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COMMUNICATION

Development and validation of LC/APCI-MS method for the quantification of oat ceramides in skin permeation studies

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Abstract

Ceramides (CERs) are the backbone of the intercellular lipid lamellae of the *stratum corneum* (SC), the outer layer of the skin. Skin diseases such as atopic dermatitis, psoriasis, and aged skin are characterized by dysfunctional skin barrier and dryness which are associated with reduced levels of CERs. Replenishing the depleted epidermal CERs with exogenous CERs has been shown to have beneficial effects in improving the skin barrier and hydration. The exogenous CERs such as phyto-derived CERs (PhytoCERs) can be delivered deep into the SC using novel topical formulations. This, however, requires investigating the rate and extent of skin permeation of CERs. In this study, an LC/APCI-MS method to detect and quantify PhytoCERs in different layers of the skin has been developed and validated. The method was used to investigate the skin permeation of PhytoCERs using Franz diffusion cells after applying an amphiphilic cream containing PhytoCERs to the surface of ex vivo human skin. As plant-specific CERs are not commercially available, well-characterized CERs isolated from oat (*Avena abyssinica*) were used as reference standards for the development and validation of the method. The method was linear over the range of 30–1050 ng/mL and sensitive with limit of detection and quantification of 10 and 30 ng/mL, respectively. The method was also selective, accurate, and precise with minimal matrix effect (with mean matrix factor around 100%). Even if more than 85% of oat CERs in the cream remained in the cream after the incubation periods of 30, 100, and 300 min, it was possible to quantify the small quantities of oat CERs distributed across the SC, epidermis, and dermis of the skin indicating the method's sensitivity. Therefore, the method can be used to investigate the skin permeation of oat CERs from the various pharmaceutical and cosmeceutical products without any interference from the skin constituents such as the epidermal lipids.

Keywords Phyto-derived ceramide · Oat ceramide · *Stratum corneum* · Skin · LC/APCI-MS · Skin permeability

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Abbreviations

CER	Ceramide
DR	Dermis
EP	Epidermis
ELSD	Evaporative light scattering detector
FA	Fatty acid
GlcCER	Glucosylceramide
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MS/MS	Tandem mass spectrometry
MF	Matrix factor
SIM	Selected ion monitoring
S/N	Signal to noise ratio
RSD	Relative standard deviation
SB	Sphingoid base
SC	*Stratum corneum*
SD	Standard deviation

Introduction

Ceramides (CERs) are essential constituent of intercellular lipid lamellae of the *stratum corneum* (SC) playing a critical role in skin health by providing a barrier and retaining the skin moisture [1]. Studies have shown that the level of epidermal CERs (Fig. S1, see Electronic Supplementary Material, ESM) is reduced in skin diseases such as atopic dermatitis [2] and psoriasis [3] and with increasing age [2, 4] resulting in defective skin barrier and skin dryness. It has also been documented that replenishing the depleted epidermal CERs with exogenous CERs has beneficial effects in improving skin barrier and skin hydration [5].

In plants, CERs are predominantly found as glucosylceramides (GlcCERs) (Fig. S2, ESM). While 4,8-sphingadienine (d18:2$^{\Delta4,8}$), 8-sphingenine (d18:1$^{\Delta8}$), and 4-hydroxy-8-sphingenine (t18:1$^{\Delta8}$) represent the dominant SBs in plant GlcCERs, sphingosine (d18:1$^{\Delta4}$), sphinganine (d18:0), and phytosphingosine (t18:1) are the dominant SBs in human skin CERs [6] (Fig. S3, ESM). The FAs in the plant GlcCERs are mostly saturated and α-hydroxylated and have a chain length of C14-C26 [7, 8]. On the other hand, skin CERs contain non-hydroxy, α-hydroxy, or ester-linked ω-hydroxy FAs [1, 8].

