Oxysterols in cosmetics—Determination by planar solid phase extraction and gas chromatography–mass spectrometry

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**Abstract**

Sterol oxidation products (SOPs) are linked to several toxicological effects. Therefore, investigation of potential dietary intake sources particularly food of animal origin has been a key issue for these compounds. For the simultaneous determination of oxysterols from cholesterol, phytosterols, dihydro-5lanosterol and lanosterol in complex cosmetic matrices, planar solid phase extraction (pSPE) was applied as clean-up tool. SOPs were first separated from more non-polar and polar matrix constituents by normal phase thin-layer chromatography and then focused into one target zone. Zone extraction was performed with the TLC–MS interface, followed by gas chromatography-mass spectrometry analysis. pSPE showed to be effective for cleaning up cosmetic samples as sample extracts were free of interferences, and gas chromatographic columns did not show any signs of overloading. Recoveries were between 86 and 113% with relative standard deviations of below 10% (\textit{n} = 6). Results of our market survey in 2016 showed that some cosmetics with ingredients of plant origin contained phytosterol oxidation products (POPs) in the low ppm range and therefore in line with levels reported for food. In lanolin containing products, total SOPs levels (cholesterol oxidation products (COPs), lanosterol oxidation products (LOPs), dihydro-5lanosterol oxidation products (DOPs)) being in the low percent range exceeded reported levels for food by several orders of magnitudes.

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1. Introduction

Sterols are a class of non-polar lipids which are found in fat of plant and animal origin. Cholesterol is the major sterol of animal fat, whereas lanosterol and dihydro-5lanosterol are components of sheep wool fat and its refined product lanolin. Phytosterols are present in plant oils, with \( \beta \)-sitosterol, campesterol and stigmast-erol being the most common congeners. Containing at least one carbon–carbon double bond, sterols are prone to reactions with oxygen resulting in the formation of sterol oxidation products (SOPs), [1–4].

SOPs, particularly cholesterol oxidation products (COPs) and phytosterol oxidation products (POPs) have repeatedly been reported to possess adverse biological properties [2,5–11]. For the assessment of countermeasures against high SOPs intake, COPs and POPs contents have been determined in a wide variety of foods, with the focus on food processing procedures suspected of increasing SOPs levels [5,10,12]. Besides food, cosmetic products may also play a role as exogenous SOPs sources as they often contain lanolin, phytosterols, the unsaponifiable extract of oils and pure vegetable oils. [5,6]. In the case of lip care products or nursing ointments, the way of absorption might even be oral [6]. Especially lanolin is known to contain high cholesterol (∼15%), lanosterol (∼7%) and dihydro-5lanosterol (∼5%) levels [4,13,14]. Furthermore, as far as we know, no data have been published on the occurrence of lanosterol oxidation products (LOPs) and dihydro-5lanosterol oxidation products (DOPs) in consumer products. These aspects render lanolin an issue of special concern.

In order to estimate human SOPs intake, sophisticated analytical methods capable of determining low levels in complex matrices e.g. cosmetics are required. Most published methods for the determination of SOPs in food include a clean-up step of the saponified or transterified lipid extract prior to gas chromatography, preferably coupled to a mass spectrometer [15,16]. The removal of matrix compounds is mostly performed with solid phase extraction (SPE) [16–19] or preparative liquid chromatography (LC) [20,21]. Both clean-up techniques, however, have a low capacity for sample extracts and overloading may lead to insufficient removal of interfering components or even to clogged SPE cartridges or LC columns. These methods also involve large volumes of organic solvents, are
time consuming and costly and do not allow for visual monitoring during the clean-up step. Visual control is especially important when samples of varying composition involving complex matrices are to be analysed as it enables minor adjustments to be made in time, if necessary.

Thin-layer chromatography (TLC) is a fast and inexpensive technique for separating, detecting and quantifying target compounds. In addition, TLC can also be applied for sample clean-up. This clean-up technique is called planar solid phase extraction (pSPE) [22]. TLC plates are single use items and therefore, reconditioning of the stationary phase is no issue. In addition, increasing the area of the application zone as well as simultaneous multiple applications is a simple way to increase sample size. Our decision to test pSPE for our study came with paraffin containing samples leading to the mentioned problems with our former SPE clean-up method [6].

Our aim was to develop a pSPE–GC–MS method for the simultaneous determination of COPs, POPs and for the first time lanosterol oxidation products (LOPs) and dihydrolanosterol oxidation products (DOPs) in cosmetics where sterols from both plant and animal origin often occur together.

2. Experimental

2.1. Chemicals and materials

7-Ketocholesterol (7-KC), 7β-hydroxycholesterol (7β-HC), cholester-5β,6β epoxide (5β-CE), cholesterol-5α,6α epoxide (α-CE), cholestan-3β,5,6β-triol (CT), 5α-cholestan, β-sitosterol (>70%), 25-hydroxycholesterol (25-CH), CI. 11005, 4-(4-nitrobenzyl)pyridine (NBPI), and polytetrafluoroethylene (PTFE) membrane filters (0.2 and 0.45 µm) were purchased from Sigma-Aldrich (Buchs, Switzerland); 7α-hydroxycholesterol (7α-CH) from Chemos (Regensburg, Germany). Dichloromethane, diethyl ether (both analytical grade), n-hexane, acetone, methanol (all GC grade), sodium methylate (30% solution in methanol, for synthesis), tetraethylenepentamine and citric acid monohydrate were from Merck (Darmstadt, Germany) and Sylon HTP (1,1,1,3,3,3-hexamethyldisilazane: trimethylchlorosilane: pyridine: v:v:v: 3:1:9) from Supelco (Gland, Switzerland). All TLC and HPTLC plates were from Merck and obtained from VWR (Dietikon, Switzerland). They were prewashed with methanol and dried in an oven at 100 °C for 10 min before use. Propylene centrifuge tubes (15 mL; 50 mL) were from SPL Lifescience (Gyeonggi-do, Korea), polypropylene pipettes from alpha laboratories (Eastleigh, UK). Lanolin was obtained from a local pharmacy.

