Determination of potentially hazardous oxidation products in cosmetics containing lanolin or 1-(1,2,3,4,5,6,7,8-octahydro-2,3,8,8-tetramethyl-2-naphthalenyl)ethanone (OTNE)

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Universität Hohenheim
Institut für Lebensmittelchemie

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2019
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# Table of contents

I. **Introduction** ........................................................................................................................................... 1

1. Definition ................................................................................................................................................ 1

2. Safety and regulation of cosmetic products .............................................................................................. 1

   2.1. Regulation (EC) No 1223/2009 .............................................................................................................. 1

   2.2. Scientific committee on consumer safety ........................................................................................... 2

   2.3. Safety of cosmetic fragrances ............................................................................................................ 2

3. Absorption of cosmetic ingredients ........................................................................................................... 2

   3.1. Dermal absorption ............................................................................................................................. 2

   3.2. Oral absorption ................................................................................................................................... 4

4. Skin sensitization and Photosensitization ................................................................................................. 4

   4.1. Skin Sensitization ............................................................................................................................... 4

   4.2. Photosensitization ............................................................................................................................. 5

   4.3. Patch-Test ......................................................................................................................................... 6

5. Oxidation of cosmetic ingredients ............................................................................................................. 7

   5.1. General introduction .......................................................................................................................... 7

   5.2. Autoxidation ...................................................................................................................................... 7

   5.3. Photooxidation .................................................................................................................................. 8

   5.4. Enzymatic oxidation .......................................................................................................................... 9

6. Analytical methods for monitoring the degree of sample oxidation ......................................................... 9

   6.1. Chemical methods ............................................................................................................................. 10

      6.1.1. Peroxide value ............................................................................................................................... 10

      6.1.2. Conductivity value ...................................................................................................................... 11

   6.2. Chromatographic methods ............................................................................................................... 11

      6.2.1. Gas chromatography .................................................................................................................. 11

      6.2.2. High-performance liquid chromatography .................................................................................. 12

7. Aims of the studies .................................................................................................................................... 13
7.1. Oxidation of sterols in lanolin ................................................................. 14
7.1.1. Lanolin/ wool wax .................................................................................. 14
7.1.2. Manufacturing of lanolin ...................................................................... 14
7.1.3. Composition of lanolin ........................................................................... 14
7.1.4. Allergic contact dermatitis from lanolin ............................................... 15
7.1.5. Analytical methods for the determination of sterol oxidation products 15
7.2. Oxidation of the fragrance 1-(1,2,3,4,5,6,7,8-octahydro-2,3,8,8-tetramethyl-2-naphthalenyl)ethanone (OTNE) in perfumes ...................................................... 16
7.2.1. Perfume: Definition and functioning ................................................... 16
7.2.2. Fragrance oxidation: State of science .................................................... 17
7.2.3. 1-(1,2,3,4,5,6,7,8-octahydro-2,3,8,8-tetramethyl-2-naphthalenyl)ethanone ............................................................................................................. 17
8. Literature ......................................................................................................... 19

II. Publications ..................................................................................................... 24

1. Determination of cholesterol oxides by gas chromatography-flame ionization detection/mass selective detection and their occurrence in lanolin-containing cosmetics and ointments. .................................................................................................................... 24
2. Oxysterols in cosmetics-Determination by planar solid phase extraction and gas chromatography-mass spectrometry ........................................................................ 32
3. Photooxidation of Octahydro Tetramethyl Naphthalenylethanone (OTNE) in Perfumes and Aftershaves .................................................................................... 42

III. Discussion ...................................................................................................... 52

IV. Summary ....................................................................................................... 57

V. Zusammenfassung .......................................................................................... 59
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-CE</td>
<td>cholesterol 5α,6α-epoxide</td>
</tr>
<tr>
<td>α-SiE</td>
<td>sitosterol 5α,6α-epoxide</td>
</tr>
<tr>
<td>APCI</td>
<td>atmospheric pressure chemical ionization</td>
</tr>
<tr>
<td>β-CE</td>
<td>cholesterol 5β,6β-epoxide</td>
</tr>
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<td>β-SiE</td>
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<tr>
<td>CaOPs</td>
<td>campesterol oxidation products</td>
</tr>
<tr>
<td>CMR</td>
<td>carcinogenic, mutagenic or toxic for reproduction</td>
</tr>
<tr>
<td>COPs</td>
<td>cholesterol oxidation products</td>
</tr>
<tr>
<td>CT</td>
<td>5α-cholest-3β,5α,6β triol</td>
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<td>DAD</td>
<td>diode array detection</td>
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<td>dihydrolanosterol oxidation products</td>
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<td>DPTMDS</td>
<td>diphenyltetramethylidisiloxane</td>
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<td>EC</td>
<td>European Commission</td>
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<td>EI</td>
<td>electron impact ionization</td>
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<td>flame ionization detection</td>
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<td>GC</td>
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<td>HPLC</td>
<td>high-performance liquid chromatography</td>
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<tr>
<td>HPTLC</td>
<td>high-performance thin-layer chromatography</td>
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<tr>
<td>HRGC</td>
<td>high-resolution gas chromatography</td>
</tr>
<tr>
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<td>high-resolution mass spectrometry</td>
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<tr>
<td>IFRA</td>
<td>International Fragrance Association</td>
</tr>
<tr>
<td>IS</td>
<td>internal standard</td>
</tr>
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<td>LC</td>
<td>liquid chromatography</td>
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<td>limit of quantification</td>
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<tr>
<td>MS</td>
<td>mass spectrometry</td>
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<td>MB</td>
<td>methylene blue</td>
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MTBE  methyl tert-butyl ether
NBP  4-(p-nitrobenzyl)pyridine
OTNE  1-(1,2,3,4,5,6,7,8-octahydro-2,3,8,8-tetramethyl-2-naphthalenyl)ethanone
OTNE-DG1 OTNE degradation product 1
OTNE-DG2 OTNE degradation product 2
OTNE-DG3 OTNE degradation product 3
PAHs  polycyclic aromatic hydrocarbons
POPs  phytosterol oxidation products
pSPE  planar solid phase extraction
PTFE  polytetrafluoroethylene
PTV  programmable temperature vaporizer
R·  alkyl radical
RB  rose bengal
ROOH  organic hydroperoxides
ROH  organic alcohol
ROO·  peroxy radical
RO·  alkoxy radical
RT  room temperature
SC  stratum corneum
SCCS  Scientific Committee on Consumer Safety
SiT  sitostanetriol
SOPs  sterol oxidation products
SPE  solid phase extraction
TEPA  tetraethylenepentamine
TiEt  titanium ethoxide
TLC  thin-layer chromatography
RIFM  Research Institute of Fragrance Materials
UV  ultra violet
ULC  ultra liquid chromatography
QC  quality control standard
<table>
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<td>VIS</td>
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<td>7-KDL</td>
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<tr>
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<tr>
<td>7,11-KL</td>
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<tr>
<td>20α-HC</td>
<td>20α-hydroxycholesterol</td>
</tr>
<tr>
<td>25- HC</td>
<td>25-hydroxycholesterol</td>
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Preliminary remarks

The research work presented in this thesis was carried out under the supervision of Prof. Dr. Wolfgang Schwack, Institute of Food Chemistry, University of Hohenheim, Stuttgart, Germany, between October 2014 and March 2019.

Parts of this doctoral thesis have already been published in international peer-reviewed journals, or were presented at international conferences as poster presentations.

Full publications


Poster presentations


Chapter II of this thesis is in form and content identical with the full publication 1-3. All images, pictures and illustrations in this work were created by the author.
Contributions

The participation and contributions of the authors to the specified full publications are as follows:

**Sonja Schrack** performed all the essential practical and analytical work. The analysis and interpretation of the obtained data as well as the preparation of the original manuscripts that lead to the specified publications was carried out by herself.

**Prof. Dr. Wolfgang Schwack** was the supervisor of this work and he proofread and corrected the manuscripts that lead to the specified publications. Prof. Dr. Wolfgang Schwack advised in the scope and the milestones of this thesis and in clarifying analytical questions. He functioned as an advisor throughout the publication process.

**Dr. Christopher Hohl**, was the local supervisor and therefore served as a first contact person in daily work. Dr. Christopher Hohl helped to link the scope of the thesis with the interests of the State Laboratory of the Canton Basel-City. He also checked the manuscripts in terms of comprehensibility.

**Dr. Markus Niederer** served as first contact person for gas chromatographic questions in daily work. **B. Roux** helped with the routine work according to the manuscript.
1. Introduction

1. Definition

According to the main European Regulation on cosmetic products (EC) 1223/2009, “a cosmetic products means any substance or mixture intended to be placed in contact with the external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance, protecting them, keeping them in good condition or correcting body odors” [1].

2. Safety and regulation of cosmetic products


The regulation (EC) 1223/2009 provides the framework of requirements for cosmetic products on the market in the European Union. Requirements thus are harmonized for all European member states with the general aim to achieve an internal market and a high health protection level when cosmetics are used normal or reasonably foreseeable.

Stating that commercially available cosmetics shall be safe for human health, chapter II article 3 is of special importance in that respect. As manufacturers are to designate a person ensuring the safety of cosmetic products (Chapter III article 10), the responsibilities are further clearly settled. Besides, the regulation requires a safety assessment for every cosmetic product to be performed and documented in a report [1]. For safety reasons, chapter IV is important covering restrictions for certain substance categories like dyes, UV filters or preservatives. Nanomaterials and substances classified as carcinogenic, mutagenic or toxic for reproduction (CMR) are also regulated in this chapter. For the same reason, cosmetics further shall not contain any substance listed in annex II, as for instance hexachloroethane, nitroglycerine and ethylene oxide [1]. Annex III lists substances with application specific restrictions. One example is given by the allergenic fragrances like limonene, linalool and coumarin [1].
2.2. Scientific committee on consumer safety

As an independent risk assessment body of the European Committee, the Scientific Committee on Consumer Safety (SCCS) is entrusted with evaluating safety aspects of non-food products like cosmetics and their ingredients.

Based on different studies, the SCCS regularly publishes opinions on risks of cosmetic ingredients [2]. Manufacturers of cosmetics should consider these recommendations for safety assessments.

2.3. Safety of cosmetic fragrances

The fragrance industry established a self-regulated system to ensure the safety of fragrances. The system is based on the Research Institute of Fragrance Materials (RIFM) and the International Fragrance Association (IFRA) [3]. RIFM generates and evaluates data on toxicological aspects of fragrances including acute oral toxicity, acute dermal toxicity, skin irritation, mucous membrane irritation, skin sensitization and elicitation, photosensitization, toxicokinetics, reproductive toxicity, genotoxicity, carcinogenicity and repeated dose toxicity [3]. Based on these results, the IFRA establishes guidelines. Cosmetic manufacturers should consider these guidelines when evaluating the safety of their cosmetic products [3].

3. Absorption of cosmetic ingredients

Absorption generally is a term for the transport of a compound into the systemic circulation. The absorption of compounds into the human body is successfully exploited by the pharmaceutical industry in treating disorders with drugs. On the other side, the absorption of hazardous compounds has to be taken into account.

3.1. Dermal absorption

Dermal absorption describes the transport of chemicals through the skin into the inner body. Dermal absorption of unwanted compounds therefore can be relevant for cosmetics applied on the skin.
Cosmetics generally are grouped into “leave-on” and “rinse-off” products. As their name suggests, leave-on cosmetics like creams are meant to stay on the skin whereas rinse-off cosmetics such as shower gels and shampoos are washed off the skin shortly after application. Due to the longer contact with the skin, dermal absorption of compounds is more relevant for leave-on cosmetics.

To understand which compounds undergo dermal absorption, the structure and function of the skin need to be discussed in more detail: With a surface of two square meters, the skin is the largest human organ [4]. The skin is mainly made up of three layers being, from top to bottom the epidermis, the dermis and the subcutis [4, 5]. The three layers are further divided in several sublayers [4]. Being the barrier between the body and the environment, the skin has a number of protective functions: The skin protects the inner body from microbiological, physical and chemical harm [4, 5]. The transmission of stimulus after mechanical or thermal stress is assured by several sensory receptors on the surface of the skin [5]. The skin also protects the body from losing heat and vital substances such as water and minerals [4, 5]. When exposed to sunlight, the skin forms melanin as a protection against excessive solar radiation [6]. Although being the protective barrier between the human body and the environment, the skin is not completely impenetrable.

The skin absorption particularly depends on the permeability of a compound through the epidermis, or, more precisely, the outer sublayer of the epidermis, the stratum corneum (SC). The SC consists of keratin-rich corneocytes, which are embedded in a lipid bilayer consisting of hydrophobic molecules with hydrophilic head groups. As most substances permeate the SC via the lipid bilayer (intercellular) (Fig. 1), absorption requires compounds to be lipophilic [7]. Due to the hydrophilic head groups of the lipid bilayer, however, diffusion of extremely apolar molecules still is impeded [7]. The SCCS, therefore, concluded that charge and lipophilicity of a given compound correlate with its skin penetration capability. A low molecular weight further enhances dermal absorption [7]. Other less significant absorption routes are transcellular where absorption occurs via skin cells (Fig. 1) [8, 9] or via sweat glands/hair follicle openings where water soluble substances may penetrate [8].
3.2. Oral absorption

Oral absorption describes the transport of compounds via mouth into the inner body. This can happen for cosmetics, either when residues of an applied product are involuntarily swallowed, or when the product is meant to come into contact with the mucous tissue of the mouth (e.g. mouthwashes). Lip care products are the best-known examples for involuntary ingestion. The SCCS estimated the average daily application amount of lip care products to be 0.057 g per person with an oral uptake of up to 100 % in the worst case [10]. Another example involving a potentially high oral uptake amount is nipple ointments. Their leaflets often state that it is not necessary to remove the applied ointment before nursing. This leads to an increased oral uptake by infants.

4. Skin sensitization and Photosensitization

4.1. Skin Sensitization

Skin sensitization (also known as allergic contact dermatitis) is an immune response occurring as eczema after the repeated contact of a sensitizing compound (allergen) with the skin [11, 12].

As the biochemical processes behind allergic contact dermatitis are located in the epidermis, only compounds capable to penetrate stratum corneum are potential sensitizers. Mentioned biochemical processes are grouped into two phases [13]. In the induction phase, the allergen first reacts with epidermal protein [11, 13]. The so formed antigen is then processed from the Langerhans cells in the epidermis [11]. As a result,
the Langerhans cells circulate to the lymph cells where the antigens come into contact with the T-cells [12, 11]. Thereby stimulated T-cells then proliferate and circulate through the body, which is when the induction phase is over [11]. In the following elicitation phase, subsequent exposure of the skin with the compound provokes the allergic response [13]. The substance again binds on epidermal protein and the formed antigen is repeatedly processed and presented from the Langerhans cells [11]. The antigen, however, is then recognized by circulating T-cells, which finally activate cellular and biochemical processes resulting in the immunological response [11].

The assessment of the skin sensitization potential of compounds is performed with animal tests like local lymph node assay and guinea pig testing [11, 13] since there are no regulatory accepted non-animal alternatives [13].

4.2. Photosensitization

After application of cosmetics on the skin, the ingredients are exposed to sunlight. As a result to that solar radiation, adverse biological reactions due to phototoxicity or photoallergy may occur [14]. The general term for both is photosensitization [14]. Photosensitization should also be considered for the safety evaluation of cosmetics for skin use.

From the chemical point of view, the initiation step for a molecule’s phototoxic and/or photoallergic response is the transition of the molecule into its excited state by absorbing energy from a UV light photon [15, 12]. It is therefore not surprising that only molecules absorbing in the UV range of natural light (290-700 nm) are considered to potentially cause phototoxic or photoallergic response.

A photoallergic reaction occurs as an allergic contact dermatitis similar to processes described in the chapter “skin sensitization”. The difference is that not the compound itself, but the excited species binds to the skin protein, thereby inducing the mentioned biochemical and immunological processes.