The dermatological benefits of oral dietary supplements containing plant-derived CERs (PhytoCERs) have been shown in earlier studies [9–11]. Nonetheless, little effort has been made to deliver PhytoCERs topically. As PhytoCERs are structurally related to SC CERs, the topical delivery of PhytoCERs can potentially stabilize SC lipid lamellae. However, further studies are needed to investigate the rate and extent of permeation of PhytoCERs from pharmaceutical formulations and cosmetic products into the various skin layers.

Previously, analytical methods have been developed for the determination of endogenous CERs in the SC including LC/APCI-MS [12, 13], LC/ESI-MS [14, 15] and AMD-HPTLC [16]. Ex vivo or in vivo CER skin permeation studies, however, require analytical methods that can selectively detect and quantify the exogenous CERs permeated into the SC without any interference from skin constituents including the epidermal lipids. Ex vivo skin permeation studies also require sensitive analytical methods as small size skin layers containing minute quantities of CERs are obtained from Franz diffusion cells. In the literature, LC-MS methods are reported for the quantification of exogenous deuterated CER [NP] [17] and a novel dimeric CER [18] in the skin. To our best knowledge, however, the penetration of PhytoCERs across the various skin layers after topical application of PhytoCER-based formulations has not yet been investigated. A method for quantitative analysis of PhytoCERs in the various skin layers is, therefore, needed.

One of the major challenges we faced in developing a method for quantitative determination of PhytoCERs in biological systems was unavailability of phyto-identical CER reference standards on the market. While the commercially available CER standards usually have 4-sphingenine (d18:1$^{\Delta4}$) SB, PhytoCERs typically contain 4,8-sphingadienine (d18:2$^{\Delta4,8}$), 8-sphingenine (d18:1$^{\Delta8}$), and 4-hydroxy-8-sphingenine (t18:1$^{\Delta8}$) SBs [19]. In LC-MS-based analysis, these structural variations could affect the MS signal intensity (associated with the stability of CERs in the ion source and under CID conditions). For instance, unlike CERs with C4-saturated SBs, CERs containing C-4 desaturated/hydroxylated SBs are readily dehydrated/deglucosylated during ionization process (the so-called in source fragmentation) (Table S1 and Figs. S4 and S5, see ESM) [19, 20]. The spectra, therefore, represent characteristic neutral losses of water and glucose moieties. Therefore, it was indispensable to isolate GlcCERs from a plant, Ethiopian oat (*Avena abyssinica*), cleave the glycosidic linkage of GlcCERs, and characterize the resulting CERs [21]. Thus, the well-characterized oat CERs (oat CER I and II, Fig. S6, ESM) were used as reference standards for the development and validation of the LC-MS method.

Atmospheric pressure chemical ionization (APCI) is an effective method for ionization of non-polar compounds and, therefore, is widely used for analysis of various lipid classes [12, 13, 22] including PhytoCERs [19, 21]. In comparison to electrospray ionization (ESI), the ionization process in APCI is less dependent on the nature of mobile phase, less prone to matrix effects, and mostly proton adducts are formed [23]. Thus, the present work is aimed at developing and validating an LC/APCI-MS method for the detection and quantification of oat CERs in the SC and other layers of the skin.

Materials and methods

Materials

Soybean GlcCER (> 99% by TLC) was obtained from Avanti Polar Lipids (Alabaster, AL, USA). CER [AP] and CER [AS] were obtained from Evonik-Industries (Essen, Germany). Formic acid, hydrogen chloride solution (4.0 M in dioxane), and 1, 4-dioxane anhydrous (99.8%) were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). HPLC grade methanol and chloroform were purchased from VWR International GmbH (Darmstadt, Germany). Isopropanol and *n*-hexane were obtained from Grüssing GmbH (Filsum, Germany). LC-MS grade methanol, silica gel 60 (0.063–0.200 mm), and TLC (silica gel 60, F_{254}, 20 cm × 20 cm) plates were obtained from Merck KGaA (Darmstadt, Germany).

Ethanol was supplied by Brüggemann GmbH & Co. KG (Heilbronn, Germany). The Ethiopian oat grain (*Avena abyssinica*, Poaceae, local name: Ajja) was purchased from the local market (Holleta area, Ethiopia) in July, 2015. CERs isolated from oat grain (oat CER I and oat CER II) were used as reference standards.