2.2. Gas chromatography–mass spectrometry (GC–MS) and liquid chromatography-high resolution mass spectrometry (LC–HRMS)

Compounds were separated and detected with a Thermo Finnigan Trace GC (Thermo Scientific, Bremen, Germany) coupled to a PolarisQ MS (Thermo Scientific) using two capillary columns, a DB17 ms (10 m × 0.25 mm ID; 0.25 µm film) and a DB5 ms (30 m × 0.25 mm ID; 0.25 µm film) (both Agilent Technologies, Santa Clara, USA) connected with a pressfit. A diphenyltetramethyldisiloxane (DPTMDS) deactivated precolumn and postcolumn (both 1 m × 0.25 mm ID) were used. Injections were performed with a PAL autosampler (CTC analytics, Zwijndrecht, Switzerland) into a programmable temperature vaporizer (PTV) injector used in the PTV large volume mode and equipped with a silcot liner (2 mm ID), Injection speed was 50 µL s⁻¹ and pre/post inject delay was 3000 ms. The injector was set at 100 °C (1 min, 50 kPa) with a split ratio of 10:1 during evaporation time. For the transfer of the analytes to the column, the temperature was raised at 14.5 °C s⁻¹ to 280 °C (1 min, 140 kPa) and the split valve was shut.

For cleaning the injector, the injector temperature was raised to 310 °C at 14.5 °C s⁻¹ (10 min, 140 kPa) with a split ratio of 10:1. Helium was used as a carrier gas at a pressure of 50 kPa for 1 min then at a pressure of 140 kPa for the remaining of the GC runtime. Initial oven temperature was set at 60 °C for 2.5 min, then raised to 320 °C at a rate of 20 °C min⁻¹ and held constant for 15 min. Full-scan mass spectra (m/z 50–650) were recorded with a 17-min delay in the electron impact ionization (EI) mode at 30 eV (trap offset 10, AGC target 50, high mass adjust 50%, wave form off). The ion source temperature was set at 220 °C, and the transfer line temperature at 280 °C. Processing and interpretation of mass spectra were carried out with Xcalibur 2.1 (Thermo Scientific). Quantification was performed with an internal standard (5α-cholestan) using the extracted ion chromatograms of main mass signals (Table 1).

The LC-high resolution mass spectrometry (HRMS) system consisted of a LTQ Orbitrap XL equipped with a heated ESI II source (Thermo Scientific), a HTS-PAL autosampler (CTC analytics). Separation was performed on a Waters Atlantis T3 column (150 mm × 3 mm; 5 µm) using a gradient elution (A: nanopure water and B: methanol). The flow rate was 200 µL min⁻¹ and gradient elution started with 95% of A and 5% of B (0–2 min). The eluent was then first steadily modified to 50% of A and 50% of B (5 min–17 min) and then modified to 5% of A and 95% of B (17–25 min). After, the eluent was reset to the initial composition for 7 min (25 min–32 min). Ionisation was performed with electrospray ionisation (ESI) in the positive mode (capillary temperature 300 °C, sheath gas flow 50 arbitrary units, auxiliary gas flow 5 arbitrary units, source voltage 4 kV) and full scan spectra were recorded in m/z range of 115–1000. Processing of data was performed with Xcalibur 2.1

2.3. Isolation of lanosterol and dihydrolanosterol

Lanosterol and dihydrolanosterol were isolated from a transesterified lanolin solution (2,7.2) by preparative liquid chromatography on a SpectraSystem including UV6000, AS3000, D4000 (Thermo Scientific, Reinach, Switzerland) coupled to a fraction collector from Gilson (Mettenstetten, Switzerland). The separation was performed on a Lichrosorb-100 RP18 column (5 µm, 250 cm × 4 mm) (Knauer, Berlin, Germany) with methanol at 1 mL min⁻¹. Detection was performed with a diode array detector at 210 nm. Eluate fractions containing lanosterol or dihydrolanosterol were collected and methanol vaporised with a nitrogen evaporator. Several milligrams of each sterol were obtained by repeating the procedure as often as needed (25 µg lanolin per run). Purities were checked with gas chromatography (GC) – flame ionisation detection (FID) and were found to be >88%.

2.4. Generation of phytosterol oxidation products (POPs) and dihydrolanosterol oxidation products (DOPs) by thermooxidation

Oxidised phytosterol and dihydrolanosterol congeners were obtained by thermooxidation. Solutions (10 mg in 10 mL acetone) of sitosterol and dihydrolanosterol, respectively, were pipetted into a 200-mL erlenmeyer flask and kept at 120 °C for 15 h using a heating plate. After cooling the flask under cold running water, the residue containing unreacted precursor and sterol oxides was dissolved in 10 mL of acetone under sonication. The POPs and DOPs solutions were then ready for identification (2.6) and quantification. For the latter, levels of congeners were determined with GC–FID using the corresponding congeners of COPs as standards. As the response factors of FID for substances having the same chemical group are often similar, this approach was deemed to be suitable. The sitosterol stock solution also contained campesterol. We therefore also expected to find campesterol oxidation products (CaOPs).
Table 1
Characterisation of sterol oxidation products (SOPs) in the mixture.