A phototoxic response in contrast to photoallergic response occurs after a single exposure and is characterized by an irritation similar to an intense sunburn [15, 16]. This is either induced, when the excited molecule reacts with an endogenous compound leading to a cytotoxic compound (direct), or, when the emittance of the surplus energy of the excited molecule induces the formation of reactive oxygen species and radicals as
secondary reaction products, which then react with endogenous molecules, leading to
the mentioned cytotoxic effects (indirect) [19, 15]. An often-reported example for
indirect phototoxicity is the formation of highly cytotoxic singlet oxygen as a secondary
reaction product [15, 17, 18]. Such an example is given by polycyclic aromatic
hydrocarbons (PAHs). PAHs absorb UV light, transferring them to their excited state.
Returning to their ground state, the molecules donate surplus energy to ambient oxygen
(triplet state) forming reactive singlet oxygen and other oxidizing molecules [19].

4.3. Patch-Test

The (photo) patch-test is a commonly used method to establish whether a certain
compound induces phototoxic/photoallergic response or skin sensitization when applied
on the skin of an individual person. The exact test procedure varies from laboratory to
laboratory.

The procedure of a German, Austrian and Swiss photopatch-test
(photoallergic/phototoxic response) group is exemplarily described [20]. The test
substances are applied on two aluminum disks, which are then fixed on the skin of the
test person’s back using an adhesive stripe. One patch serves as a control and remains
on the skin. After 24 hours, the other patch is removed from the skin, when the
uncovered skin area is irradiated with artificial sunlight in the range of 320-420 nm with
an intensity of 5-10 J/m². After irradiation, the patch is refixed on the test person’s
back. Irradiation is repeated up to three times each after time intervals of 24 hours.
Reddened irradiated skin together with a negative control reveals a
phototoxic/photoallergic response.

The procedure of the patch-test (skin sensitization) is similar to that of the photopatch-
test, with the difference that the test substances are not irradiated. A control patch
therefore is not needed and test substances are only once fixed on the test persons back.
The patch also is removed from the skin in time intervals of 24 hours up to three times,
when reddened skin reveals a contact response.
5. Oxidation of cosmetic ingredients

5.1. General introduction

The European regulation for cosmetics requires the labelling of an expiration date up to which a cosmetic product shall be safe for human health [1]. Microbial infestation, physical change or chemical reaction may cause the deterioration of product quality over time. An example for a chemical reaction is the oxidation of allylic compounds, which is the issue of this work.

The oxidation of allylic compounds with atmospheric oxygen is spin forbidden as the outstanding electron arrangement of atmospheric triplet oxygen does not allow for a direct reaction with the substrate being in singlet condition [21, 22]. Atmospheric triplet oxygen has two unpaired electrons therefore behaving like a biradical [23]. The singlet substrate, however, has an opposite spin direction causing the spin barrier [23].

5.2. Autoxidation

Autoxidation describes a radical chain reaction of allylic compounds with oxygen.

In the initiation period of the reaction, either peroxy (ROO·), alkoxy (RO·) or alkyl (R·) radicals are formed [21]. Presumably not only sunlight (chapter 5.3.), but also heat, enzymatic processes as well as the presence of heavy metals play a major role during this initial formation of radicals [21].

In the following propagation phase, R· reacts with oxygen forming ROO·, and ROO· and RO· abstract a hydrogen atom of an allylic substrate thereby forming an organic hydroperoxide (ROOH) and an alcohol (ROH), respectively. Due to the lowest reaction rate, the formation of ROOH is the rate-limiting step [23]. As the hydrogen abstraction, however, occurs selective on the most weakly bonded hydrogen atom, and the thereby formed radical is stabilized over neighboring carbon atoms, a specific hydroperoxide spectrum is the result (Fig. 2) [21].

In the branching phase, ROOH break down into RO·, ROO· and hydroxy radicals. In the chain termination phase, the combination of two radicals lead to stabile reaction products such as peroxy compounds, oligomers or polymers [21]. The overall mechanism [24] of oxidation is demonstrated in Fig. 3.
5.3. Photooxidation

Photoinduced oxidation (Photooxidation) not only requires the presence of the substrate (allylic compound) and sunlight, but also a sensitizer being capable of absorbing sunlight. For example, pigments/dyes can serve as sensitizers.

The sensitizer first absorbs sunlight, transferring it into an excited triplet state [21, 22, 23]. The energy can then be transferred onto oxygen, leading to the formation of reactive singlet oxygen, which directly reacts with the double bond of the substrate (type II sensitizer) [21, 22]. The resulting products are hydroperoxides of the substrate formed by an “ene” addition mechanism [23] (Fig. 2). In a second pathway, the excited sensitizer reacts with the substrate abstracting hydrogen and leading to the formation of substrate radicals (R·), which then follow the mechanisms of autoxidation (type I sensitizer) (3.2.) [21]. The hydroperoxides formed by autoxidation differ from those of the type II photooxidation in respect to the position of the hydroperoxide group (Fig. 2).

---

Fig. 2 Hydroperoxide patterns formed after autoxidation (A) and photooxidation with type II sensitizer (B)
5.4. Enzymatic oxidation

Lipoxygenase is an enzyme, which selectively catalyzes the oxidation of certain unsaturated fatty acids, namely those with a 1-cis,4-cis pentadiene group [21]. This catalysis has its greatest effect on the oxidation of linoleic and linolenic acid in plant, as well as arachidonic acid in animal material [21]. Similar to autoxidation and photooxidation, enzymatically formed oxidation products are hydroperoxides. Enzymatic oxidation, however, is substrate specific, selective and depends on the pH value and temperature [21].

6. Analytical methods for monitoring the degree of sample oxidation

As there is no widely applicable method to determine the extent of oxidation, different analytical methods are used. Some of them are chemical, while others have a chromatographic origin. The methods in general rely on either the determination of primary (peroxides) and secondary (aldehydes etc.) oxidation products, or on tracking declining levels of oxidation sensitive ingredients.
6.1. Chemical methods

6.1.1. Peroxide value

An often-used scale for assessing the degree of oxidation is the so called peroxide value. The peroxide value relies on the determination of the primary formed oxidation products being peroxides. The peroxide value is defined as the amount of peroxide, expressed as milliequivalents of peroxide oxygen, present in one kilogram of sample [25]. An increased level of peroxides may indicate an advanced oxidation state.

According to the autoxidation mechanism, the peroxide level is low in the induction period when only radicals but no primary oxidation products are formed. With advancing oxidation (propagation phase), the peroxide value strongly increases due to the peroxides being continuously formed. In the branching and termination phase, however, the peroxide values decrease due to hydroperoxides reacting to secondary oxidation products and/or forming stable products like oligomers and polymers. The peroxide value as a function of the time is shown in Fig. 4. Monitoring of the peroxide value gives a plot showing how and up to which phase oxidation has progressed.

The peroxide value is determined by titration, when an acidic solution of potassium iodide is first added in excess to the sample solution. The peroxides then oxidize iodide to iodine [26] (Fig. 5). After a starch solution is added, the iodine is back-titrated with a thiosulfate solution (Fig. 5). The starch is the titration indicator, because its amylose forms a blue complex even with traces of iodine. The peroxide value is calculated by the amount of reacted iodide.
ROOH + 2I + 2H⁺ → ROH + H₂O + I₂
R₁OOR₂ + 2I + 2H⁺ → R₁OH + R₂OH + I₂
I₂ + amylose + 2Na₂S₂O₃ (blue) → 2NaI + amylose + Na₂S₄O₆ (colorless)

Fig. 5 Peroxide value titration

6.1.2. Conductivity value

The determination of the conductivity value is less known, but has also been successfully used to assess the oxidative stability of specific cosmetic ingredients. The conductivity value is determined after polar oxidation products are extracted with water [27, 28].

In an essential oil study, observation showed that an increasing conductivity value directly is linked to an advanced oxidation state [27, 28]. Whereas nonpolar educts are not soluble in water, it is assumed that oxygenated compounds are partly soluble in water, therefore inducing conductivity. The formation of hydrogen bonds is considered to be a possible reason for this [27]. As not only labile primary, but also stable secondary oxidation products are thought to provoke conductivity, the conductivity value continuously increases during the oxidation process [28]. This is in contrast to the peroxide value, which decreases after reaching a maximum value.

6.2. Chromatographic methods

6.2.1. Gas chromatography

Oxidation-induced changes can be detected by gas chromatography (GC) [27], when the content of an oxidable compound is monitored over time. A decreasing content after a storage period, for example, may indicate the compound’s oxidation. Additionally, the detection of newly formed peaks may indicate the formation of oxidation products due to the advanced extent of lipid oxidation.

The GC determination of specific oxidation products also is frequently used to assess the oxidation degree. The determination of secondary oxidation products of unsaturated fatty acids of plant oils is an example for this [29]. The omega-6 (n-6) and omega 3 (n-3) polyunsaturated fatty acids, first oxidize to hydroperoxides of fatty acids, which
break down into volatile aldehydes, which are finally determined with GC. This breakdown usually occurs prior to the maximum peroxide value [24]. Best known is the oxidation of linoleic acid into different hydroperoxide isomers of linoleic acid as primary oxidation products. One is 13-hydroperoxyoctadeca-9,11-dienoic acid, which subsequently breaks down into hexanal [30] (Fig 6). Hexanal is a frequently determined marker for lipid oxidation in vegetable oils.

![Diagram of 13-hydroperoxyoctadeca-9,11-dienoic acid breakdown into hexanal and further oxidation products]

**Fig. 6** Breakdown of 13-hydroperoxyoctadeca-9,11-dienoic acid into hexanal and further oxidation products

### 6.2.2. **High-performance liquid chromatography**

In cases when the oxidation products are not GC amenable, high-performance liquid chromatography (HPLC) can provide a solution. This is successfully applied for essential oils when HPLC combined with mass spectrometry (MS) is used for the determination of fragrance hydroperoxides [27]. HPLC-MS also has been used for the identification of oxidized triglycerides in lipids [31].
7. Aims of the studies

Cosmetics are consumer products of daily use and therefore should not be detrimental to human health. The evaluation of cosmetics safety mainly is based on adverse health effects potentially arising from individual ingredients used, with a focus set on certain product categories like preservatives, dyes and on avoiding any substances classified as CMR. However, less consideration is given to unwanted compounds formed by reactions for example during manufacturing or storage of the cosmetic product or after its application on the skin.

An example for such a reaction is the oxidation of an allylic compound. Thereby formed oxidation products are well studied in the food sector, with the result that many of them are considered to pose a potential health hazard [32, 33, 34]. For cosmetics, there also have been some reports on oxidized ingredients having unwanted effects. The best-known examples are terpenes, used as fragrances, which upon oxidation lead to contact allergens [35]. In spite of its wide range, the matter has only received little attention. The oxidation of specific cosmetic ingredients in terms of consumer safety therefore defined the key issue of this work.

The major part is about the oxidation of sterols, a class of non-polar lipids, which are found in fat of plant and animal origin. As some of the sterols contain at least one unsaturated double bond, they are prone to react with oxygen resulting in the formation of sterol oxidation products (SOPs) [36]. Being linked to several diseases such as arteriosclerosis [37], their occurrence in fat rich food has been the subject of many studies [38]. Their content in cosmetics, however, never has been addressed. This is especially surprising as a widely used ingredient, lanolin, is known to contain unusually high precursor levels. Being also used in lip care products and ointments for nursing women, oral ingestion may represent a relevant uptake path.

The focus of the second part of this work is set on fragrances as only terpenes have received sufficient attention [35, 39, 40]. 1-(1,2,3,4,5,6,7,8-octahydro-2,3,8,8-tetramethyl-2-naphthalenyl)ethanone (OTNE) is the compound chosen for this work, as it is commonly used for cosmetics and contains an allylic double bond.
7.1. Oxidation of sterols in lanolin

7.1.1. Lanolin/ wool wax

Wool wax represents purified, water-free sebaceous gland fat of sheep [41, 42]. Lanolin, however, means a mixture of wool wax (65-75 %), water (20-25 %) and mineral or vegetable oil (5-10 %) [42].

The wool fat protects the sheep from external influences like sun, wind, rain or cold [41]. Assuming that lanolin may also be capable of protecting the human skin from external influences, its frequent use in cosmetics is conceivable. The usage of lanolin in lip care products is further widespread due to its consistency improving properties.

As lanolin is the only known commercially used product obtained from sebaceous glands fat, it has a characteristic composition.

7.1.2. Manufacturing of lanolin

Containing of up to 15 % wool fat, sheep wool is the basic substance for the production of lanolin [42]. The sheep wool fat is obtained from the wool either by liquid extraction with a suitable solvent and subsequent distillation [41], or more often as a by-product of the wool scouring process used in the textile industry [41]. The fat then accumulates in the soapy washing water, from where it is separated either by centrifugation or by acidification with sulfuric acid and subsequent smashing (“acid cracking”) [41]. In the case of centrifugation, the obtained raw sheep wool fat is neutralized with an alkaline solution, then extracted with aqueous alcohol, and finally purified by discoloration. In the case of “acid cracking”, the wool fat is extracted from the wool sludge with a suitable solvent. The obtained extract then is purified with an alkaline solution, after which the solvent is removed by distillation. In a final step, the raw wool fat is purified by discoloration [41].

7.1.3. Composition of lanolin

Lanolin is a highly complex mixture of esters, di-esters and hydroxyl esters [43], which are condensates of long-chain alcohols with long-chain acids [41]. The long-chain fatty acids either contain a hydroxy group or not [41]. The majority of the alcohol fraction
consists of sterols (about 74 %) with cholesterol being the major compound [~38%], followed by lanosterol [~15 %] and dihydrolanosterol [~10 %] (Fig. 7) [41,43,44].

Other alcohols have a typical acyclic aliphatic framework with sometimes even two hydroxy groups. Whereas other oils and fats contain sterols as minor compounds, lanolin contains unparalleled high sterol levels.

Depending on the geographic location, the sheep breed, the method of fat extraction and the purification level, the chemical composition of lanolin strongly fluctuates [43].

Fig. 7 Structural formula of the most abundant sterols present in lanolin

7.1.4. Allergic contact dermatitis from lanolin

Allergic contact dermatitis from lanolin and its derivatives have previously been reported [43]. Since several decades, researchers have looked for potentially allergenic compounds in lanolin and its derivatives [43] without yet finding any specific compound.

Many studies indicated that the alcoholic fraction of hydrolyzed lanolin contains the allergens [43]. One study indicated that sterols are most important for the allergic potential of lanolin [45]. A further study from Denmark tested 20 lanolin sensitive patients twice, with freshly produced and with old lanolin. Whereas about 50 percent reacted to fresh lanolin, all patients reacted to the old one, giving evidence that oxidation products are allergens [46].

7.1.5. Analytical methods for the determination of sterol oxidation products

The oxidation of sterols leads to the formation of SOPs, which were frequently studied in food. Structural formulas of the most abundant cholesterol oxidation products are exemplarily shown Fig. 8.
Most published methods for the determination of SOPs are based on GC. In a first step, the SOPs are either hydrolyzed under alkaline conditions or a transesterification step sets them free. In a clean-up step, oxysterols are separated from sterols and other accompanying matrix compounds, mostly by solid phase extraction on a silica cartridge. After derivatization of the oxysterols to the trimethylsilyl ethers, separation and detection of the congeners are performed with GC coupled to flame ionization detection or MS.

Some researchers used liquid-chromatography instead of GC. As oxysterols do not have a significant absorption in the ultraviolet range, MS is the detection method of choice in that case. [47]

7.2. Oxidation of the fragrance 1-(1,2,3,4,5,6,7,8-octahydro-2,3,8,8-tetramethyl-2-naphthalenyl)ethanone (OTNE) in perfumes

7.2.1. Perfume: Definition and functioning

A perfume is a mixture of fragrance compounds, which is mostly diluted in a water-ethanol solution. The compounds are either of natural or synthetic origin. Directly after application of a perfume, the most volatile compounds are perceived, which gives the
so-called top note [48]. The so-called heart is perceivable after five to twenty minutes and lasts for two to four hours [48]. Perception after that time is due to the so-called dry out, which gradually disappears after time [48].