Methods

Isolation and structural characterization of oat GlcCERs

The isolation and structural identification of oat GlcCERs were carried out following the methods described elsewhere [21].

Cleavage of glycosidic linkage of oat GlcCERs

The oat GlcCERs were dissolved in anhydrous 1,4-dioxane (30 mg/mL) at room temperature and the solution was mixed with 4.0 M HCl in dioxane (1:1, *v*/*v*) in a round bottom flask. The reaction mixture was stirred on a magnetic stirrer at 500 rpm for 18 h at room temperature. The reaction mixture was kept in an ice water bath and the content of the mixture was neutralized with saturated $NaHCO_3$ solution. The reaction mixture was exhaustively extracted with $CHCl_3$ (three times) on a separatory funnel to separate the CERs from the hydrophilic components of reaction mixture (including the sugar moiety). The $CHCl_3$ phase was washed with saturated NaCl solution (brine) and dried under nitrogen stream. The progress of the reaction was monitored by TLC and LC-MS analyses.

Isolation of predominant oat CERs

Isolation of the resulting oat CERs from the rest of reaction products in the $CHCl_3$ phase was achieved by TLC-guided column chromatography using a gradient elution: first elution with $CHCl_3$ followed by a second elution with $CHCl_3$/MeOH (9:1, *v*/*v*). The two predominant oat CERs collected from the second elution were separated by preparative LC/APCI-MS using a 1260 Infinity LC (Agilent Technologies, Waldbronn, Germany) coupled to a 6120 series single quadrupole mass spectrometer. The detail LC-MS conditions are described elsewhere [21].

LC/APCI-MS method development

An LC-MS/MS system consisting of UHPLC coupled on-line to a triple quadrupole MS (QqQ-MS, TSQ Quantum Ultra, Thermo Fisher Scientific, Bremen, Germany) equipped with HESI source with an APCI probe was used for development and validation of the analytical method [24]. The oat-derived CERs were separated on a YMC-Pack ODS-AQ column (150 × 2.0 mm I.D., S-3 μm, 200 Å pore size, YMC Europe GmbH, Dinslaken, Germany) under the following chromatographic conditions: mobile phase comprised eluent A (aqueous + 0.1% *v*/*v* formic acid) and eluent B (MeOH + 0.1% *v*/*v* formic acid in), flow rate was set to 0.3 mL/min, column temperature was 40 °C, injection volume was 10 μL, and total run duration was 40 min. The separation relied on a multi-step gradient elution accomplished as follows: 5 min isocratically at 90% eluent B, followed by sequential linear gradients to 95 and 100% eluent B in 5 and 20 min, respectively. The post-run equilibration time was 10 min. The MS was operated in the positive ion mode using selected ion monitoring (SIM, *m*/*z* 554 and *m*/*z* 610) and the following APCI settings: source vaporizer temperature of 450 °C, capillary temperature of 275 °C, source (discharge) current of 6 μA, sheath gas pressure of 55 psi, and ion sweep gas pressure of 30 psi.

Extraction of SC lipids

The SC lipids were extracted from a full-thickness skin sample and the skin of six volunteers. The full-thickness human skin from which the subcutaneous tissue was removed was extracted with 5 mL of *n*-hexane/ethanol 2:1 (*v*/*v*) at room temperature overnight. The extract was filtered through a 0.45 μm syringe filter (PERFECT-FLOW® PTFE, WICOM Germany GmbH, Heppenheim, Germany) and dried under a nitrogen stream at 40 °C. The SC lipids from the skin of human subjects were extracted following a protocol described elsewhere [16]. Briefly, round glass cylinders were kept over the volar forearm of the six volunteers and tightly pressed to the skin to prevent lateral leakage; the cylinders were filled with 5 mL of *n*-hexane/ethanol 2:1 (*v*/*v*) and the lipid was extracted for 5 min over extraction area of 6.158 cm^2. The SC extracts were taken with micropipette and dried under nitrogen stream at 40 °C. The residues were reconstituted in 1 mL of MeOH, filtered through 0.45 μm syringe filter, and stored at − 25 °C until use.