<table>
<thead>
<tr>
<th>name</th>
<th>abbreviation</th>
<th>retention time (min)</th>
<th>main mass signals (m/z)</th>
<th>concentration (µg mL⁻¹)</th>
<th>type</th>
</tr>
</thead>
<tbody>
<tr>
<td>7α-hydroxysitosterol</td>
<td>7α-HSi</td>
<td>20.47</td>
<td>484</td>
<td>0.15</td>
<td>POPs</td>
</tr>
<tr>
<td>7β-hydroxysitosterol</td>
<td>7β-HSi</td>
<td>22.03</td>
<td>484</td>
<td>0.45</td>
<td>POPs</td>
</tr>
<tr>
<td>sitosterol-5α,6α-epoxide</td>
<td>α-SE</td>
<td>23.78</td>
<td>412, 502, 394</td>
<td>1.15</td>
<td>POPs</td>
</tr>
<tr>
<td>sitosterol-5β,6β-epoxide</td>
<td>β-SE</td>
<td>23.46</td>
<td>412, 502, 394</td>
<td>0.73</td>
<td>POPs</td>
</tr>
<tr>
<td>7α-hydroxycamposterol</td>
<td>7α-HCa</td>
<td>19.79</td>
<td>470</td>
<td>0.017</td>
<td>POPs</td>
</tr>
<tr>
<td>7β-hydroxycamposterol</td>
<td>7β-HCa</td>
<td>21.13</td>
<td>470</td>
<td>0.047</td>
<td>POPs</td>
</tr>
<tr>
<td>sitostanol</td>
<td>SIT</td>
<td>21.29</td>
<td>431, 484, 559, 474</td>
<td>0.63</td>
<td>POPs</td>
</tr>
<tr>
<td>7-ketocampester</td>
<td>7-KCa</td>
<td>25.08</td>
<td>486, 381, 396, 469</td>
<td>0.65</td>
<td>POPs</td>
</tr>
<tr>
<td>7-ketositosterol</td>
<td>7-Ksi</td>
<td>26.66</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7α-hydroxycholesterol</td>
<td>7α-HC</td>
<td>18.98</td>
<td>456</td>
<td>3</td>
<td>COPs</td>
</tr>
<tr>
<td>7β-hydroxycholesterol</td>
<td>7β-HC</td>
<td>20.06</td>
<td>456</td>
<td>3</td>
<td>COPs</td>
</tr>
<tr>
<td>cholesterol-5α,6α-epoxide</td>
<td>α-CE</td>
<td>21.48</td>
<td>384, 474, 366</td>
<td>10</td>
<td>COPs</td>
</tr>
<tr>
<td>cholesterol-5β,6β-epoxide</td>
<td>β-CE</td>
<td>21.22</td>
<td>384, 474, 366</td>
<td>10</td>
<td>COPs</td>
</tr>
<tr>
<td>cholesteroltril</td>
<td>CT</td>
<td>21.62</td>
<td>403, 456, 531, 546</td>
<td>10</td>
<td>COPs</td>
</tr>
<tr>
<td>7-ketocholesterol</td>
<td>7-KC</td>
<td>23.31</td>
<td>472, 367, 382, 455</td>
<td>10</td>
<td>COPs</td>
</tr>
<tr>
<td>7-keto-3,11-dihydroxy-dihydrolanosterol</td>
<td>7-KDD</td>
<td>22.74</td>
<td>602, 376, 512</td>
<td>0.72</td>
<td>DOPs</td>
</tr>
<tr>
<td>11-keto-3,7-dihydroxy-dihydrolanosterol</td>
<td>11-KDD</td>
<td>24.25</td>
<td>602, 587, 559</td>
<td>0.64</td>
<td>DOPs</td>
</tr>
<tr>
<td>7,11-diketodihydrolanosterol</td>
<td>7,11-KD</td>
<td>24.25</td>
<td>528, 472, 429</td>
<td>2.3</td>
<td>DOPs</td>
</tr>
<tr>
<td>7-ketodihydrolanosterol</td>
<td>7-KD</td>
<td>25.25</td>
<td>514, 499</td>
<td>1.8</td>
<td>DOPs</td>
</tr>
</tbody>
</table>

The m/z used for quantification were highlighted in bold.

For validation purposes and identification studies, thermooxidation was also performed with lanosterol and cholesterol. In the case of lanosterol, a shorter reaction time of only 20 min was chosen as the substance was found to be significantly more reactive.

2.5. Standard solutions

2.5.1. Sterol oxidation products mixture (SOPs mixture)
A SOPs mixture containing both commercially purchased COPs and in-house generated POPs and DOPs was prepared in acetone and used for calibrations (Table 1).

2.5.2. Internal standard for quantification
A 0.25 µg mL⁻¹ solution of 5α-cholestan in n-hexane (IS solution) was used as an internal standard (IS) for quantification.

2.5.3. Recovery standard for quantification (correction factor)
Before work-up, samples were spiked with a recovery standard (25-HC) either with 100 µg mL⁻¹ or 10 mg mL⁻¹ depending on expected SOPs levels. The spiking aliquot was chosen to result in a theoretical 25-HC concentration of 0.5 µg mL⁻¹ in the solution used for GC–MS. When the 25-HC concentration determined in a given sample solutions deviated by more than 10% from this value, congener concentrations were multiplied with a correction factor defined by the set point divided by the actual point concentration. The system control standard contained 25-HC at the set point concentration.

2.5.4. System control standard
A system control standard (QC) in hexane, containing SOPs at half the concentrations of the SOPs mixture and 25-HC at 0.5 µg mL⁻¹ was prepared. After derivatisation, QC was regularly analysed every ten injections with GC–MS.

2.6. Identification of dihydrolanosterol oxidation products (DOPs)
The identification of DOPs formed during thermooxidation of dihydrolanosterol was performed with high-performance thin-layer chromatography (HPTLC) as follows: 200 µL were applied (spray application) on two Lichrospher 60 F254 10 cm × 10 cm (200 µm layer thickness) plates with an automatic TLC sampler ATS4 (CAMAG, Muttenz, Switzerland). The band length was 6 mm and we used a distance of 12.0 mm from the lower and the plate edge. DOPs were separated using two-dimensional development in a 10 cm × 10 cm horizontal developing chamber (CAMAG): (i) n-hexane: diethyl ether (1:5, v/v) was used up to a migration distance of 80 mm, (ii) after rotating the plate by 90° to the left, the second development was performed with n-hexane: diethyl ether (1:1, v/v) up to a migration distance of 60 mm. After leaving to dry, one plate was derivatised in a dipping chamber (CAMAG) with a solution of 2% nitrobenzyl pyridine (NBP) in acetone (speed: 2 cm s⁻¹, time: 2 s) followed by drying of the plate (10 min, 100 °C) on the plate heater (CAMAG). After cooling, the plate was dipped (1 s) into a 1% solution of tetraethylenepentamine (TEPA) in acetone, after which epoxides became visible as purple spots.