7.2.2. Fragrance oxidation: State of science

Fragrances with a carbon-carbon double bond generally should be considered as oxidable. However, only terpene oxidation has received distinct attention with autoxidation considered to be the major mechanism [49,35]. Some of the terpenes’ oxidation products, especially the hydroperoxides, are suspected to be allergic [49, 35].

Probably the best studied terpene oxidation products are those of limonene [50] (Fig. 9). Besides, the oxidation of geraniol [51], linalool [39] and β-caryophyllen [52] has been the topic of some studies.

**Fig. 9** Structural formulae of limonene (A) and its main primary oxidation products cis/trans limonene 2-hydroperoxide (B) and cis/trans limonene 1-hydroperoxide (C), respectively secondary oxidation products carvone (D), cis/trans limonene epoxide (E) and cis/trans carveol (F)

7.2.3. 1-(1,2,3,4,5,6,7,8-octahydro-2,3,8,8-tetramethyl-2-naphthalenyl)ethanone

OTNE is a synthetic fragrance obtained as a mixture of at least three isomers (Fig. 10). As OTNE frequently is used in cosmetics for personal care, it finds its way into waste water and, therefore, traces are also found in surface water, where it can be either
persistent, or degraded. Besides photodegradation, digestion by microbes is a potential degradation pathway. The photodegradation potential of OTNE on the model of soil surface has been investigated in a previous study [53] showing that OTNE decomposes under the influence of sunlight, probably due to photooxidation. As a result, the oxidation of OTNE in cosmetic products or after the cosmetics application on the skin seems to be obvious. Due to stabilizing effects like a large organic rest and a highly substituted hydroperoxide bearing carbon atom, potentially formed hydroperoxides at least in theory, might be stable.

Fig. 10 Structural formulae of 1-(1,2,3,4,5,6,7,8-octahydro-2,3,8,8-tetramethyl-2-naphthyl)ethanone (OTNE)
8. Literature


II. Publications

1. Determination of cholesterol oxides by gas chromatography-flame ionization detection/mass selective detection and their occurrence in lanolin-containing cosmetics and ointments.

Determination of cholesterol oxides by gas chromatography-flame ionization detection/mass selective detection and their occurrence in lanolin-containing cosmetics and ointments.
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Determination of cholesterol oxides by gas chromatography-flame ionization detection/mass selective detection and their occurrence in lanolin-containing cosmetics and ointments

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Keywords: cholesterol oxidation products, gas chromatography-flame ionization detection, gas chromatography-mass spectrometry, lanolin

Synopsis
OBJECTIVE: Cholesterol oxides (COPs) are thought to be of toxicological relevance in cholesterol-containing foods. For cholesterol-containing cosmetics and the like, no information is available up to this date. Therefore, the first of two main aims of this study was to develop and validate a method for determining COPs in lanolin-containing cosmetics such as lipsticks and fatty creams as well as in nipple ointments. The second aim was to study the occurrence of COPs and their concentration levels in the respective product classes.

METHODS: The procedure is based on a published method for food comprising some necessary modifications. Sample preparation consisted of transesterification, solid-phase extraction and silylation of target compounds. Separation of the derivatized COPs and their quantification were performed with gas chromatography (GC) using a flame ionization detector (FID) or a mass spectrometer (MS).

RESULTS: The successful validation and the trouble-free application during the market survey showed that the method was fit for purpose. Total COP levels found were in the low per cent range (up to 3%) and surprisingly high, being many orders of magnitude higher than those published for foods.

CONCLUSION: To our knowledge, we present for the first time a method for the determination of COPs in non-food consumer products. Furthermore, our study demonstrates that lanolin-containing cosmetics may be an additional exogenous source of COPs. We further show evidence, that at least part of the COPs are already formed on the sheep’s wool.

Résumé
OBJECTIF: Les oxides de cholestérol (COP) sont considérés toxico- logiquement pertinents dans les aliments contenant du cholestérol. Pour les produits cosmétiques et assimilés contenant du cholestérol, aucune information est disponible à cette date. Par conséquent, le premier des deux principaux objectifs de cette étude était de développer et de valider une méthode pour déterminer les COP dans des produits cosmétiques contenant de la lanoline, tels que les rouges à lèvres et les crèmes grasses ainsi que dans les pommades pour mamelons. Le deuxième objectif était d’étudier l’apparition des COP et leurs niveaux de concentration dans les catégories de ces produits respectifs.

MÉTHODES: La procédure est basée sur une méthode publiée pour les aliments, comprenant quelques modifications nécessaires. La préparation des échantillons est composée de transétherification, extraction phase solide et silylation des composés cibles. La séparation des COP dérivatisés et leur quantification a été réalisée par chromatographie en phase gazeuse (CG) en utilisant un détecteur à ionisation de flamme (FID) ou un spectromètre de masse (MS).

RÉSULTATS: La validation réussie et l’application sans problème lors de l’étude des produits du marché a montré que la méthode était adaptée à son objet. Les niveaux des COP totaux se trouvaient dans les valeurs de faible pourcentage (jusqu’à trois pour cent) et étonnamment élevés, étant de plusieurs ordres de grandeur plus élevés que ceux publiés pour les aliments.

CONCLUSION: A notre connaissance, nous présentons pour la première fois une méthode pour la détermination des COP dans les produits de consommation non alimentaires. En outre, notre étude démontre que la lanoline contenue dans les produits cosmétiques peuvent être une source exogène supplémentaire de COP. Nous montrons en outre qu’au moins une partie des COP sont déjà formés sur la laine des moutons.

Introduction
Cholesterol is widely known to be a constituent of the human diet which can be detrimental to health [1]. On the other hand, cholesterol is ever-present in mammalian cells and plays a vital part in the human body as a precursor of bile acids and steroid hormones [2-4]. Cholesterol oxides, also known as cholesterol oxidation products (COPs), are a group of more than 30 known congeners [5]. They are formed when cholesterol undergoes a heat or light induced radical reaction with oxygen, or an enzymatic oxidation [4-6]. Whereas some hydroxylated congeners might also be of physiological relevance, COPs as a class have more often been reported to exhibit adverse biological activities, namely inhibition of cholesterol synthesis, alteration of membrane function and cytotoxicity [3,4,6,7]. They are thought to be involved in diseases including diabetes, atherosclerosis, cancer, neurodegenerative processes and kidney failure [1,4].

Hence, from the early 1980s onwards, COPs have been analysed in a wide variety of cholesterol-containing food, such as meat,
sausages, eggs and dairy products [5]. Concentration levels of COPs were shown to be influenced by food processing and storage conditions, as well as by constituents of the food matrix: cholesterol autoxidation is minimised by low transition metal contents, modest cholesterol levels and the presence of highly saturated fats [6]. Eight congeners: cholesterol 5α,6α-epoxide (5α,6α-CE), cholesterol 5β,6β-epoxide (5β,6β-CE), 5α-cholestan-3β,5α,6β-triol (CT), 7α-ketocholesterol (7α-KC), 20α-hydroxycholesterol (20α-HC), 7α-hydroxycholesterol (7α-HC), 7β-hydroxycholesterol (7β-HC) and 25-hydroxycholesterol (25-HC) have been commonly reported. Five of these (5α-CE, 5β-CE, CT, 7-KC and 7β-HC) are formed only by radical reactions, whereas congeners with oxidised side chains are mainly produced enzymatically [4]. For 7α-HC and 25-HC, both pathways are possible [4]. In human biological samples, nine COPs were identified [4]. Besides seven of those mentioned above (7α-HC, 7β-HC, 5α-CE, 5β-CE, CT, 7-KC and 25-HC), two others, 24-hydroxycholesterol (24-HC) and 27-hydroxycholesterol (27-HC) were found [4]. In vivo transformation of cholesterol as an endogenous source is not thought to fully explain the presence of all COPs in human plasma and tissues [4]. The contribution of COPs by diet intake, however, remains unclear [4].

Determination of trace amounts of COPs in complex matrices such as food and human biological samples only became feasible with the widespread use of high-resolution gas chromatography (HRGC) in the 1980s. The described sample preparations basically consist of an extraction of lipids with an organic solvent, trans-terification or saponification, clean-up of extracts, enrichment of COPs and derivatisation. Detection of COPs was performed either with a flame ionization detector (FID) or with mass spectrometry (MS).

High-performance liquid chromatography (HPLC) coupled to a photodiode array detector has also been successfully used for the analysis of COPs. As the epoxides 5α-CE and 5β-CE do not absorb UV light, a refractive index detector was necessary [8].

To our knowledge, food is the only consumer product where the occurrence of COPs has been reported. Some cosmetics would also lend themselves to COPs screening, namely those with ingredients of animal origin. In this respect, the most widespread ingredient also having a high proportion of cholesterol is lanolin, a synonym for highly refined sheep wool fat [9]. Our first tentative screening of a few products very quickly showed that lanolin-containing cosmetics gave striking COP signals. Therefore, we focused on developing a validated method for the analysis of COPs, which we then used for a preliminary market survey.

Materials and Methods

Chemicals

7-KC, 7α-HC, 5α-CE, 5β-CE, CT, cholesterol, 5α-cholestan and 25-HC were purchased from Sigma-Aldrich (Buochs, Switzerland). 7α-HC was purchased from Chemos (Regensburg, Germany). Dichloromethane, tert-butyl methyl ether (MTBE; both analytical grade), acetonitrile, n-hexane, isopropanol (all GC grade), citric acid monohydrate and sodium methydsulphate (30% solution in methanol, for synthesis) were purchased from Merck (Darmstadt, Germany). Reagent for derivatisation: Sylon HPF (HMDS + TMCS + Pyridin; 3 : 1 : 9) was obtained from Sopelco (Glaral, Switzerland). Acetonitrile (LC/MS grade) was purchased from Biosolve (Valkenswaard, the Netherlands).

Solutions

All solutions were stored at room temperature (RT).

Stock solutions

Stock solutions in acetonitrile (1 mg mL⁻¹) were individually prepared for 7-KC, CT, 5α-CE, 5β-CE, CT, 7β-HC, 7α-HC, cholesterol, 25-HC and 5α-cholestan (IS).

Standard solutions

An internal standard solution (IS solution) was prepared by diluting the IS stock solution with n-hexane to a concentration of 25 μg mL⁻¹.

A standard COPs mixture containing six COP congeners at a concentration of 100 μg mL⁻¹ for 7-KC, 5α-CE, 5β-CE and CT plus 30 μg mL⁻¹ for 7α-HC and 7β-HC was prepared by mixing aliquots of each stock solution and diluting with n-hexane.

A cholesterol standard solution was obtained by evaporating 100 μl of the cholesterol stock solution under a stream of nitrogen and redissolving the residue in 1000 μl of internal standard solution resulting in a cholesterol concentration of 100 μg mL⁻¹.

Standard solutions for calibration

Standard solutions for calibration were obtained by diluting the standard COPs mixture with n-hexane. Concentrations were between 50 and 3 μg mL⁻¹ (7-KC, 5α-CE, 5β-CE, CT), respectively, 15 and 1 μg mL⁻¹ (7α-HC, 7β-HC) with five calibration points. Five hundred microliters of each calibration solution was evaporated under a nitrogen stream, derivatized and diluted in 500 μl of IS solution. For GC-MS, levels of calibration solutions were five times lower and each derivatized solution was twice washed with 2 ml of water before injection.

Solutions for calibrating cholesterol were obtained by diluting the cholesterol standard solution with the IS solution resulting in concentrations from 100 to 3 μg mL⁻¹ with six calibration points.

Quality control standards

Quality control standards (QC) were obtained by diluting the standard COPs mixture with n-hexane. COP concentrations were between 7.5 and 25 μg mL⁻¹ for GC-FID and 3 and 10 μg mL⁻¹ for GC-MS. After derivatization, the mixture was stable for 6 months (GC-FID), respectively, 1 week (GC-MS) when stored at RT.

Samples

In 2014, 30 samples of lanolin-containing lip-care sticks (28), fatty creams (4) and niple ointments (6) were taken from the Swiss and German market. The focus was on products with labelled declarations indicating lanolin to be a major constituent.

Storage effects on COP levels were studied by comparing one consent sample from the market survey with a 7- and an 8-year-old sample from the same brand.

For COPs screening, 47 lanolin-free lip-care sticks were also taken from the market.

For clarifying the origin of COPs, five sheep wool samples were obtained from hobby breeders.

Procedure

Sample preparation was based on a method published for the determination of phytosterol oxidation products (POPs) in food using...
the following modifications which were either crucial for analysing cosmetics or which further improved method performance [10]. In brief, the method consists of four steps: transesterification, solid-phase extraction clean-up (SPE), derivatization and separation/ quantification with GC. The major modifications consisted of spiking with 25-HC as a work-up control before transesterification, substitution of chloroform with dichloromethane and of water with saturated sodium chloride solution, adding an additional filtration step at the end of this step, as well as using a higher column capacity for the SPE clean-up and washing derivatized solutions with water prior to GC-MS.

Extraction of fat from sheep wool

Sheep wool was weighed into a 100-ml pear shaped flask. Fat was then extracted with n-hexane/isopropanol (3:2; v:v) for 30 min at RT using a ratio of 1:20 (w:v) wool to solvent. The solvent was evaporated with a rotary evaporator.

Sample preparation and transesterification

Samples of 30 mg (cottons, wool fat) or 60 mg (lip-care products, fatty creams) were weighed into a 15-ml screw-capped glass centrifuge tube. Fifty microlitres of the 25-HC stock solution were added together with 2 ml of a mixture containing 10% sodium methylate in methanol and MBTH (4:1; v:v). The tube was vortexed (10 s), followed by extraction under sonication in an ultrasonic bath at 40°C for 30 min. The mixture was then left to stand at RT for 30 min. Two millilitres of a saturated sodium chloride solution and 4.75 ml of dichloromethane were added, then vortexed (20 s), shaken (30 s) and centrifuged for 10 min (872 g). The upper phase was removed and discarded with a Pasteur pipette; then, 2 ml of 1% of citric acid in deionized water were added. The mixture was vortexed (20 s) and centrifuged for 5 min (872 g). After discarding the aqueous phase, the remaining dichloromethane phase was filtered through a microfilter with a 0.45 μm polytetrafluoroethylene (PTFE) membrane.

Determination of cholesterol

Five hundred microlitres of the dichloromethane extract were evaporated to dryness under a stream of nitrogen and redissolved in 1000 μl of the IS solution. This solution was used for analysis with GC-FID or GC-MS.

Verification of 7-KC levels using liquid chromatography (LC) – double array detection (DAD) – tandem mass spectrometry (MS/MS) for method validation

One hundred microlitres of the dichloromethane extract were mixed with 900 μl acetonitrile and used for analysis with LC-DAD-MS/MS.

Solid-phase extraction clean-up (SPE)

An amine-modified silica cartridge filled with 1000 mg of filling material, with a capacity of 6 ml (Machery Nagel, Düren, Germany) was conditioned with 5 ml of n-hexane. Six hundred microlitres of the dichloromethane extract were loaded onto the cartridge. Washing was first carried out with 5 ml of n-hexane followed by 20 ml of n-hexane/MBTH (1:1; v:v) at approximately 2 ml min⁻¹ (not to run dry during these steps). The retained COFs were eluted with 5 ml of acetone and collected in a 5-ml glass tube.

Derivatization

The SPE eluate was narrowed down to dryness at RT in a nitrogen evaporator (Vapotherm Basis Mobil I, Berley, Germany). After raising the evaporator temperature to 65°C, 100 μl of the silylation reagent were added to the residue and left to react for 75 min. The reagent surplus was removed by narrowing down again (65°C). The residue was then dissolved in 1000 μl of IS solution and ready for injection into the GC-FID system. In the case of GC-MS, 2 ml of deionized water were added. The mixture was shaken, vortexed, and the aqueous phase was discarded. After repeating the process, the organic solution was ready for injection.