Method validation

The LC/APCI-MS method was validated for selectivity, linearity, matrix effect, accuracy, precision, detection limit, quantitation limit, and carry-over effect according to the EMA guideline on validation of bioanalytical methods, 2012 [25].

Calibration curve and linearity Calibrations curves were constructed using different concentrations of the two oat

CERs (30–1050 ng/mL) in MeOH. The linearity range was tested based on the average peak areas versus the concentration (ng/mL) of oat CERs using linear regression analysis and calibration curve parameters (correlation coefficient, slope, and intercept) were calculated. The concentrations of the calibration standards were back-calculated from the peak areas using the regression equations and the mean accuracy values were determined.

Limits of detection and quantification The limits of detection (LOD) and quantification (LOQ) were estimated according to the 1996 Analytical Detection Limit Guidance [26]. The LOD and LOQ of the method were estimated at spike level of 25 ng/mL (selected from a series of dilutions based on S/N ratio). The peak areas of seven replicas of oat CER solutions (25 ng/mL) were determined and the corresponding concentrations were back-calculated. From the average and standard deviation (SD) of the back-calculated concentrations, the LOD (LOD = t value × SD, where t is Student's t value for 6 degrees of freedom ($n = 7$)) and LOQ (LOQ = 10 × SD) of the method were estimated. Finally, the spike level requirements were checked: whether the S/N ratio is in the range of 2.5 to 10, the spike level is in the appropriate range (which is LOD < spike level < 10 × LOD) and the percentage recoveries (= average/spike level × 100) are reasonable.

Precision and accuracy The within-run precision and accuracy were determined by analyzing in a single run five samples per level at four concentration levels covering the calibration curve range (at the LOQ (30 ng/mL), three times the LOQ (90 ng/mL), medium (400 ng/mL), and at 75% of the upper calibration curve range (800 ng/mL)). The four concentration levels, five samples for each level in three runs analyzed on three different days, were used for the validation of between-run precision and accuracy. While the precision of the method was expressed as relative standard deviation (RSD), the accuracy was reported as a percentage of the nominal value (percent recovery). Precision and accuracy were determined at the four concentrations prepared independently.

Selectivity The selectivity of the method was determined using the full-thickness skin and SC extracts obtained from different sources. The interference of the constituents of each of the extracts in the analysis of the target CERs was separately evaluated.

Carry-over The carry-over effects of the method were assessed by injecting blank samples after running concentrated sample of oat CERs spiked in the skin lipid extract (three times) and observing the occurrence of MS signals within the retention windows of the target CERs.

Matrix effect The matrix effect was investigated using skin lipid extracts obtained from six different persons spiked with oat CERs. For each CER, the matrix factor (MF), ratio of the peak area in the presence of matrix (matrix spiked with CERs) to the peak area in the absence of matrix (pure solution of CERs), was calculated for each lot of matrix and the mean and RSD of the MF were obtained. The spiking was done at low (3 × lower LOQ) and high (close to the upper LOQ) concentrations.

Application of the method for ex vivo skin permeation studies

Preparation of oat CER-based cream Oat CERs (10 mg) were incorporated into 4 g of an amphiphilic cream (Basiscreme, *Deutscher Arzneimittel Codex* (DAC), Caesar & Loretz GmbH, Hilden, Germany).