The second plate was evaluated under UV 254 nm with the Reprostar 3 (CAMAG). All spots were individually marked with a pencil and cut out with the plate cutter (CAMAG). The silica gel of each spot was scrapped off with a spatula and extraction of DOPs was performed for 5 min with 1 mL of acetone in an ultrasonic bath.

After filtration through a polytetrafluoroethylene (PTFE) filter tip, each DOP extract was divided into three aliquots and analysed with (i) GC–MS (ii) GC–MS after derivatisation to TMS ethers and (iii) LC-high resolution mass spectrometry (HRMS). The whole procedure for the identification studies is sketched in Fig. 1.

As DOPs were also used as references for identifying LOPs, isolation of oxidised lanosterol congeners was not performed. In order to obtain full scan spectra of LOPs congeners, the derivatised thermooxidised lanosterol solution was analysed with GC–MS.

Interpretation of data was performed with Xcalibur 2.1 for calculating the molecular formula and Masslib 9.2 (MSP Kofel, Zollikofen, Switzerland) as a library with reference EI spectra.

2.7. Cosmetic sample procedure

2.7.1. Samples
A total of 46 samples of cosmetic products and ingredients (23 vegetable oils respectively body oils, 6 creams, 9 lip care products and 8ointments) were obtained from the Swiss and German market. Sample selection was based on product labels declaring lanolin, a vegetable oil, a sterol, or unsaponifiable matter of an oil as an ingredient.

For validation studies, three SOPs-free samples (lip care product, cream and paraffin wax) were obtained from the Swiss market.
2.7.2. Transesterification

200 mg of samples were weighed into a 15-mL propylene centrifuge (PP-tube) tube and added with 2 mL of dichloromethane. Then, an aliquot of the 25–HC recovery standard solution (2.5.3) was spiked with 25 μL of the Cl.I11005 marker solution (1 μg mL⁻¹ in aceton). The tube was shaken and vortexed for 10 s. For aqueous samples, 1 mL of water was added, too. After shaking and centrifuging (5 min, 872 g), the aqueous phase was removed with a pipette (PP). 1.2 mL of a 10% sodium methyate solution in methanol was then added to the dichloromethane extract. The tube was vortexed (10 s), shaken and placed in an ultrasonic bath for 30 min (RT). The tube was then left to stand for a further 30 min. A saturated sodium chloride solution (2 mL) was added, the tube was briefly shaken and centrifuged for 5 min (872 g). After removal of the upper phase, the washing procedure was repeated with 1 mL of an aqueous solution of 2% citric acid. In rare cases with the dichloromethane phase being still cloudy, the step was performed once again with water. For paraffin containing samples, the dichloromethane extract was left to stand for 10 min at 4 °C and then filtered through a 0.1 μm PTFE filtertip into a 2-mL GC vial.

2.7.3. Planar solid phase extraction (pSPE)

An aliquot of the dichloromethane extract from transesterification was applied in rectangular areas (4 mm x 3 mm/4 mm x 10 mm for paraffin containing samples) on a TLC silica 60 plate MS grade F₂₅₄ (20 cm x 10 cm; layer thickness 200 μm) with the AT₅₄ (CAMAG). Application parameters were set to: 30 μL s⁻¹ filling speed, 200 mL predosage volume, 200 mL retraction volume, 890 mL s⁻¹ dosage speed, 2 s rinsing vacuum time, 2 s filling vacuum time, 3 rinsing cycle and 2 filling cycles, 13.0 mm distance from the lower edge, 10.0 mm distance from the left edge, and 13.0 mm track distance. In general, 50–200 mL were used for samples with ingredients of plant origin, 20 μL for samples declaring lanolin as the respective main ingredient. When samples consisted mostly of lipid soluble compounds like paraffins, a sample application on one track led to overloading of the plate. Therefore, the sample volume was split onto two tracks and the extracts combined after pSPE. Chromatography was performed in a 20 cm x 10 cm horizontal developing chamber (CAMAG) using a three step development process: (i) n-hexane up to a migration distance of 85 mm, and after air-drying (ii) n-hexane: diethyl ether (1:2; v/v) up to a migration distance of 85 mm, and then (iii) acetone up to the marker zone (Cl.I11005) to focus oxysterols in the solvent front. Analytes were then extracted with acetone at 0.25 mL min⁻¹ for 60 s by the TLC-MS interface (CAMAG). The acetone was then narrowed down to dryness at RT in a nitrogen evaporator (Barkey, Leopoldshöhe, Germany). HPTLC instruments were controlled by winCATS Software 1.4.3 (CAMAG).

2.7.4. Derivatisation

The temperature of the nitrogen evaporator was set to 65 °C and 100 μL of Sylon HTP were added to the GC vial containing the dry extract and left to react for 75 min. Then, the reaction surplus was removed under a gentle stream of nitrogen followed by dissolving it in an aliquot of IS solution. In general, vegetable oils were dissolved in 200 μL whereas lanolin containing samples were dissolved in 2 mL IS solution. Samples with lanolin labelled as the first ingredient were diluted once more with IS solution (dilution factor 10). The solution was then filtered through a 0.1 μm PTFE filtertip into a GC vial with a glass insert and was then ready for GC-MS analysis.

3. Results and discussion

3.1. Optimisation of gas chromatography–mass spectrometry (GC–MS) procedure

The GC–MS parameters consisted basically of our method for the determination of COPs in cosmetics which was modified for the current subject [6]. Modifications included substituting the split injector by a programmed temperature vaporizing injector (PTV), adapting the GC oven temperature programme and using a correction factor for quantification if needed.

We used an inlet temperature (100 °C) being above the solvent boiling point but under those of the target compounds at normal pressure [23–25]. This allowed for large sample volume injections and hence lower detection limits. The most important setting in this context was a rather low helium start pressure (50 kPa) as high pressures resulted in an insufficient solvent evaporation. The transfer of the analytes to the analytical column was then achieved by increasing the PTV inlet temperature to 280 °C and the helium pressure to 140 kPa. In addition, the split valve was shut during this step further increasing sensitivity. Cleaning of the injector required an injector temperature of 310 °C. For large volume injection, the injection speed was crucial: low speed resulted in significantly less sensitivity (factor 10 to 100). The optimal speed for good results proved to be 50 μL s⁻¹. These injection parameters resulted in a linear relationship between the injection volume and analyte signals enabling large volume injections to be performed. For the routine procedure, an injection volume of 8 μL proved to be sufficient.