Gas chromatography (GC)-mass spectrometry (MS)

Compound separation and detection were performed with a Thermo Finnigan Trace GC (Thermo Fisher, Bremen, Schiller, Switzerland) coupled to a Polarrq MS (Thermo Fisher). Two capillary columns, a DB-17 ms 10 m × 0.25 mm I.D., 0.25 μm film (J&W, 123-196222, Agilent Technologies, Palo Alto, CA, U.S.A.) and a DB-5 ms fused silica column 30 m × 0.25 mm I.D., 0.25 μm film (J&W, 123-196222, Agilent Technologies) were switched in series with a P511 autosampler (CTC Analytics, Züringen, Switzerland) into a split injector set at 230°C and with a split ratio of 10:1. Helium was used as a carrier gas at a constant pressure of 140 kPa. Initial oven temperature was set at 60°C for 1 min, then raised to 280°C at a rate of 25°C min⁻¹ and finally raised to 320°C at 2°C min⁻¹ and then held constant for 1 min. Full-scan mass spectra (m/z 50-650) were recorded with a 15-min delay using electron impact ionization (EI) at 70 eV (trap offset 10, GC/AC target 50, high mass adjust 50%, wave form off). The ion source temperature was at 220°C, and the transfer line temperature was at 280°C. Processing and interpretation of mass spectra were carried out with Xcalibur V.1.4 (Thermo Fisher). Quantification was performed using the base peak signals of the full scan spectra (Table I).

Gas chromatography (GC)-flame ionization detection (FID)

Compound separation and detection were carried out with a GC8000 (Carlo Erba, Austin, TX, U.S.A.) and a FID. The same column combination was used as mentioned for GC-MS. Injections of 2 μl were performed with an A200S autosampler (Perkin Elmer Instruments, Bremen, Schiller, Switzerland) into a split injector set at 230°C and with a split ratio of 10:1 (top flow 1 ml min⁻¹, bottom flow 10 ml min⁻¹). Helium was used as a carrier gas at a constant pressure of 140 kPa. Initial oven temperature was at 220°C for 3 min, then raised to 280°C at a rate of 25°C min⁻¹ and finally raised to 120°C at 2°C min⁻¹ and held constant for 1 min.

Table I Base peaks of cholesterol oxidation product TMS-ethers and of cholesterol

<table>
<thead>
<tr>
<th>Referring congener</th>
<th>Base peaks (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-KC</td>
<td>456</td>
</tr>
<tr>
<td>7-HE</td>
<td>458</td>
</tr>
<tr>
<td>7-CE</td>
<td>384.474</td>
</tr>
<tr>
<td>7-CE</td>
<td>384.474</td>
</tr>
<tr>
<td>7-CT</td>
<td>403.531</td>
</tr>
<tr>
<td>7-KC</td>
<td>472.367</td>
</tr>
<tr>
<td>7-CHO</td>
<td>271.377</td>
</tr>
<tr>
<td>7-CHO</td>
<td>131.456</td>
</tr>
<tr>
<td>7-CHO</td>
<td>388.301</td>
</tr>
</tbody>
</table>

*Unders siliconized.
5 min. The detector temperature was set at 320°C. Processing of data was carried out with chromcard for USB (Thermo Fisher).

Liquid chromatography–flame ionization detection (LC-FID)–MS/MS

The LC-DAD-MS/MS system consisted of a Rheos UHPLC Allegro pump with a vacuum solvent degasser unit, a column oven, an autosampler, a PDA detector (Thermo Fisher) and a TSQ Quantum Access Triple Quad equipped with an atmospheric pressure chemical ionization (APCI) interface (Thermo Fisher). Processing of data was carried out with Xcalibur LC Devices 2.2.0. (Thermo Fisher).

The analytical column was a Nucleodur Sphera 1.8 mm, 100 × 3 mm (Macherey Nagel, Düren, Germany) set at 40°C. Mobile phase A was water, B was methanol, and C was acetonitrile. The flow rate was 500 μL min⁻¹ and gradient elution started with 10% of A, 45% of B and 45% of C (0–8 min) and then reset to the initial composition for 3.40 min (8.20–12 min). Absorption spectra were taken between 290 and 400 nm. The mass spectrometer had the following settings: collision gas pressure 1 bar, auxiliary gas 5 arbitrary units and sheath gas 40 arbitrary units. Scan event 1 was recorded in full-scan mode from m/z 100–500 (scan time 0.33 s, 1 microscan, skimmer offset 10). Scan event 2 was recorded in selected reaction monitoring with the parent ion at m/z 401.3 and the centromion at m/z 383.2 (width 0.4, time 0.05 min).

Results and discussion

Method description

Screening of lanolin-containing samples with GC-MS revealed six prominent COPs (7a-HC, 7α-HC, CT, β-CE, 7-α-CE and 7-KC), which were also the most often reported congeners in food samples and are known to be formed by autoxidation reactions involving radicals. GC-FID and GC-MS were both suitable for determining COPs. Due to the high levels of target compounds found and due to the robustness of the method, GC-FID proved to be suitable for most samples. GC-MS was used for those few samples, where COP levels were low or matrix-dependent interferences occurred. Washing of derivatized solutions was mandatory for subsequent GC-MS determination. All target compounds were quantified with 5α-Cholestane as an internal standard.

Sample work-up was checked using 25-HC, being a congener not found in lanolin and lanolin-containing products. Spiking 25-HC at the beginning of preparation allowed for the supervision of the whole process. Results of sample runs were assessed to be reliable when the ratio of 25-HC to 5α-cholestane (converted into the same amount in the measurement solution) were between 0.8 and 1.1.

Besides using a standard to check the sample work-up, the most important adaptations of our method to previously published studies from the food sector were as follows: using a SPE cartridge filled with twice the amount of filling material which resulted in substantial improvements (~50%) of the recoveries of the less polar epoxides, mandatory filtration of dichloromethane extracts to prevent loss of 25-HC and mandatory washing of the derivatized solutions before analysing with GC-MS.

Peak assignment in sample extracts was performed by comparing mass spectra and retention times with those of standards. Figure 1 shows a GC-FID and a GC-MS chromatogram of a COPs standard mixture.

Method validation

Quality assurance was based on a validation of the GC-FID method and for everyday work by analyzing a control standard run in each sample series. A deviation level of 20% of the quality control standard was accepted before recalibration was needed.

Remaining components of the matrix can potentially interfere with the target compounds. Selectivity was therefore checked by comparing quantification data obtained by GC-FID with those of GC-MS using base peak signals. In addition, full-scan spectra derived from sample extracts were scrutinized for relevant interferences. No problems occurred showing that selectivity was very good.

Accuracy tests were based on four aspects: (1) Verification of determined levels of the main congener (7-KC) by LC-DAD-MS/MS, (2) Verification of total COP levels of an ointment and a lipstick sample by an external laboratory, (3) Determination of recoveries for all six COPs by spiking and (4) Isolation of formation of COPs during analysis by performing COPs determination (a) on blank samples spiked with cholesterol and (b) on COP-positive samples under an oxygen-free atmosphere (argon).

Verifications showed that data for 7-KC obtained by LC-DAD-MS/MS was in very good agreement (Δ < 5%) with those of GC-FID. In addition, total COP levels determined by the external laboratory were within measurement uncertainty of our data and therefore in good agreement (deviation of total COP levels: 1% for the lipstick and 17% for the ointment sample). Recovery rates for the six congeners were established both on six lip-care samples and on an ointment sample, the lip-care products being free of COPs and the ointment containing COP levels in the per cent range. Spiking levels for the lip-care products were chosen to be slightly above the limit of quantification.

Recoveries for all congeners were sufficient (between 73% and 111%, Fig. 2) on the low and the high end of the concentration range. Tests regarding artificial formation of COPs were negative as no COPs were found in the cholesterol spiked samples and concentration levels of COPs using the inert gas atmosphere did not differ from those with air contact.

The limits of quantification (LOQ) were adjusted to the expected COP level ranges by varying the size of sample aliquots being 60 mg for lip-care products and fatty creams, respectively 30 mg for ointments and raw wool fat samples. The lowest concentration for calibration therefore gave LOQs of 500 mg kg⁻¹ (7-KC, β-CE, 7-α-CE, CT) and 150 mg kg⁻¹ (7α-HC, 7β-HC) for the cosmetic products, respectively 1000 and 300 mg kg⁻¹ for wool fat samples and for ointments. In rare cases, where COPs were present below LOQ levels, sample preparation was repeated using lower dilution factors.

For all COP congeners, coefficients of determination (r²) were excellent over the whole measurement range (r² > 0.99; Table II). For cholesterol which was not derivatized, correlation was still on an acceptable level (r² > 0.97).

Potential variation of matrix effects due to differing sample compositions was compensated using an internal standard for quantification. In addition, the GC-FID system proved to be rugged showing no change in performance after 400 sample injections. For the GC-MS system, however, being more prone to drifts in sensitivity, a system control standard consisting of low amounts of cholesterol and BS (both 0.5 mg) was applied as a precautionary measure. The influence of small variations in procedure execution was checked by having the same sample analysed by two persons.
independently. Deviation of results for all six congeners, being lower than 5%, was therefore irrelevant. In conclusion, our routine method can be classified as rugged.

Precision was determined as repeatability with six determinations of an ointment sample (total COP level 3%) and of a lipstick sample (total COP level 0.6%). The congener-specific precisions, expressed as relative standard deviations, were between 2.3% and 8.8% for the ointment and 5.0% and 13.0% for the lipstick sample.

Estimation of uncertainty was based on Eurochec/ICTAC Guide CG4 [11]. The expanded uncertainty with a level of confidence of 95% was between 25% and 27% for all six congeners, the main source of uncertainty being the accepted deviation level of 20% from the quality control standard.

COP levels in lanolin containing products
Another aim of this study was to determine the COP levels in lanolin-containing products currently sold on the market. For this

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**Table II** Linearity range (μg mL⁻¹) and coefficient of determination

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Linearity range (μg mL⁻¹)</th>
<th>Coefficient of determination (r²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7α-HC</td>
<td>1.15</td>
<td>0.999</td>
</tr>
<tr>
<td>7β-HC</td>
<td>1.15</td>
<td>0.999</td>
</tr>
<tr>
<td>α-CBE</td>
<td>4.50</td>
<td>0.998</td>
</tr>
<tr>
<td>β-CBE</td>
<td>4.64</td>
<td>0.999</td>
</tr>
<tr>
<td>CT</td>
<td>3.50</td>
<td>0.999</td>
</tr>
<tr>
<td>7-KC</td>
<td>3.50</td>
<td>0.999</td>
</tr>
<tr>
<td>cholesterol</td>
<td>3.100</td>
<td>0.971</td>
</tr>
</tbody>
</table>

---

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*International Journal of Cosmetic Science, 1–7*
purpose. 24 lanolin-containing cosmetics (lip-care products and creams) and six nipple ointments were taken from the Swiss and German market for COPs determination.

Total concentrations of COPs (Table III) depended on the product category. Lip-care products and creams were both between 0.2 and 0.9 g 100 g\(^{-1}\). Nipple ointments, however, showed higher levels, ranging from 3 to 3 g 100 g\(^{-1}\). This was due to lanolin being the only ingredient for all ointments except for one.

Congenomer levels profiles were dominated by 7-KC for all samples. This congenomer mostly contributed about 50% to the total COP levels. The portions of all the other congenomers varied depending on the matrix. Nevertheless, certain patterns were discernible: 7a-HC and 7b-HC proportions were congruent. In most cases, b-CE levels were at least twice as high as those of a-CE.

To find out whether the occurrence of COPs was only due to the ingredient lanolin, 47 lanolin-free lip-care products were screened for COPs and their precursor cholesterol. Neither COPs nor cholesterol were detected in any sample. As to our knowledge, cholesterol is not currently used as an ingredient in cosmetics, the occurrence of COPs can probably be solely attributed to lanolin.

As the endogenous formation of COPs does not fully explain the presence of COPs in human blood and tissues, previously published studies hinted at the contribution of diet as an exogenous absorption source for COPs [2]. The most relevant congenomers reported for food were the same six compounds found in our study with 7-KC also showing the highest concentrations. Published data for cholesterol containing food show that the vast majority of total COP levels was between 0.1 and 10 mg kg\(^{-1}\) [2]. In comparison, our study revealed concentrations being several orders of magnitude higher: for lip-care products and fatty creams 10\(^2\)-10\(^4\) times and for ointments 10\(^3\)-10\(^4\) times higher. Therefore, our study shows for the first time, that cosmetics, namely lanolin-containing products, are a possible exogenous source of COPs.

On the current market, various qualities of lanolin are offered, differing in their purity degree [9]. Some grades have to be conform to the specifications mentioned in either the US and/or European Pharmacopoeia. Average total COP content of such respectively claimed samples (4 of 6 ointments studied) was 2.7 g 100 g\(^{-1}\). An additional lanolin sample labelled as ‘100% pure lanolin’ only had a third of the average. This demonstrates that it is technically feasible to produce lanolin with only a fraction of the current average COP content.

Origin of COPs in Lanolin

As a commodity lanolin is exceptional, being the only commercially used secretion of sebaceous glands and showing outstandingly high cholesterol levels. How and when do COPs get into lanolin? Manufacturing can be one reason as cleaning of the sheep wool fat often includes chemical bleaching. A prolonged storage under air contact is another possibility. Exposure of sheep wool fat to sunlight and air on the animal will also facilitate photodegradation of cholesterol. This in situ formation of COPs on sheep proved to be a major cause, as fat samples extracted from five raw wool samples of different origin had total COP levels between 0.4 and 1.9 g 100 g\(^{-1}\). Variations can probably be explained by differing exposure conditions to sunlight.

In comparison with the wool fat samples, the ointments consisting of 100% lanolin had higher COP levels (up to 3 g 100 g\(^{-1}\)). This gives evidence that COPs are additionally formed during the refining process.

In addition, a prolonged storage may also elevate concentrations. Determination of COPs in a 7- and an 8-year-old sample of an ointment showed twice, respectively, thrice as high concentrations compared to a sample of the same product stemming from our current market survey (Table IV). Cholesterol levels on the other hand were inversely proportional (two to three times lower) in the old samples due to the continued degradation of cholesterol during storage.

**Table IV** Cholesterol oxidation product (COP) and cholesterol levels (g 100 g\(^{-1}\)) in two old samples and a freshly bought one

<table>
<thead>
<tr>
<th>Sample age (years)</th>
<th>7a-HC</th>
<th>7b-HC</th>
<th>a-CE</th>
<th>b-CE</th>
<th>CT</th>
<th>7-KC</th>
<th>Total COPs</th>
<th>cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.23</td>
<td>0.24</td>
<td>0.18</td>
<td>0.35</td>
<td>0.17</td>
<td>1.50</td>
<td>2.67</td>
<td>13.0</td>
</tr>
<tr>
<td>7</td>
<td>0.97</td>
<td>0.89</td>
<td>0.55</td>
<td>1.08</td>
<td>0.51</td>
<td>4.30</td>
<td>8.20</td>
<td>4.90</td>
</tr>
<tr>
<td>8</td>
<td>0.72</td>
<td>0.73</td>
<td>0.52</td>
<td>0.90</td>
<td>0.42</td>
<td>2.59</td>
<td>5.98</td>
<td>7.24</td>
</tr>
</tbody>
</table>

**Conclusion**

To our knowledge, no study has ever been reported on levels of COPs in non-food products. Method validation and the application of the method for a market survey demonstrated that the method described is fit for purpose. The levels we found in lanool.
lin-containing cosmetics and ointments dwarf those reported in food. Analysed sheep wool fat samples demonstrated that a substantial portion of COPs are already formed on the animal, presumably by solar irradiation. Data obtained from the market survey reveal lanolin as a possible exogenous source of COPs. As lanolin is an essential constituent of cosmetics and ointments and can be produced with only a fraction of the current COPs, replacement of current grades with higher products could be taken into account.

