td>
<td>9.0</td>
<td>11.40</td>
</tr>
<tr>
<td>1</td>
<td>60 / 160 / 7 / 10 EtOH</td>
<td>20.93</td>
<td>2.93</td>
<td>8.89</td>
<td>13.97</td>
<td>12.5</td>
<td>9.60</td>
</tr>
<tr>
<td>2</td>
<td>60 / 160 / 5 / 10 MeOH</td>
<td>19.12</td>
<td>2.99</td>
<td>7.72</td>
<td>15.22</td>
<td>17.0</td>
<td>9.34</td>
</tr>
<tr>
<td>4</td>
<td>40 / 200 / 5 / 7.5 MeOH</td>
<td>20.06</td>
<td>2.91</td>
<td>7.33</td>
<td>16.88</td>
<td>20.5</td>
<td>10.56</td>
</tr>
<tr>
<td>3</td>
<td>40 / 160 / 4 / 5 MeOH</td>
<td>18.29</td>
<td>2.25</td>
<td>7.89</td>
<td>15.73</td>
<td>19.5</td>
<td>10.38</td>
</tr>
</tbody>
</table>

Bulk and resilience were assessed in order to determine the behaviour of non-extracted and extracted fibres when pressure was applied. Non-significant differences are observed in the bulk and resilience of the non-extracted and extracted fibres despite the detection of a slight increase in bulk in the values of assay 1, where the fibres were extracted using ethanol as modifier.

Whiteness and yellowness indexes observed for the non-extracted and extracted fibres using ethanol show similar values. However, a slight decrease in the whiteness index and a small increase in the yellowness index were observed in the fibres extracted using methanol as modifier. This is consistent with the results obtained in an earlier work [18], where higher differences between extracted and non-extracted fibres in both indexes were found when ceramides were obtained with methanol at pilot plant solvent extraction.
Therefore, a lower alteration of the extracted fibres was achieved using SFE with ethanol as modifier than using methanol as extraction solvent.

The results obtained in the evaluation of fibre length show an increase in all extracted fibres with respect to non-extracted fibres. This increase is higher in the fibres extracted using methanol than ethanol. Moreover, these differences are much more marked in assays 3 and 4, in which a lower temperature and percentage of methanol was used in the extraction. The increase in fibre length could indicate a higher breaking resistance of the extracted fibres. This has been demonstrated by an increase in abrasion resistance in fibres extracted with organic solvent extraction, in which similar amounts and composition of IWL were obtained [18].

Therefore, alkaline solubility was determined in order to evaluate a possible reinforcement of the wool fibres due to SFE. It seems that the alkaline solubility is diminished in the extracted fibres with respect to non-extracted fibres. Moreover, a marked reduction in the alkaline solubility in extracted fibres using 10% of ethanol or methanol should be noted. This decrease could be due to the highest temperature and the highest percentage of modifier used in the extraction process. These results are consistent with the increase in fibre length detected in extracted fibres.

4. Conclusions

Large amounts of ceramides were obtained when wool fibres were extracted with SFE at laboratory level using 10% of methanol or ethanol as modifiers, at 60 °C, 160 atm and 5-7 cell volumes of CO₂. Under these conditions, approximately 0.30% of ceramides on wool weight with 10% of methanol and approximately 0.25% of ceramides on wool weight using 10% of ethanol were obtained. Moreover, the temperature and the percentage of modifier could be reduced to 40°C and 5%, respectively, when methanol was used as modifier. In this case, smaller amounts of ceramides (approximately 0.23% on wool weight) were obtained. Similar results were achieved at pilot plant level when all these conditions were extrapolated to this level.

In general, the different extraction conditions slightly modify the wool fibre properties. Only lower whiteness and higher yellowness indexes were obtained in fibres extracted with SFE, and these extracted fibres had greater length and lower alkaline solubility than non-extracted fibres. This could be attributed to a higher breaking resistance of the extracted fibres.

It can be concluded that this work presents an extraction procedure using supercritical CO₂ at laboratory and pilot plant levels to obtain large amounts of ceramides, minimising the use of organic solvents. Although fibre properties extracted under different conditions are slightly modified, these fibres can be used for textile purposes.
Acknowledgements

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5. References