The most prominent modification of the original GC method concerned adding an isothermal GC oven period at 320 °C when the SOPs started to elute.

A correction factor for quantification was used as matrix constituents are known to interfere with quantification [26]. We therefore spiked samples with a recovery standard of 25–HC before the clean-up procedure. 25–HC was chosen because it was never found in samples analysed and behaved like the target SOPs during...
analysis. Quantification included adjusting target compound levels with a correction factor (2.53). Adjustment of levels was necessary due to matrix constituents influencing the pSPE procedure and thus causing minor shifts of the target zone. This might result in an incomplete extraction by the TLC–MS interface as its head size is too small. Determination of recovery rates showed that amounts lost were similar for target compounds and the recovery standard. The approach for level adjustment was therefore suitable.

3.2. Planar solid phase extraction (pSPE)

For pSPE method development, a suitable combination of the stationary and mobile phases as well as the development mode had to be established which would (i) wash away interfering nonpolar components into the solvent front, (ii) separate sterols from their oxidised species, and (iii) focus target compounds in one zone with polar matrix components remaining on the start zone.

Tests quickly showed that normal phase silica gel with multiple developments in the same direction gave the best results. Nonpolar matrix components, namely fatty acid methyl esters and alkanes (paraffins) migrated to the front (Rf ≈ 70–100) with n-hexane (Fig. 2). Then, the majority of sterols were separated from the oxidised congeners by using a mobile phase consisting of n-hexane/diethyl ether (1:2). Disperse orange 3 (C.I. 11005) spiked prior to sample work-up was found to be a suitable marker migrating above the oxidised and slightly below the unoxidised sterols. Finally, a mobile phase had to be found which would move all target compounds with the solvent front, focussing them in a target zone localised by the marker (C.I. 11005). We achieved this with acetone performing the last development step until the front reached the lower edge of the marker zone. Most polar matrix components remained on the start zone as acetone is too nonpolar for those matrix constituents.

For the TLC–MS extraction of oxysterols, acetone at a flow rate of 0.25 mL min⁻¹ gave best recoveries. For big zones which could not be totally covered by the TLC–MS interface head during extraction, we used 25-HC as a recovery standard to adjust results (2.5.3.).

The assessment of clean-up efficacy of every sample analysed can directly be seen on the plate as nearly all substances were detectable with the documentation system Reprostar 3 (Fig. 2). Samples analysed during the market survey were checked for interferences and overladen full scan mass spectra. None of these proved to be too big a challenge for our clean-up procedure.

Compared to existing clean-up methods, the advantages of pSPE are: The use of visual control allowing for timely adjustments of sample volume if necessary. The use of an inexpensive single-use stationary phase (HPTLC plate) where the potential build up of matrix residues on the phase due to repeated use is no issue. The possibility of cleaning up several extracts on parallel tracks simultaneously.

3.3. Identification and description of lanosterol oxidation products (LOPs) and dihydrolanosterol oxidation products (DOPs)

To our knowledge, no data have been published on the occurrence of lanosterol and dihydrolanosterol oxidation products in consumer products, namely cosmetics, although the sterol educts are main components of the ingredient lanolin [27]. As neither data on mass spectra of congeners nor commercial standards were available, we applied thermostoxination for the in-house generation of reference standards (2.4). We then screened lanolin samples for peaks which were also found in our reference standards. Those peaks in common then underwent structural elucidation.

The underlying theory of our investigations was the well-known mechanism of the non-enzymatic cholesterol oxidation [5,28], which explains the formation of hydroxy and keto oxysterols (OH/keto on carbon atom next to the double bond) as well as epoxy congeners (epoxy on the two carbon atoms of the double bond).

3.3.1. Comparison of congeners of lanosterol oxidation products (LOPs) and dihydrolanosterol oxidation products (DOPs)

The precursors lanosterol and dihydrolanosterol differ only in lanosterol having one additional double bond on C25/C26 [29,30] (Fig. 3). Consequently, the monoisotopic masses differ by two amu. Oxidation could theoretically occur on both double bonds (C9/C10 and C24/C25) of lanosterol. However, full scan spectra of derivatised LOps and DOPs congeners were nearly identical differing only by the m/z of 2 (Fig. 3). This gave evidence that in both educts only the C₉/C₁₀ double bond underwent oxidation as the exocyclic double bond probably would result in different fragmentation patterns. Due to their reactivity, the thermogenesis of oxidised lanosterol congeners proved to be tricky. Monitoring of the process showed not only their rapid formation but also degradation, possibly caused by the formation of sterol and/or oxysterol dimers and trimers. [31]. Most likely, this side reaction occurs much faster at the side chain double bond of lanosterol rendering the generation of concentrated monomeric LOPs in solutions difficult. Consequently, DOPs were used as model substances for identifying LOPs as they could easily be synthesised with a good yield for each congener.

3.3.2. Identification of lanosterol oxidation products (LOPs) and dihydrolanosterol oxidation products (DOPs)

We first determined the type of functional groups present in DOPs congeners. The NBP derivatisation of the congeners with HPTLC (2.6) showed that congeners of interest probably did not contain an epoxide group as no characteristic purple spot was visible. Consequently, congeners having a keto and/or a hydroxy group on C7 and/or C11 should be found. As CH bonding energies on C7 and C11 are almost identical [29], the probability of hydrogen abstraction followed by the addition of oxygen was similar for both C atoms.