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References
2. Oxysterols in cosmetics-Determination by planar solid phase extraction and gas chromatography-mass spectrometry

Sonja Schrack, Christopher Hohl and Wolfgang Schwack (2016):
Oxysterols in cosmetics-Determination by planar solid phase extraction and gas chromatography-mass spectrometry
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Oxysterols in cosmetics—Determination by planar solid phase extraction and gas chromatography–mass spectrometry

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A B S T R A C T
Sterol oxidation products (SOPs) are linked to several toxicological effects. Therefore, investigation of potential dietary uptake sources particularly food of animal origin has been a key issue for these compounds. For the simultaneous determination of oxysterol from cholesterol, phytosterols, dihydrocholesterol 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% (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), dihydrolanosterol 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 dihydrolanosterol are components of sheep wool fat and its refined product lanolin. Phytosterols are present in plant oils, with β-sitosterol, campesterol and stigmasterol 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 cholesteryl 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 dihydrolanosterol (~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 dihydrolanosterol 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 transesterified 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 dihydroxysterol 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), cholesteryl-5β,6β epoxy (5β,6β-CE), cholesterol-5α,6α epoxy (5α,6α-CE), cholestane-3β,5α,6α-triol (CT), 5α-cholane, 7α-sitosterol (> 70%), 25-hydroxycholesterol (25-HC), CI. 11005, 4-[4-nitrobenzyl]pyridine (NBP), and polytetrafluoroethylene (PTFE) membrane filters (0.2 and 0.45 μm) were purchased from Sigma-Aldrich (Buchs, Switzerland); 7α-hydroxycholesterol 7α-HC from Chemos (Regensburg, Germany). Dichloromethane, diethyl ether (both analytical grade), hexane, acetone, methanol (all GC grade), sodium methylate (30% solution in methanol, for synthesis), tetrathylpentenamine and citric acid monohydrate were from Merck (Darmstadt, Germany) and Sylona HIT (1,1,1,3,3,3-hexamethyldisilazane: trimethylchlorosilane: pyridine; v:v:v; 3:1:1) from Supelco (Gland, Switzerland). All TLC and HPTLC plates were from Merck and obtained from VWR (Dietikon, Switzerland). They were prevashed 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, Brechbühl, Schlieren, Switzerland) 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 presplit. A diphenylteatriemethylsiloxane (DPTMS) deactivated precolumn and postcolumn (both 1 m × 0.25 mm ID) were used. Injections were performed with a PALS autosampler (CTC analytics, Zwingen, Switzerland) into a programmable temperature vaporizer (PTV) injector used in the PTV large volume mode and equipped with a silcosteel 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, 1400 kP 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, 1400 kP) 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 1400 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 30%, waveform 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-Cholane) 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 HITS-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 dihydroxysterol

Lanosterol and dihydroxysterol were isolated from a transetherified lanolin solution (2.7.2) by preparative liquid chromatography on a Spectrasytem using UV9000, AS3000, D4000 (Thermo Scientific, Reinach, Switzerland) coupled to a fraction collector from Gilson (Mettmenstetten, Switzerland). The separation was performed on a Lichrosorb-100 RP18 column (5 μm, 250 mm × 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 dihydroxysterol 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 found to be >88%.

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

Oxidised phytosterol and dihydroxysterol congeners were obtained by thermooxidation. Solutions (10 mg in 10 mL acetone) of sitosterol and dihydroxysterol, 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 ultrasound. The DOPs and DOX 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 (CaOxFs).
### 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-hydroxyisopistosterol</td>
<td>7α-HIS</td>
<td>20.47</td>
<td>484</td>
<td>0.15</td>
<td>POPs</td>
</tr>
<tr>
<td>7β-hydroxyisopistosterol</td>
<td>7β-HIS</td>
<td>22.03</td>
<td>484</td>
<td>0.45</td>
<td>POPs</td>
</tr>
<tr>
<td>sitosterol-3α,6α-epoxide</td>
<td>α-SIE</td>
<td>23.78</td>
<td>412, 502, 394</td>
<td>1.15</td>
<td>POPs</td>
</tr>
<tr>
<td>sitosterol-5β,6α-epoxide</td>
<td>β-SIE</td>
<td>23.46</td>
<td>412, 502, 394</td>
<td>0.73</td>
<td>POPs</td>
</tr>
<tr>
<td>7α-hydroxychampsterol</td>
<td>7α-HCa</td>
<td>19.70</td>
<td>470</td>
<td>0.017</td>
<td>POPs</td>
</tr>
<tr>
<td>7β-hydroxychampsterol</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, 404, 559, 474</td>
<td>0.63</td>
<td>POPs</td>
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<tr>
<td>7α-hydroxycholesterol</td>
<td>7α-CH</td>
<td>25.08</td>
<td>466, 381, 306, 460</td>
<td>0.65</td>
<td>POPs</td>
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<tr>
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<td>5.5</td>
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<td>10</td>
<td>POPs</td>
</tr>
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<td>α-CE</td>
<td>21.22</td>
<td>384, 474, 366</td>
<td>10</td>
<td>POPs</td>
</tr>
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<td>cholesterol-5β,6α-epoxide</td>
<td>β-CE</td>
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<td>403, 456, 531, 546</td>
<td>10</td>
<td>POPs</td>
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<tr>
<td>cholesterol</td>
<td>CT</td>
<td>21.62</td>
<td>472, 367, 382, 455</td>
<td>10</td>
<td>POPs</td>
</tr>
<tr>
<td>7α-tocopherol</td>
<td>7α-TC</td>
<td>23.31</td>
<td>602, 376, 312</td>
<td>0.72</td>
<td>DOPO</td>
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<td>7β-tocopherol</td>
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<td>23.74</td>
<td>602, 587, 559</td>
<td>0.64</td>
<td>DOPO</td>
</tr>
<tr>
<td>11-keto-3,7-dihydroxy-cholesterol</td>
<td>11-KDD</td>
<td>24.26</td>
<td>528, 472, 429</td>
<td>2.3</td>
<td>DOPO</td>
</tr>
<tr>
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<td>7α-TD</td>
<td>25.25</td>
<td>514, 499</td>
<td>1.8</td>
<td>DOPO</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 DOPOs 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-cholane 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 μg 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 (DOPOs)

The identification of DOPOs formed during thermooxidation of dihydrolanosterol was performed with high-performance thin-layer chromatography (HPTLC) as follows: 200 μL were applied (spray application) on two Lichrotherm 60 F254 10 cm x 10 cm (200 μm layer thickness) plates with an automatic TLC sampler A54 (CAMAG, Muttenz, Switzerland). The band length was 6 mm and we used a distance of 12.0 mm from the lower and the left plate edge. DOPOs were separated using two-dimensional development in a 10 cm x 10 cm parallel developing chamber (CAMAG) in 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, 120°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 DOPOs was performed for 5 min with 1 mL of acetone in an ultrasonic bath. After filtration through a polytetrafluoroethylene (PTFE) filter tip, each DOPO 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 DOPOs 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 Masslab 5.2 (MSP Kofel, Zollikofen, Switzerland) as a library with reference El 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 8 ointments) 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 C1,11005 marker solution (1 µg·mL⁻¹ in acetone). 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, 927g), the aqueous phase was removed with a pipette (PF). 1.2 ml of a 10% sodium methyleate 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 (927g). 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 filter 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) on 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 preadsorption volume, 200 mL retraction volume, 890 mL s⁻¹ dosage speed, 2 s filling 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 µL 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 (C1, 11005) to focus oxyesters 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 (Barley, 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 filter 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 basically consisted 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 SO₅'s 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 (2.5.3). 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 (br, 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, focusing 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 during extraction, we used 25-HC as a recovery standard to adjust results (2.3.3).

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 overloaded 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 esters are main components of the ingredient lanolin [27]. As neither data on mass spectra of congeners nor commercial standards were available, we applied thermooxidation 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 C24/C25 [29,30] (Fig. 3). Consequently, the monoisotopic masses differ by two amu. Oxidation could theoretically occur on both double bonds (C25/C26 and C24/C25) of lanosterol. However, full scan spectra of derivatised LOPs and DOPs congeners were nearly identical differing only by a m/z of 2 (Fig. 3). This gave evidence that in both educts only the C24/C25 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 oysterol 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 NBF 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 DOP congeners, LC-HRMS enabled the determination of the exact monoisotopic mass with a Δ±2 ppm (Table 2). The isotopic patterns of C7 and C11 showed that all congeners consisted of about 30 carbon atoms. The molecular formula was calculated using the elements C, O, N, H and Cl (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 C7 stemming from the exocyclic 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 derivatised 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-ketohydrolanosterol (7-KD) and consequently allocated the corresponding LOPs congener to 7-ketolanosterol (7-KD).
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 reflection and transmission (RT).

Fig. 3. Structure formulae and mass signals (m/z) of the derivatised (values in bold) and the underderivatised 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_{3}H_{5}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_{3}H_{5}O_{3}</td>
<td>456</td>
<td>528</td>
<td>1</td>
</tr>
<tr>
<td>7-KDD</td>
<td>458,400</td>
<td>C_{3}H_{5}O_{3}</td>
<td>458</td>
<td>602</td>
<td>2</td>
</tr>
<tr>
<td>7,11-KDD</td>
<td>458,359</td>
<td>C_{3}H_{5}O_{3}</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_{3} and on the C_{11} carbon atom. Theory was supported by the comparison of the underderivatised 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 (underderivatised) and 500 (acylated) for the molecular radical ion (M^{+}) and at m/z 441 (underderivatised) and
485 (acylated) for the fragment [M-15]⁺. A further characteristic fragment was at m/z 428 (derivatised) and 472 (acylated) for [M-28]⁺ (Fig. 3). We therefore identified the DOPs congener to be 7,11-diketodihydrolanosterol (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 C7. Therefore, according to theory, C7 and C11 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 α/β 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-dihydrolanosterol (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 derivatised form with a library spectrum of 7-KDD which was α-deuterated at C7 and β-acylated on C5. The two main peaks of the derivatised 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 the 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-dihydrolanosterol and the analogous LOPs congener to 7-keto-3,11-dihydroxylanosterol (7,11-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-hydroxysterigmatosterol (7α-HS7), 7β-hydroxysterigmatosterol (7β-HS7), 7,11-diketolanosterol (7,11-KL), 7-ketolanosterol (7-KL) were determined with the help of calibration curves of the corresponding sterol (7α-HS7, 7β-HS7) or dihydrolanosterol (7,11-KD, 7-KD) congeners.

3.4.1. Selectivity

Thermooxidation of dihydrolanosterol, stiosterol, 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, 5α-epoxy, 5β-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 stiosterol 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 stiosteranol (ST) 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 quantitation (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 5α-/5β-epoxides having the flattest slope (Table 1).

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). LODs 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, CT, 7-KC, 7,11-KD, 7-KD) determined by the validated SPE-GC-MS 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 COPs, 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 stiosterol per sample weight were analysed. No oxidised sterols were found proving that sample handling was fit for purpose.
Table 3
Calibration and performance data for validated steroid oxidation products (SOPs).

<table>
<thead>
<tr>
<th>Substance</th>
<th>Linearity range (ng µL⁻¹)</th>
<th>Intercept ± SD ¹</th>
<th>Slope ± SD ²</th>
<th>Coefficient of determination (r²)</th>
<th>LOQ (mg kg⁻¹)</th>
<th>LOD (ng kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7a-HC</td>
<td>0.002-0.08</td>
<td>±0.005 ±0.001</td>
<td>44 ±2</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 ±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>7a-HS</td>
<td>0.005-0.2</td>
<td>±0.001 ±0.003</td>
<td>22 ±0.1</td>
<td>0.9990</td>
<td>0.05</td>
<td>0.003</td>
</tr>
<tr>
<td>7β-HS</td>
<td>0.01-0.5</td>
<td>±0.1 ±0.01</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>7a-HCa</td>
<td>0.003-0.02</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>0.000-0.7</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>ST</td>
<td>0.02-0.6</td>
<td>±0.05 ±0.007</td>
<td>3 ±0.02</td>
<td>0.9992</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 ±0.001</td>
<td>4 ±0.03</td>
<td>0.9991</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>7-KD</td>
<td>0.06-0.8</td>
<td>±0.1 ±0.006</td>
<td>95 ±6.0</td>
<td>0.9994</td>
<td>0.6</td>
<td>0.04</td>
</tr>
<tr>
<td>7-HSi</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>

¹ Substance abbreviations are explained in Table 1.
² SD1: standard deviation of the intercept.
³ SD2: standard deviation of the slope.

Fig. 4. Recovery results from a lip care (n=2), paraffin (n=2) and cream (n=2) sample of steroid oxidation products (SOPs) after p6F-P-GC-MS. Spiking levels were between 0.02 and 10 mg kg⁻¹.

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 phytosterols 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 [8]. Remarkably, DOPs levels were higher than LOPs levels, although lanosterol contents were twice the dihydrosterol contents. Our studies on thermooxidation, however, showed us that the generation of LOPs proved to be more difficult than SOPs from other steroids because of subse-
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 phytoester 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 oxysterols 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 LOLs and DOPs was helpful in a locating unknown peaks in lanolin containing samples. We first found these 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.

Acknowledgements

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References

3. Photooxidation of Octahydro Tetramethyl Naphthalenylethanone (OTNE) in Perfumes and Aftershaves

Sonja Schrack, Christopher Hohl and Wolfgang Schwack (2018):
Photooxidation of Octahydro Tetramethyl Naphthalenylethanone (OTNE) in Perfumes and Aftershaves

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Photooxidation of Octahydro Tetramethyl Naphthalenylethanone in Perfumes and Aftershaves

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ABSTRACT

For consumer safety reasons, cosmetics that are exposed to light are evaluated with respect to phototoxity and/or photoallergy (photosensitization). For perfumes/aftershaves, however, these tests are not performed with the cosmetic product but only with single fragrance compounds, which may influence the outcome. In the presence of a sensitizer, photoinduced oxidation of unsaturated fragrances might result in the formation of unwanted products. Therefore, using real samples, we studied the photo degradation of the common fragrance octahydro tetramethyl naphthalenylethanone (OTNE) under in vitro irradiation, during indoor storage, and after application on skin. OTNE and its photoproducts were determined by liquid chromatography (LC) coupled to diode array detection (DAD). Whereas OTNE itself was photostable, irradiation in the presence of a sensitizer and of after-shave/perfume samples resulted in a strong degradation. Photodestruction was identified as the major degradation reaction for all three trials. OTNE photodegradation products were characterized by LC-high-resolution mass spectrometry (LC-HRMS) and by high-performance thin-layer chromatography (HPTLC) after derivatization with titanium ethoxide and nitrobenzyl pyridine. Both HPTLC and HRMS data indicate that OTNE hydroperoxides are formed during irradiation.

INTRODUCTION

Fragrance compounds are key ingredients of cosmetic perfume and aftershave formulations. These compounds are usually combined to create a characteristic scent (1). As some fragrance molecules have a significant absorption band in the ultraviolet range (UV) (290–400 nm), sunlight-induced reactions may occur during storage, as well as after application on the skin. The resulting photoproducts can have phototoxic and/or photoallergic properties (2,3), which is why photosensitization is of special concern for the safety assessment of fragrances (2). Evaluation is mainly based on the intensity of the absorption spectrum and a specific photopatch test of the fragrance, as well as on test data from related compounds (2). Photoreactions are generally only studied on individual fragrances and not on mixtures as represented by a perfume or an aftershave, therefore limiting their practical value for the safety evaluation. The photodegradation of a compound with an absorption band of minor intensity cannot be excluded, as other accompanying compounds may enhance such reactions. For example, sunlight exposure is known to oxidize terpenes in essential oils (4), even without them having any significant absorption in the range of solar radiation, and is the main cause of the oils’ spoilage (4). At least some of the oxidized terpenoids are known skin sensitizers underlining the necessity of clarifying the relevance of fragrance oxidation in cosmetics exposed to sunlight (5). To our knowledge, only terpenes have received sufficient attention in this respect (6–8).

A preliminary screening of perfumes and aftershaves showed that the synthetic fragrance octahydro tetramethyl naphthalenylethanone (OTNE) is frequently used (in about 90% of our samples) in rather high concentrations. OTNE, a mixture of at least three isomers (Fig. 1), belongs to the alkyl cyclic ketone fragrance group, which generally is not considered to have any phototoxic or phototoxic potential (9). A high photodegradation rate of OTNE, however, has been reported in soil surface (10) and the atmosphere (11), probably due to photooxidation. We therefore studied OTNE photodegradation in perfumes and aftershaves, with the main focus set on screening for potentially unwanted products of photooxidation.

MATERIALS AND METHODS

Chemicals and materials. Benzoyl peroxide, dicumyl peroxide, bis[1-tert-butylperoxy)ethyl]benzene, titanium ethoxide, rose bengale (C1 45410), 4,4'-nitrobenzylpyridine, benzidine, acrylamide, alcohols, allyl glycidyl ether, 3-amino-9-ethyl-carbazole, 4-aminoazobenzene, 4-aminobiphenyl, α-naphtalene, benzo(a)anthracene, resorcinol, diglycidyl ether, 4-chloroazulene, epichlorohydrin, 4-chloro-2-methylaniline, benzyl chloride, 3,3-dichlorobenzidine, 3,3-dimethyldibenzidine, 3,3-dimethylbenzidine, 2,4-dinitrotoluene, glycidol, 1,2-epoxy-3-phenylpropene, safrole, nicotine and polyethylene glycol (PEG) membrane filters (0.45 µm) were purchased from Sigma-Aldrich (Buchs, Switzerland). Hydrogen peroxide (30% solution in water), tetrachloroethylene, benzene, Muant perfume test strips, α-hexane, acetone, methanol (all solvents GC grade) and high-performance thin-layer chromatography (HPTLC) plates silica gel F254 (20 cm × 10 cm), with a layer thickness of 0.2 mm and a concentration zone of 25 mm, were from Merck (Darmstadt, Germany). The HPTLC plates were prewashed with methanol and dried in an oven at 100°C for 10 min before use. Benzoyl peroxide (10 mg µL−1 in acetone/ethanol) and caprotil and 1,3-dichloro-2-propanol were purchased from VWR (Dietikon, Switzerland). Clear glass vials (7.5 mL) with screw caps made of polypropylene with polyethyleneterephthalate coating and 2 mL glass vials

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were from infactrum (Goldau, Switzerland). OTNE was obtained as a mixture of the isomers shown in Fig. 1 from Givrand SA (Vernier, Switzerland).

**Samples.** Three OTNE-containing perfume and nine OTNE-containing aftershave samples were purchased from the Swiss market.

**Reference solutions.** OTNE-sol containing 0.2 mg mL⁻¹ OTNE in ethanol/water (8:2, v:v), OTNE-RB and OTNE-MB contained 1 mg mL⁻¹ OTNE and 1 μg mL⁻¹ rose benzene (RB) or methylene blue (MB), respectively.

To study the mechanistic oxidation pathway, a solution containing 1 mg mL⁻¹ sodium azide in ethanol/water (8:2, v:v) was added to the solutions to be irradiated (perfume/aftershave sample or OTNE reference) up to a level of 10 μg mL⁻¹.

**In vitro irradiation.** One milliliter of the test solution was pipetted into a 7.5 mL glass vial. The closed vial was deposited horizontally in an Atlas (Mörfelden-Walldorf, Germany) CPS+ simulator test, equipped with a 1.5 kW xenon lamp and a daylight filter (wavelength range: 200-800 nm). As the simulator was ventilated during operation, the vial was fixed with an adhesive tape. The irradiation time was always 1 h, with an irradiation intensity of 750 W m⁻², delivering a total dose of 2700 kJ m⁻² (equivalent to 1.3 h midday summer sun in central Europe).

**Peroxide test.** Irradiated and nonirradiated OTNE reference solutions or samples were screened for peroxides by dipping the test strip for one second into the solution.

Liquid chromatography-diode array detection (LC-DAD). The LC-DAD system was a Dionex ultimate 3000 system comprising an RS pump, RS autosampler, a RS column compartment and a diode array detector (Thermo Scientific). The injection volume was 2 μL for OTNE reference solutions and 1 μL for the irradiated/nonirradiated sample solutions. UV/VIS spectra were scanned from 200 to 800 nm with a scan rate of 10 Hz. The determination of degradation rate of OTNE (200 nm) was performed on an Acquity Shield RP-18 column (50 mm x 3 mm; 1.7 μm) (Waters, Baden, Switzerland) using a gradient elution (A: aqueous buffer of sodium dihydrogen phosphate (1.2 g L⁻¹) adjusted to pH 2.7 with 1 N oxalic acid, B: methanol and C: acetonitrile). The flow rate was 1.2 mL min⁻¹, and gradient elution started with 85.5% A and 15% B (0-15 min), followed by three linear ramps to 45% A and 50% B (15-9 min), 28.5% A and 65% B (9-14 min), and finally to 1% A and 99% B (14-14.3 min) with a hold time of 1.7 min (14.3-16 min). The eluent was then reset to the initial composition for 0.2 min (16-16.2 min) followed by an equilibration time of 2.7 min.

The determination of the degradation products of OTNE (200 nm) was performed on a Kinexet C18 column (100 mm x 3 mm; 2.6 μm) (Waters, Baden, Switzerland) using a gradient elution (A: water, B: methanol). The flow rate was 1 mL min⁻¹ and gradient elution started with 60% B (0-1.5 min). The eluent was then steadily modified to 50% B (1.5-7 min) with a hold time of 1.7 min (7.0-8.7 min). The eluent was then reset to the initial composition within 0.2 min (8.7-8.9 min). Equilibration time before the next injection was 2.7 min.

**Liquid chromatography–high-resolution mass spectrometry (LC-HRMS).** The HRMS system consisted of a Q Exactive System equipped with an atmospheric pressure chemical ionization (APCI) source (Thermo Scientific, Reinach, Switzerland). The LC system was a Dionex ultimate 3000 system comprising of RS pump, RS autosampler and RS column compartment (Thermo Scientific). Separation was performed on a Nucleosil Sphera RP column (200 mm x 3 mm; 3 μm) (Macherey-Nagel, Düren, Germany) using acetonitrile in nanopure water and B: 0.1% formic acid in methanol at a flow rate of 0.6 mL min⁻¹, starting with 50% B (0-2 min), followed by a linear gradient to 100% B (2-9 min) and a hold time of 4.5 min (9-13.5 min). After the run, the eluent was reset to the initial composition for 0.5 min (13.5-14.0 min). Equilibration time was 3 min. Samples were diluted in ethanol/water (8:2, v:v) before injection (10-fold). Injection volume was 2 μL for the OTNE reference and the irradiated/nonirradiated sample solutions. Ionization was performed in the positive mode (capillary temperature 220°C, spray voltage 5 V, sheath gas flow 30 arbitrary units, auxiliary gas flow 20 arbitrary units), and full scan spectra were recorded in the m/z range of 50-350. The Orbitrap resolution was set to 70 000. MS data processing was performed with Xcalibur 2.4 (Thermo Scientific).

**High-performance thin-layer chromatography.** Sample application, plate development and scanning of plates before dipping were performed with two plates to be used for the two different derivatization procedures. Perfume/aftershave samples and OTNE reference solutions were applied with an automatic TLC sampler AT34 (CAMAG, Muttenz, Switzerland) with the following settings: spray band (4 mm), 22 tracks (10 mm distance from the lower edge, 14 mm distance from the left edge, and 4 mm track distance). Dosage speed was set to 800 nL s⁻¹, and nozzle temperature was room temperature. Application volumes were 10-20 μL for perfume/aftershave solutions and 25 μL for the OTNE reference solutions. Plates were developed in a horizontal developing chamber with n-hexane/acetonitrile (4:3; v:v) up to a migration distance of 55 mm. After development, the plates were scanned (Scanner 3, CAMAG) in the multi wavelength mode from 200 to 450 nm (50 nm steps).

Derivatization was then performed in a dipping chamber (CAMAG), speed: 5 cm s⁻¹. One plate was dipped into a solution of 2% 4-[(4-nitrobenzyl)pyridine (NBP) in acetonitrile (time: 0 s) followed by drying of the plate (10 min, 100°C) on a plate heater (CAMAG). After cooling, the plate was dipped (time: 1 s) into a 10% solution of tetrahydroxyperamine in acetonitrile and subsequently dried (1 min, 100°C). The second plate was dipped into a solution of 5% sulfuric acid in methanol containing 1% titanium ethoxide (Ti(II)E) (time: 0 s). The plate was left in a flame cupboard for 5 min. Plates were documented with a Reprostar 3 (CAMAG) under visible light and scanned with a Scanner 3 (CAMAG) in the absorbance mode at 550 nm and 400 nm for NBP and Ti(II)E, respectively. Scan conditions were the same for both procedures: Scan speed was 40 nm s⁻¹, data resolution was 100 μm step⁻¹ and the slit dimension was set to 4 mm x 0.3 mm. Spectra of the peaks (200-700 nm) were recorded in the absorbance mode using a speed of 100 μm s⁻¹, a data resolution of 5 μm step⁻¹ and a slit dimension of 3 mm x 0.45 mm. The quick scan range was situated slightly above the solvent front for all tracks.

**Suitability tests for titanium ethoxide and 4-[(4-nitrobenzyl)pyridine derivatizations after HPTLC.** The suitability of the both derivatization methods was verified with test substances of two categories (Table 1). The peroxides consisted of hydroperoxides (ROOH), peroxides (ROOR) and hydrogen peroxide (H₂O₂). The alkylating agents contained miscellaneous chemicals classified as carcinogens or mutagens (cld were grouped in compounds having direct alkylating properties or not. HPTLC conditions for these experiments were the same as described above, except for minor modifications. The application volume of the OTNE
Table 1. Reference solutions of peroxides and alkylation agents*

<table>
<thead>
<tr>
<th>Peroxides</th>
<th>mg mL⁻¹</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrogen peroxide</td>
<td>0.2</td>
<td>Ethanol/water 9:1</td>
</tr>
<tr>
<td>Benzylic peroxide</td>
<td></td>
<td>Ethanol</td>
</tr>
<tr>
<td>Dicumyl peroxide</td>
<td>1</td>
<td>Ethanol</td>
</tr>
<tr>
<td>bis[1-(tert-butylperoxy)-1-methylethyl]-benzene</td>
<td>1</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Cumene hydroperoxide</td>
<td>1</td>
<td>Ethanol</td>
</tr>
<tr>
<td>tert-butyl-hydroperoxide</td>
<td>1</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Alkylation agents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acrylamide (12)</td>
<td>1</td>
<td>Acetone</td>
</tr>
<tr>
<td>Alachlor (2-chloro-N-(2,6-diethylphenyl)-N-(methoxy)acrylamide) (13)</td>
<td>1</td>
<td>Acetone</td>
</tr>
<tr>
<td>Allyl glycidyl ether (14)</td>
<td>1</td>
<td>Acetone</td>
</tr>
<tr>
<td>Resorcinol diglycidyl ether (15)</td>
<td>1</td>
<td>Acetone</td>
</tr>
<tr>
<td>Captafol (2,1,2,2,5-tetrachloroethylsulfanyl)-3,4,7,8-Tetrahydronisidine-1,3-dione (16)</td>
<td>1</td>
<td>Acetone</td>
</tr>
<tr>
<td>Epichlorohydrin (17)</td>
<td>1</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Benzylic chloride (18)</td>
<td>1</td>
<td>Acetone</td>
</tr>
<tr>
<td>Glycidol (19)</td>
<td>1</td>
<td>Acetone</td>
</tr>
<tr>
<td>1,2-epoxy-3-phenoxypropane</td>
<td>1</td>
<td>Acetone</td>
</tr>
</tbody>
</table>

*Negatively tested chemicals (no direct alkylation properties): benzene (21), benzidine (22), 3-amino-9-ethyl-carbazole (22), 4-aminoazobenzene (22), 4-aminobiphenyl (22), o-anisidine (22), benzo(a)anthraene (23), benzo(j)fluoranthene (24), 4-chloro-2-methylanthracene (22), 4-chloronaphthalene (22), 1,3-dichloro-2-propanol (24), 3,3-dichlorobenzidine (22), 3,3-dimethylbenzidine (22), 3,3-dimethoxybenzidine (22), 2,4-dinitrophenol (25), safrole (26), and nicotine (27).

The reference solution was 1 mL. Plates were not scanned in the multiple wavelength mode before derivatization. Due to the very low boiling point of tert-butylhydroperoxide, the respective reference solution was manually applied with a pipette only after plate development, immediately before derivatization. The same procedure also applied to H₂O₂, which otherwise would migrate to the solvent front during development.

Tests on thermal induced oxidation of OTNE. Two milliliters of OTNE-sol and an OTNE-rich afterglow sample were each filled in 7.5 mL glass vials. The open vials were placed in a thermal chamber thermostated at 80°C for 80 min. Due to partial evaporation of the solvent, vials were filled up to the initial volume (2 mL) using ethanol/water (8:2; v:v) before LC-DAD analysis.

Photooxidation on the skin. In vivo studies on the skin, four afterglow samples (0.5-2% OTNE) were used, which proved to be free of photoproduts. The upper arms of four test persons were cleaned with a paper towel soaked in acetone. On both arms, an 8 cm x 5 cm rectangle was drawn with a pencil, and an aliquot of 100 mL afterglow sample was spread on both rectanges. One of the two rectangles served as a nonirradiated reference, with the sample residues being immediately recovered from the skin with three cotton swabs soaked in ethanol/water (8:2; v:v). The other aliquot was left on the skin, while the test persons stayed 1 h indoors plus 1 h outdoors. Thereafter, the exposed rectangle was also treated with three cotton swabs soaked in ethanol/water (8:2; v:v) in an ultrasonic bath for 15 min. After filtration (0.2 μm PTFE filter), the solutions were analyzed by LC-DAD (Kinetics C18 column); the injection volume was 8 μL.

### Results and Discussion

#### Photooxidation of OTNE

With a degradation of <1% as determined by LC-DAD, OTNE in reference solution (OTNE-sol, 1 mg mL⁻¹) turned out to be highly photostable. However, in the presence of the photosensitizers rose bengale or methylene blue (1 μg mL⁻¹), 1-h in vitro irradiation of OTNE solutions resulted in a strong photodegradation of 34% (RB) and 45% (MB). Chromatograms then showed newly formed peaks, attributable to degradation products. With comparably short peak retention times on the RP 18 column, these products turned out to be more polar than OTNE. Besides a couple of tiny peaks, three distinct new peaks could clearly be detected and these degradation products were numbered according to elution order (OTNE-DG1, OTNE-DG2, OTNE-DG3) (Fig. 2). As both sensitizers gave comparable results, we used rose bengale (RB) for further degradation trials, which also is the standard sensitizer used by the fragrance industry.

LC-DAD screening of twelve OTNE containing perfume and afterglow samples, irradiated for 1 h with a dose of 2700 kJ m⁻², led to an OTNE degradation of up to 33%, although no sensitizer was added (Table 2). The main OTNE photoprodut (OTNE-DG2) was found in all irradiated samples, whereas OTNE-DG3 only was detectable in nine of the samples (Table 2) and the minor product OTNE-DG1 never was found with LC-DAD. To confirm that these newly formed peaks actually were OTNE photoproducts, irradiated samples were spiked with irradiated OTNE-RB (8:2; v:v). Subsequent LC-DAD analysis gave coeluting and increased peakies of both OTNE-DG2 and OTNE-DG3, exemplarily shown in Fig. 3 for an afterglow sample. Based on a calibration with OTNE, concentrations of 0.02-2.40 and 0.01-0.4 μmol mL⁻¹ for OTNE-DG2 and OTNE-DG3, respectively, were determined in the studies perfume/afterglow samples (Table 2). Taking experimental uncertainties into account, OTNE degradation more or less equals the formation of OTNE-DG2 plus OTNE-DG3 (Table 2).

#### Photooxygenation

As degradation of OTNE in reference solutions only occurred in the presence of a sensitizer and degradation products formed were more polar than OTNE, photooxidation was assumed to be the major degradation pathway for these trials. The results further show that oxidation of OTNE also occurred in perfumes/afterglows upon irradiation even without adding a sensitizer. Most likely, some matrix compounds of the perfumes/afterglows also act as sensitizers, boosting OTNE photooxidation.