After the HPTLC separation of the DOPs congeners, LC-HRMS enabled the determination of the exact monoisotopic mass with a Δ±5 ppm (Table 2). The isotopic patterns of C12 and C13 showed that all congeners consisted of about 30 carbon atoms. The molecular formula was calculated using the elements Si, O, N, H and C (maximum number of atoms set to 100) (Table 2). GC–MS of underivatised congeners confirmed the monoisotopic mass obtained by LC–MS (Table 2). Derivatisation converted hydroxy to TMS ether groups thereby increasing the monoisotopic mass by 72 for each converted hydroxy group. The comparison of the m/z of monoisotopic masses of the derivatised and underivatised form gave the number of hydroxy groups present in the molecule (Table 2). All congeners possessed a hydroxy group on C13 stemming from the educt dihydrolanosterol.

7-KD has only one hydroxy group (C7) stemming from the basic structure. As the calculated molecular formula contained two oxygen atoms (Table 2), a keto group at C7 or C11 was the most likely explanation. Thus, we compared the full scan spectra of the underivatised and the derivatised substance with spectra of two compounds found by the library search program. Both reference spectra had the dihydrolanosterol structure lacking the 3-hydroxy group but showing a keto group on C7 or C11 instead. Our spectra of derivatised and underivatised substances characteristically had only two main peaks being the m/z of the monoisotopic mass and of the monoisotopic mass minus 15 (Fig. 3). This pattern was solely found in the substance with the keto group at C7, whereas the C11 congener showed stronger fragmentation with a main mass signal in the low mass range (<300). We thus identified the substance to be 7-ketodihydrolanosterol (7-KD) and consequently allocated the corresponding LOPs congener to 7-ketolanosterol (7-KL).
Fig. 2. Work up procedure for a paraffin containing lip care sample: Planar solid phase extraction (pSPE) steps including sample application, the three-fold development (Step 1: n-hexane. Step 2: n-hexane: diethyl ether (1:2; v/v). Step 3: acetone up to the marker) and the extraction with TLC–MS interface. Plates were documented under 254 nm, 366 nm or under white light with a combination of remission and transmission (RT).

Fig. 3. Structure formulae and mass signals (m/z) of the derivatised (values in bold) and the underivatised lanosterol oxidation products (LOPs) and dihydrolanosterol oxidation products (DOPs).

Table 2
Structural elucidation of dihydrolanosterol oxidation products (DOPs).

<table>
<thead>
<tr>
<th>DOPs</th>
<th>Exact mass (m/z) (LC-HRMS)</th>
<th>Calculated molecular formula</th>
<th>Mass (m/z) (GC–MS)</th>
<th>Mass of TMS ether (m/z) (GC–MS)</th>
<th>number of derivatised hydroxy groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-KD</td>
<td>442.374</td>
<td>C_{30}H_{52}O_{2}</td>
<td>442</td>
<td>514</td>
<td>1</td>
</tr>
<tr>
<td>7,11-KD</td>
<td>456.359</td>
<td>C_{30}H_{52}O_{2}</td>
<td>456</td>
<td>528</td>
<td>1</td>
</tr>
<tr>
<td>7-KDD</td>
<td>458.375</td>
<td>C_{30}H_{52}O_{2}</td>
<td>458</td>
<td>602</td>
<td>2</td>
</tr>
<tr>
<td>11-KDD</td>
<td>458.375</td>
<td>C_{30}H_{52}O_{2}</td>
<td>458</td>
<td>602</td>
<td>2</td>
</tr>
</tbody>
</table>

For 7,11-KD, the calculated molecular formula contained three oxygen atoms, whereas derivatisation results gave only one hydroxy group on C_3 (Table 2). As oxidation only should occur on the C atoms next to the double bond, 7,11-KD probably had the keto groups on the C_7 and on the C_11 carbon atom. Theory was supported by the comparison of the underivatised 7,11-KD spectrum obtained by GC–MS with a library spectrum of the acetylated form of 7,11-KD: Both spectra showed the same characteristic peaks at m/z 456 (underivatised) and 500 (acetylated) for the molecular radical ion (M⁺) and at m/z 441 (underivatised) and...
485 (acylated) for the fragment [M-15]+. A further characteristic fragment was at m/z 428 (underivatised) and 472 (acylated) for [M-28]+ (Fig. 3). We therefore identified the DOPS congener to be 7,11-diketodihydronanolosterol (7,11-KD) and the LOPS congener in analogy to be 7,11-diketolanosterol (7,11-KL).

Two DOPS congeners revealed the same monoisotopic mass containing three oxygen atoms, two of them belonging to two hydroxy, the third therefore to a keto group. The basic structure of DOPS has a hydroxy group situated on C3. Therefore, according to theory, C7 and C1, either contained the hydroxy group or were part of the keto group and vice versa, leading to constitutional isomers. In principle, these congeners could also be stereo isomers with the functional groups bound on the same C atoms but with the hydroxy group having a different αβ position instead. This option, however, was dismissed as the full scan spectra of the two congeners were totally different.

The full scan spectrum of 11-KDD was in fact found in the spectra library and matched our spectrum obtained by GC–MS. We therefore assigned the congener to be 11-keto-3,7-dihydroxy-dihydronanolosterol (11-KDD) and the corresponding LOPS congener to be 11-keto-3,7-dihydroxy-lanosterol (11-KDL). As the second congener was thought to be 7-KDD, we compared our spectra of the underivatised form with a library spectrum of 7-KDD which was α-deuterated at C11 and β-acylated on C3. The two main peaks of the underivatised 7-KDD and the deuterated substance, being the m/z corresponding to the monoisotopic mass and a fragment, had a Δm of 43 amu (m/z 458 and 501) and of 1 amu for the fragment (m/z 304 and 305) as would be expected. In addition, the spectrum of our derivatised compound was consistent with fragments having a Δm of 72 amu for each converted hydroxy group.

We therefore assigned the second DOPS congener to be 7-keto-3,11-dihydroxy-dihydronanolosterol and the analogous LOPS congener to 7-keto-3,11-dihydroxylanosterol (7-KDL).

3.4. Method validation

Quality assurance consisted of the validation procedure and of using a quality control standard every ten injections during routine analysis. Validation could not be performed for all SOPS found in samples due to lacking references. Therefore, levels of 7α-hydroxystigmasterol (7α-HSt), 7β-hydroxystigmasterol (7β-HSt), 7,11- diketolanosterol (7,11-KL), 7-ketolanosterol (7-KL) were determined with the help of calibration curves of the corresponding sitosterol (7α-HSi, 7β-HSi) or dihydrolanosterol (7,11-KD, 7-KD) congeners.