Photoinduced oxidation potentially leads to the formation of reactive oxygen species (ROS). The members of the ROS group are oxygen-containing radicals, hydroperoxides (ROOH), peroxides (ROOR) or hydrogen peroxide (H₂O₂), which generally are of special concern in respect to consumer safety (28). Some of them are highly reactive, therefore short-lived, making their detection difficult. Therefore, a peroxide test strip was used as a quick detection method. It is based on the conversion of peroxide oxygen in the presence of an organic redox indicator through the enzyme peroxidase, leading to blue oxidation products (29,30). According to the product information of the test, organic peroxides are hardly detected by this test. After irradiation in the sun simulator, all perfume/afterglow samples as well as OTNE-RB gave a strong positive response, resulting in a strong blue color. The formation of peroxides showed proof of the assumed photooxidation as degradation reaction.

Two photochemical oxidation pathways leading to hydroperoxides are known: Photooxidation of type I is a radical reaction, whereas type II includes the formation of singlet oxygen in an
intermediate stage (31). Figuring out, whether type I or II occurred, is challenging, as the greatest difference between both reaction products is the position of the hydroperoxide group. Sodium azide, however, is known to specifically quench singlet oxygen hindering type II photooxidations (32). Therefore, OTNE-RB, OTNE-MB and the perfume/aftershave samples were irradiated in the presence and the absence of sodium azide. The addition of sodium azide to OTNE-RB/OTNE-MB resulted in an OTNE photodegradation being 35% lower compared to solutions without sodium azide along with a proportionally reduced formation of degradation products (Fig. 2). This was in line with the expectations as rose bengal and methylene blue are well-known to enhance the type II photooxidation (33,34). In perfumes/aftershaves, however, sodium azide did not affect the degradation of
Table 2. Degree of octahydro tetramethyl naphthalenyl/ethaneone (OTNE) photodegradation after 1-h irradiation in a sun simulator and formation of its photoproducts, OTNE-DG2 and OTNE-DG3, in OTNE reference solution with the sensitizer rose bengal (RB) and in perfume/aftershave samples (with no sensitizer added).

<table>
<thead>
<tr>
<th>Sample</th>
<th>OTNE (μmol mL⁻¹)*</th>
<th>OTNE degradation (%) (μmol mL⁻¹)</th>
<th>OTNE degradation products (μmol mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTNE-RB</td>
<td>4.3</td>
<td>34 (1.45)</td>
<td>0.98</td>
</tr>
<tr>
<td>Perfume/aftershave 1</td>
<td>88</td>
<td>1.9 (1.66)</td>
<td>1.40</td>
</tr>
<tr>
<td>Perfume/aftershave 2</td>
<td>26</td>
<td>3.9 (1.04)</td>
<td>0.73</td>
</tr>
<tr>
<td>Perfume/aftershave 3</td>
<td>57</td>
<td>1.1 (0.62)</td>
<td>0.46</td>
</tr>
<tr>
<td>Perfume/aftershave 4</td>
<td>360</td>
<td>1.0 (3.53)</td>
<td>0.70</td>
</tr>
<tr>
<td>Perfume/aftershave 5</td>
<td>32</td>
<td>3.4 (1.04)</td>
<td>0.83</td>
</tr>
<tr>
<td>Perfume/aftershave 6</td>
<td>15</td>
<td>5.6 (0.83)</td>
<td>0.42</td>
</tr>
<tr>
<td>Perfume/aftershave 7</td>
<td>22</td>
<td>2.9 (0.62)</td>
<td>0.17</td>
</tr>
<tr>
<td>Perfume/aftershave 8</td>
<td>2.7</td>
<td>0.8 (0.02)</td>
<td>0.02</td>
</tr>
<tr>
<td>Perfume/aftershave 9</td>
<td>34</td>
<td>4.3 (1.45)</td>
<td>1.10</td>
</tr>
<tr>
<td>Perfume/aftershave 10</td>
<td>3.6</td>
<td>15 (0.52)</td>
<td>0.22</td>
</tr>
<tr>
<td>Perfume/aftershave 11</td>
<td>14</td>
<td>33 (4.4)</td>
<td>2.40</td>
</tr>
<tr>
<td>Perfume/aftershave 12</td>
<td>1.5</td>
<td>5 (0.03)</td>
<td>0.06</td>
</tr>
</tbody>
</table>

*Initial concentration. †Based on a calibration with octahydro tetramethyl naphthalenyl/ethaneone. ‡Limit of detection: 0.004 μmol mL⁻¹.

Figure 3. Liquid chromatography-diode array detection chromatogram (200 nm) of a nonirradiated (A) and irradiated (B) after shave sample. The zoomed chromatogram section shows the irradiated sample (continuous line) and the irradiated sample spiked with irradiated octahydro tetramethyl naphthalenyl/ethaneone (OTNE)-RB reference solution (dotted line).

OTNE, nor the formation of its photoproducts. Consequently, in contrast to the irradiation of reference solution, type I photooxidation of OTNE most likely occurred in the samples, indicating that the type of photooxidation in the reference solution is different from that of the studied perfume/aftershave samples. Vice versa, the studied samples obviously did not contain type II photosensizers.

To check whether OTNE oxidation also can be a thermal process, OTNE-sol and the sample 1 (Table 2) were treated at 80°C for 80 min under constant ambient air contact. However, OTNE oxidation products could not be detected (Fig. 2). The thermooxidation potential of OTNE thus proved to be negligible, substantiating UV radiation to be the oxidation inducer.

Photooxidation of OTNE using real settings

Oxidation on the skin. The formation of OTNE oxidation products on the skin is likely to occur as fragrances are exposed to sunlight after application on the skin. To study the in vivo formation of OTNE oxidation products, the samples 1, 2, 3, and 9 (Table 2), showing no evidence of OTNE peroxides, were twice applied on the skin of a test person, where after one application was immediately extracted from the skin and served as a reference. The other application was exposed to light indoors and outdoors (each 1 h) and then recovered from the skin. Subsequent analysis with LC-DAD showed the references to be free of OTNE degradation products. Analysis of the in vivo irradiated aftershave resulted in the same OTNE degradation products (OTNE-DG2 and OTNE-DG3) as found after artificial UV irradiation of OTNE reference solutions. Levels, however, were two to four times lower. These levels can be seen as minimum values as they do not consider incomplete recovery of the OTNE degradation products from the skin during extraction and potential absorption of target compounds by the skin. Furthermore, these trials were performed on autumn days with solar irradiation doses between 540 and 2500 kJ m⁻² thus well below the dose used for artificial irradiation simulating a midsummer day (2700 kJ m⁻²). The results nevertheless demonstrate that photons-induced oxidation of OTNE on the skin has to be expected and leads to the question whether photoxoygenated OTNE, in analogy to oxygenated species of limonene and linalool, might be a contact allergen (8,35,36).

Oxidation during storage. In four of the samples, OTNE-DG2 was found even before irradiation trials with estimated concentrations of 0.004 and 0.33 μmol mL⁻¹. These samples were also tested positive with the peroxide test strip, revealing peroxide levels of 0.5–10 mg L⁻¹, expressed as H₂O₂. Thus, photooxidation obviously had already occurred during storage. This is not surprising, as the samples had been stored in their original glass flasks in a well-lit room, resulting in some exposure to light.
The eight samples with no signs of photodegradation had been stored in the dark. For further testing, the negative samples were individually transferred to a clear glass vial and left for 5 days in a well-lit room. Subsequent analyses revealed OTNE-DG2 in four samples in levels of 0.011 and 0.05 μmol mL⁻¹. The peroxide test strip also gave a positive response for these four with peroxide levels of 0.5–2 mg L⁻¹.

Therefore, photooxidation can even occur during storage indoors if perfume/aftershave were filled in transparent glassware.

Characterization of OTNE degradation products by liquid chromatography high-resolution mass spectrometry

Analysis of the irradiated OTNE-RB solutions with LC-HRMS revealed similar spectra for the three OTNE photoproducts, although relative abundances of the mass signals varied slightly (Fig. 4). The found exact mass of the protonated molecules (m/z 267.1951) fitted well with the common molecular formula of the photoproducts (C₁₅H₂₃O₃) with a mass deviation of 1.5 ppm, indicating that hydroperoxides of OTNE had been formed. The in-source fragmentation resulted in both loss of water (m/z 249.1846) and hydrogen peroxide (m/z 233.1898), but with different intensities.

Identical LC-HRMS results were obtained from the irradiated perfume/aftershave samples, exemplarily shown in Fig. 5 for an aftershave (sample 1) and a perfume (sample 3). Thus, the photoinduced formation of OTNE-DG2 and OTNE-DG3 in perfumes/aftershaves with no sensitizer added could be confirmed. Although OTNE-DG1 was not detected in irradiated samples after LC-DAD analysis, a respective peak at m/z 267.1951 was found in some samples with LC-HRMS (Fig. 5), indicating that traces of even this photoproduct are potentially formed during perfumes/aftershave irradiation.

Characterization of OTNE degradation products by high-performance thin-layer chromatography

Screening for potentially unwanted oxidation products in irradiated samples was performed by HPTLC using two derivatizing reagents, one for the detection of peroxides and the other one as reactant for alkylating agents.

As a mobile phase, toluene proved to give the most efficient separations for the oxidized fragrances, but due to its high boiling point it is difficult to evaporate it from the HPTLC plate also containing rather volatile target compounds. A mixture of n-hexane and acetone (4:3; v/v) was therefore chosen as the overall best mobile phase. HPTLC plates with a concentration zone allowed to apply sample solutions on an inert surface, thus preventing oxidation of fragrances on the plate. As a second measure to prevent oxidation on the plate, derivatization was performed immediately after development.

Detection of peroxides. For the detection of peroxides, the indicator reagent titanium ethoxide was adapted from a standard photometric test (37). According to the instructions of this test, H₂O₂ and hydrogen peroxide containing substances form yellow titanium complexes. To our knowledge, TiEt has not been reported as a derivatizing reagent for HPTLC. Hydroperoxides (ROOH) were of particular interest in this context, as the hydroperoxides of the two monoterpenes limonene and linalool are known contact allergens (4,8,35,36).

As photoinduced oxidation of fragrances may result in the generation of ROOH, ROOR and H₂O₂, the TiEt derivatizing reagent was tested with reference solutions of different commercially available peroxides (Table 1). The response of H₂O₂ was tested without separation on the silica plate, as the compound cannot be chromatographed on a normal phase silica plate. All ROOH compounds and H₂O₂ led to yellow zones. Only one ROOR (dicumylperoxide) of three tested references did so, too, perhaps due to the standard being contaminated by hydroperoxides. A color reaction of TiEt with ROOR therefore is generally not to be expected. Spectra of titanium-peroxide complexes were highly identical irrespective of the reaction partner and therefore easy to distinguish from spectra of any potentially interfering
Figure 5. High-resolution extracted ion chromatograms (m/z 267.1951 + 249.1846 + 233.1898) of an irradiated perfume and aftershave as compared to a OTNB-RB reference solution.
matrix compounds. The limit of detection (LOD) was estimated by decreasing the volume of the hydroperoxide reference solutions applied onto the plate down to a resulting signal-to-noise ratio of 3 for the 400 nm scan. The LOD was determined to be 80 ng zone⁻¹ for cumenehydroperoxide and 200 ng zone⁻¹ for tert-butylhydroperoxide.

Detection of alkylating agents. Alkylating agents may react with nucleophilic groups of skin proteins, which might explain their skin sensitizing potential (38,39). Furthermore, they can also react with nucleophilic DNA bases, thereby initiating mutagenic/carcinogenic processes. NBP has often been used as a reaction model for DNA bases (18,40,41), when alkylating agents react with NBP giving purple colored adducts. The suitability of the NBP derivatization method was checked with 26 chemicals classified as carcinogen or mutagen (Table 1). Based on information from literature, we grouped them to either having directly alkylating properties or not. The method proved to be suitable, as all chemicals with direct alkylating properties formed a purple blue zone (application: 1 µg per zone), whereas the others were not detectable by the NBP reagent. Besides visible inspection, the plates were scanned at the specific absorption wavelength of the NBP adduct (550 nm), followed by recording the spectra of the colored zones. The limit of detection was estimated by decreasing the volumes of the reference solutions applied on the plate down to a resulting signal-to-noise ratio of 3 (scan 550 nm). The LODs were determined to be between 5 and 170 ng zone⁻¹ with a mean LOD of 40 ng zone⁻¹.

Peroxides have no alkylating properties, suggesting that they do not give a positive response with NBP. However, peroxides are able to decompose into radicals, which then may react with skin proteins (42). Furthermore, chemical carcinogenesis of some organic peroxides is thought to be induced by those radicals (43). It is therefore not surprising that the two ROOH references used in our experiments were previously described as potential tumor promoters (44). In addition, aliphatic hydroperoxides of fragrances are well-known skin sensitizers (42). Apparently, the decomposition of some organic peroxides, especially hydroperoxides, may occur rapidly, leading to the question whether aliphatic radicals are also formed during NBP derivatization at high temperatures (100°C) resulting in a positive response. Therefore, the ROOH, H₂O₂, and ROOR references (Table 1) were tested with the HPTLC-NBP method. In contrast to H₂O₂ and the ROOR references, the ROOH references indeed gave purple blue zones.

Concluding from these results, the detection of a compound with both TIEI and NBP would most likely classify them as alkylating hydroperoxides.

Screening of OTNE reference. To assess the formation of either peroxides or/and alkylating agents during irradiation, the irradiated and nonirradiated OTNE reference solutions were analyzed by HPTLC as described. After separation, plates were first scanned in a multiple wavelength mode, followed by the derivatization procedures.

Similar to LC-DAD results, the irradiation of OTNE-RB led to three new peaks detectable in the 200 nm scan (Fig. 6). Three peaks with the same hRₑ values were also obtained with NBP (Fig. 6). The peak intensity profiles of the two derivatization techniques, however, did not fully match. The middle peak (OTNE-DG2) probably represents a peroxide as TIEI derivatization also revealed a strong signal with the same hRₑ value.

![Figure 6. High-performance thin-layer chromatography (HPTLC) scan results before derivatization at 200 nm, after derivatization with nitrobenzylpiridine (NBP) at 550 nm, and after derivatization with TIEI at 400 nm of the octahydro tetramethyl naphthalene-ketone reference solution spiked with the sensitizer rose benigala (A) and a perfume sample (B).](image)

(Fig. 6). Unfortunately, the three peaks were not well resolved after TIEI derivatization. Nevertheless, these results agreed well with the LC-HRMS results supporting previous evidence that the OTNE photoproducts are hydroperoxides.

Screening of samples. In the irradiated perfumes/aftershaves, however, OTNE photoproducts were not clearly found with NBP/TIEI. This was in contrast to LC-DAD results, where OTNE photoproducts were detected in all irradiated samples. As peaks with hRₑ values similar to those of the oxygenated OTNE were found instead, OTNE photoproducts were most likely not chromatographically separated from other oxidized compounds of the samples. Furthermore, the unknown peaks also showed highly correlating hRₑ values for both derivatization procedures and mostly were not detectable in the nonirradiated samples, giving evidence that peroxides of several other fragrances had been formed during perfume/aftershave irradiations. As an example, scan results of a perfume sample are shown in Fig. 6. Irradiation of the perfume resulted in two new HPTLC zones/peaks with both TIEI and NBP: the hRₑ values were identical. NBP derivatization also showed an additional peak in both the irradiated and nonirradiated perfume, which was not detectable by TIEI.
CONCLUSIONS

Our study showed that the common fragrance compound OTNE, known to be photostable in solvent in its pure form, undergoes considerable photodegradation in the presence of a sensitizer and in a perfume/after shave matrix. The experiments clearly revealed photoinduced oxidation as the major degradation reaction. Furthermore, OTNE photooxidation even occurred during indoor storage of samples, and an in vivo test demonstrated that photoxidation also may happen after application on the skin. Our HPTLC screening method proved to be suitable in detecting potentially unwanted photoproducts in irradiated aforesomes and perfumes. As this study shows for OTNE, photostability tests of individual fragrances in pure solvents are inadequate models for tests in finished cosmetic products.

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REFERENCES

III. Discussion

Considering the widespread use of cosmetics and the negative effects, which are known to arise from certain cosmetic ingredients, the safety of cosmetic products is of special importance. This is why the Regulation (EC) No. 1223/2009 provides that the manufacturer of a cosmetic product is obliged to assess the safety of their cosmetic products in a safety report. As the requirements in this regulation reflect the current scientific knowledge with just a few studies reporting on negative effects of cosmetic ingredients in the past few years, there is a need for more research in this field. This is in contrast to the food sector, where numerous studies have been published on food ingredients of concern. The overriding objective of this work, therefore, was to contribute knowledge to the safety aspects of cosmetic ingredients by investigating the oxidation of certain frequently used cosmetic ingredients. A focus was set on the formation of potentially critical compounds and the development of analytical methods.

The oxidation of allylic compounds to potentially hazardous compounds has received less attention in cosmetics, although cosmetic products contain unsaturated organic compounds, for example in the form of fatty acids or aroma compounds. The lack of analytical methods suitable for determining the oxidation state might be a reason for the few studies. As the peroxide content increases in the early state of a compound’s oxidation, this parameter is often used to assess the oxidation state. The peroxide value, however, has its limits, which is due to a decreasing peroxide content in an advanced oxidation state. As a result, the oxidation of a compound needs to be considered individually by determining, for example, specific oxidation products or decreasing levels of the oxidable compound.

The oxidation of cholesterol into cholesterol oxidation products (COPs) formed the first part of this work. As COPs are thought to induce several disorders, their levels have been reported for different types of fatty food. Cosmetics, however, have never been addressed up to this date. By adapting a method from the food sector, COPs were determined in cosmetics (chapter II, 1). The analytical method included a transesterification step for setting free esterified alcohols (also COPs). The following SPE procedure was used for sample preparation. COPs were then derivatized to trimethylsilyl ethers, which were separated by gas chromatography. Detection of silylated COPs was performed by either flame ionization detection or mass
spectrometry. Quantification was performed by internal calibration using reference substances. Spiking with 25-HC as a control standard at the beginning of preparation, allowed for the supervision of the whole process.

The method was used for lanolin containing cosmetic products, namely lip care products, nipple ointments for nursing women and fatty creams, due to the lanolins’ extraordinary content of the precursor cholesterol. Analysis revealed six COP congeners (7α-HC, 7β-HC, CT, β-CE, α-CE and 7-KC), which were also the most often reported COPs in food samples. These congeners are known to be formed by autoxidation reactions involving radicals. Total COPs content (sum of the congeners) was in the low percent range: for lip care products and creams between 0.2 and 0.9 % and for nipple ointments between 1 and 3 %. The higher COPs levels in nipple ointments were due to lanolin being the only ingredient for most ointments. Compared to levels found in food, COPs levels were several orders of magnitude higher ($10^2$ to $10^4$). The application note of the ointments for nursing women "does not have to be washed off before nursing" also makes it clear that the absorption route is oral just like lip care products. As a consequence, lanolin containing cosmetics, in addition to food, represent a further uptake source for COPs. As some oxidized compounds are known contact allergens, and COPs can come in contact with the skin, tests should therefore be performed to elucidate, if COPs can provoke allergies. Some published studies indeed reported on allergenic properties of the lanolin alcohol fraction.

The second part of this work was about the determination of oxidized congeners of other sterols (Chapter II, 2), as properties similar to those of COPs are to be expected. For this purpose, a new analytical method had to be developed, as the method described in Chapter II 1- especially the SPE clean-up procedure- was not suitable in this case. COPs were first extracted using dichloromethane. A dye serving as a marker and 25-HC serving as a recovery standard were added to the sample solution. COPs were then released with transesterification. The transesterified extract was cleaned up with planar solid phase extraction (pSPE). After applying an aliquot onto the silica plate, chromatography first was performed with n-hexane and, after air drying, with n-hexane:diethyl ether (1:2,v:v). Nonpolar interfering matrix compounds thereby migrated from the start zone towards the upper plate edge, while the polar oxidized sterols and the marker compound remained on the start zone. The plate was then developed with acetone, which caused the marker compound and the oxidized sterols to migrate out of
the start zone. The oxidized sterols were focused into one zone, made visible by the marker (disperse orange 3) compound. The marker proved to be suitable by migrating above the oxidized and slightly below the unoxidized sterols. The target analytes were then extracted from the plate using an automatic elution instrument (TLC-MS interface). To check clean-up efficacy, the plate was visualized using a suitable documentation system (Reprostar3). With this, clean-up efficacy of every sample analyzed was directly assessed. After evaporation of the extraction solvent (acetone), the oxidized sterols were derivatized and then determined by GC-MS. A great advantage of this method is due to the nature of thin-layer plates being disposable articles: the stationary phase does not have to be reconditioned and larger sample quantities can be applied. The limit of determination can furthermore be greatly reduced by multiple application of the sample solution. Quantification was based on COPs, as no reference standards of the other oxidized sterols were available. As matrix constituents interfered with quantification, a correction factor was used. Sample preparation was checked with the recovery standard 25-HC. Recoveries were between 86 % and 113 %, depending on the congener. Limits of detection were between 0.003 and 0.250 mg kg$^{-1}$, also depending on the congener.

The method was then applied to various fat containing cosmetics. In addition to COPs in lanolin containing cosmetics, oxidized congeners of plant sterols (POPs) were found in cosmetics containing plant oils or phytosterols. Identification of peaks was based on comparison of obtained mass spectra and retention times of peaks with those found in literature. POPs contents were in the low ppm range. Lanosterol and dihydrolanosterol (besides cholesterol) are main components of the ingredient lanolin. A study on the potential formation of oxidation products of these sterols was performed. As no data on mass spectra of congeners were available, thermooxidation was applied for an in-house generation of reference standards. Lanolin containing samples were then screened for the peaks found in the thermooxidized reference standards. Only peaks in common underwent structural elucidation with high-performance thin-layer chromatography (HPTLC) and liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS). Full scan spectra of formed oxidation products of dihydrolanosterol and lanosterol were identical, with the exception that m/z differed by 2. This is not surprising, as the monoisotopic masses of the precursors lanosterol and dihydrolanosterol also differ by the same amount, which is due to lanosterol having one additional double bond on C24/C25. The oxidation of lanosterol therefore probably
occurred not on the C24/C25 double bond. As the thermoinduced formation of LOPs was more tricky, DOPs were used as model compounds for identifying LOPs and DOPs. For identification, LOPs and DOPs were first separated with HPTLC. The separated congeners were scrapped off from the plate, followed by a triple analysis with a) gas chromatography, b) gas chromatography after derivatization to trimethylsilyl ethers and c) LC-HRMS. Based on the exact monoisotopic mass, results of LC-HRMS allowed for a conclusion on the molecular formulae of the congeners. The gas chromatographic analysis confirmed the monoisotopic mass. As the derivatization converted hydroxy to trimethylsilyl ether groups, the monoisotopic mass thereby increased by 72 for each converted hydroxy group. The comparison of the monoisotopic masses of the derivatized and nonderivatized oxysterol revealed the number of hydroxy groups present in the molecule. In this way, four LOPs congeners and four DOPs congeners were identified for the first time in cosmetic products. Levels reached up to 0.3 %. As COPs are thought to cause various health disorders, similar properties of POPs, DOPs and LOPs need to be considered.

The oxidation of fragrances was studied in the third part of this work (Chapter II, 3). The subject is not new as the oxidation of terpenes into known contact allergens has been studied before. However, the oxidation of other fragrances was hardly investigated, although all fragrances with a double bond are oxidable in theory. In order to extend our knowledge in this field, the oxidation of a synthetic fragrance frequently used in perfumes/aftershaves, octahydro tetramethyl naphthalenyl ethanone (OTNE), was studied. OTNE commonly is used in rather high concentrations. A study reported a high photodegradation rate of OTNE in soil surface, probably due to photooxidation. Based on this, photooxidation of OTNE into potentially hazardous compounds in analogy to terpene oxidation products was considered in perfumes/aftershaves.

For this, OTNE was thrice diluted in a perfume matrix (water: ethanol mixture). Rose bengale (photosensitizer) was added to one, methylene blue (photosensitizer) in another trial. In the third trial no sensitizer was added. Photooxidation was induced by irradiation in an artificial sunlight simulator. The irradiated solutions were analyzed with liquid chromatography coupled to diode array detection (LC-DAD). Whereas OTNE with no photosensitizer added was rather photostable, OTNE strongly degraded about 40 % in the presence of a photosensitizer. Chromatograms showed three newly formed peaks attributable to oxidation products of OTNE. Twelve OTNE containing
perfumes/aftershaves then were irradiated with no sensitizer added. OTNE degradation reached 30 % even though no sensitizer was added. The same OTNE oxidation products (two of the three) as formed in the reference with the sensitizers were found. These results showed that OTNE photooxidation can occur in real samples. Formed OTNE oxidation products were then characterized with LC-HRMS. Obtained exact monoisotopic masses of the protonated oxidation products indicated the formation of hydroperoxides of OTNE. In addition, selective derivatization methods after separation with HPTLC were developed. Titanium ethoxide was adapted from a standard photometric test for peroxides and used for the first time as a derivatizing agent after HPTLC. Derivatization after HPTLC additionally was performed with nitrobenzyl pyridine (NBP), which is actually used for the detection of alkylating agents. The suitability of NBP for the detection of some peroxides has been determined with several reference compounds. Both derivatization methods supported the assumption that peroxides of OTNE were formed through photooxidation. This was further supported by the determination of peroxides using a peroxide test strip.

An in-vivo test further showed that OTNE photooxidation even occurs after the application of the OTNE containing perfume on the skin. Three OTNE containing perfumes were applied twice on the skin of a test person. One trial served as a reference and was immediately extracted from the skin. The other trial was extracted from the skin after exposure to sunlight indoors and outdoors (each 1 h). Analysis with LC-DAD revealed OTNE oxidation products to be present in the irradiated trials, but not in the non-irradiated reference.

A storage study further showed that OTNE oxidation products can also be formed indoors under normal storage conditions. For this, eight OTNE containing perfumes, which had been stored in the dark, were transferred into clear glass vials. The samples were left to stand for 5 days in a well-lit room. Subsequent analysis with LC-DAD revealed OTNE oxidation products in these samples.

It could be shown on the basis of two examples, that the oxidation of cosmetic ingredients is omnipresent. Negative health effects caused by the oxidation products formed cannot be excluded, which is why this topic will be considered in further studies. It is desirable in the long term that the oxidation of cosmetic ingredients becomes a mandatory part of the safety assessment.
IV. Summary

Cosmetic products are important consumer goods in the "non-food" sector. Shampoo, shower gel and day cream are regularly used by consumers. In order to ensure the safety of cosmetic products, their placing on the market is defined by legal provisions. In the member states of the European Union the cosmetics Regulation (EC) No. 1223/2009 applies, in which, for example, the use of certain critical ingredients is generally prohibited or application-related limited. Critical compounds, however, can also be formed by secondary reactions of the cosmetic ingredients. An example for a secondary reaction is the oxidation of an unsaturated organic compound. Thereby formed oxidation products with potentially adverse properties are well known from the food sector. As the oxidation of cosmetic ingredients, however, has less been studied, the oxidation of selected cosmetic ingredients with respect to the formation of potentially critical compounds was investigated within the framework of this thesis.

The oxidation of cholesterol to various cholesterol oxidation products (COPs) was investigated in a first step. COPs are known from the food sector and are suspected of causing certain diseases such as arteriosclerosis. Cosmetic products have not yet been tested for COPs, although a versatile ingredient used only in cosmetic products, lanolin, contains above-average levels of the cholesterol, which is the precursor. For the detection, COPs were initially released by transesterification. This was followed by sample preparation in the form of solid phase extraction (SPE), derivatization and subsequently analysis by gas chromatography using either flame ionization detection or mass spectrometry. Total COPs contents in cosmetics containing lanolin, namely lip care products, fat creams and ointments for nursing women were in the low percent range (up to 3 %) and were thus several orders of magnitudes higher than the contents found in food.

The SPE procedure, however, reached its limits in certain matrices and was not suitable for the determination of oxidized congeners of other sterols. The analytical procedure therefore was adjusted in so far as SPE was replaced by planar solid phase extraction. Using normal phase thin-layer chromatography, the oxidized sterols were first separated from more non-polar matrix compounds. After, the target compounds were focused into one zone. The zone was then extracted from the plate using an automatic elution...
instrument (TLC-MS interface), followed by evaporation of the extraction solvent. Besides COPs, oxidized congeners of lanosterol (LOPS), dihydrolanosterol (DOPs) and plant sterols (POPs) were thereby found in different cosmetics. POPs contents were in the low ppm range and thus comparable to those in food. DOPs and LOPs are oxidation products of lanosterol and dihydrolanosterol, which are specific sterols of lanolin, and, in the framework of this thesis, have been detected for the first time in levels of up to 3000 ppm.

The oxidation of fragrances was studied in the second part of this work. The subject is not new as the oxidation of terpenes to contact allergens has been studied in earlier studies. The oxidation of other fragrances was hardly investigated. In order to extend our knowledge in this field, the oxidation of a synthetic fragrance frequently used in perfumes, octahydro tetramethyl naphthalenyl ethanone (OTNE) was studied. For this, OTNE was dissolved in a perfume matrix (water-ethanol mixture) and a photosensitizer (sens) was added. Photooxidation was then induced by irradiation in an artificial sunlight simulator. Formed oxidation products were characterized by liquid chromatography coupled to high resolution mass spectrometry and selective derivatizations after separation with high-performance thin-layer chromatography (HPTLC): Besides nitrobenzyl pyridine (NBP), titanium ethoxide was adapted from a standard photometric test for peroxides and used for the first time as a derivatizing agent after HPTLC. Obtained results indicated that peroxides of OTNE were formed during oxidation. This was subsequently confirmed by the determination of peroxides using peroxide test strips. In irradiated OTNE containing real samples (perfumes/aftershaves), OTNE oxidation products were even found without adding a photosensitizer. It was found out that the OTNE oxidation even occurs, when perfumes are stored indoors under normal temperature and light conditions. An in-vivo test showed that OTNE oxidation can be expected on the skin after application of a perfume.
V. Zusammenfassung


Im ersten Schritt wurde die Oxidation von Cholesterin zu verschiedenen Cholesterinoxidationsprodukten (COPs) untersucht. COPs sind aus dem Lebensmittelbereich bekannt und stehen im Verdacht, bestimmte Krankheiten wie Arteriosklerose hervorzurufen. Kosmetische Mittel wurden bisher nicht auf COPs untersucht, obwohl ein nur in kosmetischen Produkten vielseitig eingesetzter Inhaltsstoff, Lanolin, überdurchschnittlich hohe Gehalte am Vorläufer Cholesterin enthält. Für den Nachweis wurden COPs zunächst durch Umesterung freigesetzt. Danach folgte eine Probenaufbereitung in Form einer Festphasenextraktion (SPE), die Derivatisierung und eine Analyse mit Gaschromatographie, gekoppelt an einen Flammionisationsdetektor oder Massenspektrometer. Gesamt COPs Gehalte in kosmetischen Mitteln mit Lanolin, nämlich Lippenpflegeprodukte, Fettcremen und Salben für stillende Frauen, waren im niedrigen Prozentbereich (bis 3 %) und lagen damit um einige Größenordnungen über den in Lebensmitteln gefundenen Gehalten.

Die SPE Methode kam bei bestimmten Matrizes an ihre Grenzen und war zudem nicht für die Bestimmung oxidierter Kongenere anderer Sterole geeignet. Die Methode wurde deshalb angepasst, indem die SPE durch eine planare Festphasenextraktion ersetzt wurde: Durch Normalphasen-Chromatographie wurden die oxidierten Sterole zunächst