3.4.1. Selectivity

Thermooxidation of dihydrolanosterol, sitosterol, campesterol, and cholesterol was performed to show that validated SOPS could undoubtedly be classified as oxidation products of the corresponding sterols.

Identification of SOPS found in samples was performed by comparison of main mass signals and retention time (RT) with the SOPS mixture (Table 1). In addition, main peaks of COPs and POPs were compared to previously published studies [6,18]. As described under 3.3, LOPS and DOPS identification was more difficult because no published data were available as a reference. Nevertheless, structure elucidation was performed as far as possible (3.3).

According to literature [18], the order of elution of COPs and POPs followed a common pattern with the 7α-hydroxy congener eluting first followed by the 7β-hydroxy, 5,6α-epoxy, 5,6β-epoxy, triol, and 7-keto congener. In addition, a substance with the same full scan spectra as the triol derivative and eluting after the 7α-hydroxy congener occurred in both the thermooxidised cholesterol and the thermooxidised sitosterol solution. This indicates that there may be two different triol isomers. Although the later eluting triol congener was more often found in samples, the early eluting sitostanetriol (SiT) congener was used for validation studies and quantification performance due to a higher signal in the SOPs mixture.

Chromatographic separation was achieved for all congeners except for the coeluting 7,11-KD and 11-KDD. As the m/z of the main peaks differed without interferences from other fragments, proper identification and quantification was possible (Table 1).

No matrix interference was observed neither in spiked matrices used for recovery tests nor in samples from the market survey showing that selectivity was very good.

3.4.2. Linearity, limit of detection (LOD), limit of quantification (LOQ)

Coefficients of determination were excellent over the whole measurement range being ≥ 0.9990 confirming linearity. It is noteworthy, that slope values depend on the congener group as the values for 7α-/7β-hydroxy congeners are higher than those for 7-keto and triol congeners as well as 5,6α-/5,6β-epoxides having the flattest slope (Table 3).

For limit of detection (LOD) estimation, the lowest standard solution used for calibration was diluted step-by-step (dilution factor two and up to six times) until a signal to noise ratio of three was reached for each analyte. Limits of detection were between 0.003 and 0.250 mg kg⁻¹ depending on the congener, whereas 7α-/7β-hydroxy congeners showed the lowest and the epoxy-congeners the highest LODs (Table 3). A comparison with published LODs shows, that our values were similar or better.

We set the limit of quantification (LOQ) to be the lowest point of the calibration curves (Table 3). LOQs and linearity ranges were adjusted to expected SOPs levels. In general, COPs, LOPS, DOPS were found in relative high contents in lanolin containing products (low percent range) whereas POPs stemming from ingredients like vegetable oils, phytosterols or unsaponifiable of vegetable oils showed much lower levels (low ppm range).

3.4.3. Precision and accuracy

Accuracy was first checked with a method comparison on four lanolin containing samples (two ointments and two lip care products) with total levels of COPs in the mid-range being between 6000 and 9000 mg kg⁻¹. The levels of the eight congeners (7α-/HC, 7β-/HC, α-CE, β-CE, CF, 7-KC, 7,11-KD, 7-KD) determined by the validated SPE–GC–FID method [6] and the described pSPE–GC–MS method differed by 15 percent at the most (3–15%). These deviations can be neglected as the precision of the pSPE method was in a similar range and both methods differed in respect to extraction, transesterification, sample clean-up and the detector.

Recoveries (between 86 and 113%) (Fig. 4) were determined by performing the sample procedure plus GC–MS quantification twice on three blank matrices (lip care product, cream, paraffin wax) (n = 6, application volume pSPE: 200 μL). As the bulk of all three matrices is dissolved during the extraction procedure, no difference in recovery rates between analytes spiked or in situ is to be expected. Results were comparable to a previous study on the determination of POPs using similar spiking levels [26]. Spiking levels depended on the levels we expected in samples and were between 0.02 mg kg⁻¹ and 5 mg kg⁻¹ for POPs, respectively between 0.7 mg kg⁻¹ and 10 mg kg⁻¹ for COPs and LOPS. Method precision expressed as RSD (%) of recoveries were below 10% (Fig. 4).

As a check for possible artificial formation of SOPs during analysis, two blank lip care samples each spiked with one milligram cholesterol and sitosterol per sample weight were analysed. No oxidised sterols were found proving that sample handling was fit for purpose.
3.5. SOPs in cosmetic samples

For the assessment of SOPs levels in cosmetics currently sold on the market, a survey of 42 samples obtained from the Swiss and German market was performed. Samples selected contained either a vegetable oil, or phytostersols or lanolin or a combination of them. COPs, DOPs and LOPs were found in samples containing lanolin (Table 4). COPs contents were in the low percent range and therefore in line with our previous study [6]. Remarkably, DOPs levels were higher than LOPs levels, although lanosterol contents were twice the dihydrolanosterol contents. Our studies on thermooxidation, however, showed us that the generation of LOPs proved to be more difficult than SOPs from other sterols because of subsequent formation of dimers and trimers. This fact may also apply to cosmetics.

POP were detected in vegetable oils or samples with phytosterols as ingredients. Levels in vegetable oils, being in the low ppm

### Table 3
Calibration and performance data for validated sterol oxidation products (SOPs).

<table>
<thead>
<tr>
<th>Substance</th>
<th>Linearity range (ng μL⁻¹)</th>
<th>Intercept ± SD1(²)</th>
<th>Slope ± SD2(²)</th>
<th>Coefficient of determination (r²)</th>
<th>LOQ (mg kg⁻¹)</th>
<th>LOD (mg kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7α–HC</td>
<td>0.002–0.08</td>
<td>+0.005 ± 0.01</td>
<td>44 ± 0.4</td>
<td>0.9991</td>
<td>0.02</td>
<td>0.003</td>
</tr>
<tr>
<td>7β–HC</td>
<td>0.02–0.8</td>
<td>−0.5 ± 0.2</td>
<td>27 ± 0.2</td>
<td>0.9992</td>
<td>0.2</td>
<td>0.007</td>
</tr>
<tr>
<td>β–CE</td>
<td>0.09–3.0</td>
<td>−0.1 ± 0.07</td>
<td>2 ± 0.02</td>
<td>0.9990</td>
<td>1.0</td>
<td>0.05</td>
</tr>
<tr>
<td>α–CE</td>
<td>0.09–3.0</td>
<td>+0.06 ± 0.05</td>
<td>2 ± 0.01</td>
<td>0.9993</td>
<td>1.0</td>
<td>0.09</td>
</tr>
<tr>
<td>CT</td>
<td>0.09–3.0</td>
<td>−0.4 ± 0.2</td>
<td>6 ± 0.04</td>
<td>0.9991</td>
<td>1.0</td>
<td>0.05</td>
</tr>
<tr>
<td>7-KC</td>
<td>0.3–10.0</td>
<td>−0.5 ± 0.1</td>
<td>6 ± 0.03</td>
<td>0.9993</td>
<td>3.0</td>
<td>0.03</td>
</tr>
<tr>
<td>7α–HSi</td>
<td>0.005–0.2</td>
<td>−0.001 ± 0.03</td>
<td>22 ± 0.1</td>
<td>0.9990</td>
<td>0.05</td>
<td>0.003</td>
</tr>
<tr>
<td>7β–HSi</td>
<td>0.01–0.5</td>
<td>−0.1 ± 0.03</td>
<td>17 ± 0.1</td>
<td>0.9992</td>
<td>0.1</td>
<td>0.004</td>
</tr>
<tr>
<td>α–SiE</td>
<td>0.03–0.5</td>
<td>−0.02 ± 0.006</td>
<td>3 ± 0.03</td>
<td>0.9991</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>β–SiE</td>
<td>0.02–0.4</td>
<td>+0.01 ± 0.003</td>
<td>2 ± 0.02</td>
<td>0.9995</td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td>7α–HCa</td>
<td>0.002–0.02</td>
<td>−0.008 ± 0.004</td>
<td>27 ± 0.4</td>
<td>0.9997</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>7β–HCa</td>
<td>0.006–0.7</td>
<td>0.007 ± 0.004</td>
<td>24 ± 0.2</td>
<td>0.9995</td>
<td>0.06</td>
<td>0.03</td>
</tr>
<tr>
<td>SIT</td>
<td>0.02–0.6</td>
<td>0.05 ± 0.007</td>
<td>3 ± 0.02</td>
<td>0.9990</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>7-KDD</td>
<td>0.02–0.7</td>
<td>−0.08 ± 0.04</td>
<td>14 ± 0.1</td>
<td>0.9990</td>
<td>0.2</td>
<td>0.007</td>
</tr>
<tr>
<td>7,11-KD</td>
<td>0.07–2.3</td>
<td>+0.04 ± 0.04</td>
<td>5 ± 0.04</td>
<td>0.9991</td>
<td>0.7</td>
<td>0.05</td>
</tr>
<tr>
<td>11-KDD</td>
<td>0.02–0.6</td>
<td>−0.04 ± 0.02</td>
<td>6 ± 0.08</td>
<td>0.9990</td>
<td>0.2</td>
<td>0.01</td>
</tr>
<tr>
<td>7-KCa</td>
<td>0.04–0.7</td>
<td>+0.002 ± 0.01</td>
<td>4 ± 0.03</td>
<td>0.9991</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>7-KC</td>
<td>0.06–0.8</td>
<td>+0.1 ± 0.06</td>
<td>9.5 ± 0.07</td>
<td>0.9994</td>
<td>0.6</td>
<td>0.04</td>
</tr>
<tr>
<td>7-KSI</td>
<td>0.17–6.0</td>
<td>−0.2 ± 0.08</td>
<td>4.4 ± 0.04</td>
<td>0.9991</td>
<td>1.8</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Fig. 4. Recovery results from a lip care (n = 2), paraffin (n = 2) and cream (n = 2) sample of sterol oxidation products (SOPs) after pSPE-GC–MS. Spiking levels were between 0.02 and 10 mg kg⁻¹.

### Table 4
Occurrences of sterol oxidation products (SOPs) in different sample categories.

<table>
<thead>
<tr>
<th>Sample category</th>
<th>n</th>
<th>mg/kg POPs</th>
<th>LOQ (mg kg⁻¹)</th>
<th>LOD (mg kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vegetable oils</td>
<td>23</td>
<td>1–51</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>creams</td>
<td>6</td>
<td>0–21</td>
<td>0–48</td>
<td>1–230</td>
</tr>
<tr>
<td>fat ointments</td>
<td>8</td>
<td>–</td>
<td>7–3000</td>
<td>90–23000</td>
</tr>
<tr>
<td>lip care products</td>
<td>9</td>
<td>1–5</td>
<td>22–1500</td>
<td>600–9000</td>
</tr>
</tbody>
</table>
range, were consistent to a previously published study [15]. Up to this date, no data have been available on POPs levels in cosmetics enriched with phytosterol or the unsaponifiable matter of vegetable oils.

4. Conclusion

The developed pSPE–GC–MS method, in which pSPE was applied for the first time for oysteroids in cosmetics, proved to be a sensitive and efficient method. The possibility of visual monitoring clean-up during pSPE is especially advantageous as small adjustments can be made in-time, if necessary. In cosmetics, we found COPs in lanolin containing products in the low percent range (up to 2.3%) being in line with a previous study [6]. POPs levels determined in our study (1–25 ppm) were in the order of magnitudes published for food [15]. In addition, the in-house generation of LOPs and DOPs was helpful in allocating unknown peaks in lanolin containing samples. We first found those substances in consumer products namely lanolin containing cosmetics in contents of up to 0.3 percent. Additionally, COPs contents in lanolin containing products being up to 2.3% exceeded reported levels for food by several orders of magnitudes.

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