Ex vivo skin permeability studies The applicability of the method was studied using an excised human skin in which the subcutaneous layer was removed. The skin was stored at − 20 °C until use. The skin was first defrosted and mounted on a Franz diffusion cell (Crown Glass Company, Somerville, NJ, USA). A defined amount of the formulation, 20 mg of amphiphilic cream containing oat CERs, was applied evenly on the outer surface of the skin (3.1416 cm^2) facing the donor compartment which was kept on filter gauze (Sartolon polyamide, pore size 0.45 μm; Sartorius Stedim Biotech GmbH, Gottingen, Germany). The acceptor compartment contained distilled water and was stirred continuously. The cell was kept at 32 °C using circulating water. The permeation experiments were carried out for 30, 100, and 300 min (skin sample from one source was used for each penetration time). Following the incubation period, the formulation remaining on the surface of the skin was removed by a cotton swab and three 6-mm-diameter discs (0.2827 cm^2) were cut out using a Kromayer punch (Stiefel-Laboratorium, Offenbach, Germany). The discs were sectioned into different slices using a cryomicrotome (Jung, Heidelberg, Germany). While the upper 10-μm-thick slice represents the SC, the next four 20-μm-thick slices were considered as viable epidermal layer (EP) (two slices for each epidermal sub-layers EP1 and EP2). Each of the DR sub-layers (DR1 to 3) were represented by five 40-μm-thick slices. The details of sample preparation procedure have been described elsewhere [24]. The quantity of oat CERs in each skin slice, the total quantity of oat CERs distributed across the various skin sub-layers, total quantity of oat CERs

recovered, and the extraction recovery were calculated as follows:

$$\text{Quantity of oat CERs in each skin slice} = \left(\text{quantity of oat CERs in each slice}/0.2827\ \text{cm}^2\right) \times 3.1416\ \text{cm}^2$$

$$\text{Total quantity of oat CERs distributed across the various sub-skin layers} = \left(\text{total quantity of oat CERs in the skin slices}/0.2827\ \text{cm}^2\right) \times 3.1416\ \text{cm}^2 = \left[\text{quantity in (SC + EP1 + EP2 + DR1 + DR2 + DR3 + RT)}/0.2827\,\text{cm}^2\right] \times 3.1416\,\text{cm}^2$$

where RT is the remaining skin tissue

$$\text{Total quantity of oat CERs recovered} = \text{quantity of oat CERs in(various skin sub-layers + cotton swab + filter gauze + acceptor fluid)}$$

$$\text{Extraction recovery}(\%) = (\text{total quantity of oat CERs recovered}/\text{nominal quantity of oat CERs applied}) \times 100$$

Results and discussion

Preparation of oat CERs reference standards

As reported in our previous work [21], about 12 GlcCER species were identified by LC/APCI-MS/MS in oat grain. The two predominant oat GlcCER species were found to contain 8-sphingenine (d18:1$^{\Delta 8}$) linked to hydroxypalmitic acid (d18:1/h16:0) and hydroxy arachidic acid (d18:1/h20:0). As the CERs are needed for the SC delivery as a replacement of the native CERs lost due to some skin diseases and aging, cleavage of the sugar moiety in the GlcCER species was necessary. Hence, the glycosidic linkage of oat GlcCERs was cleaved under strongly acidic conditions. The molecular formulae of oat CERs assigned by positive mode HR/ESI-MS at *m*/*z* 554.5133 (calculated 554.5143, with mass error of 1.8 ppm, oat CER I ($C_{34}H_{67}NO_4$)) and *m*/*z* 610.5784 (calculated 610.5769, with mass error of 2.5 ppm, oat CER II ($C_{38}H_{75}NO_4$)) were identical to the ones identified by LC/APCI-MS/MS (Figs. S7 and Fig. S8, ESM) [21]. The purity of the CERs was found to be > 99% as determined by HPLC-ELSD (a representative HPLC-ELSD chromatogram of oat CER is shown in Fig. S7, ESM). The well-characterized oat CERs were used as reference standards for the development and validation of the method.

Method development

During method development, the effects of mobile phase composition (water/MeOH, varied from 0:100 to 10:90) and formic acid concentration (0.1, 0.2, and 0.3% (*v*/*v*) in both water and MeOH) on selectivity of target oat CERs in SC extracts were investigated. A better selectivity was obtained on YMC-Pack ODS-AQ column using a gradient of 95 to 100% eluent B (MeOH + 0.1% formic acid) in 20 min. A baseline-separation of oat CERs from the SC components was achieved by isocratic elution (90% eluent B for 5 min) followed by sequential linear gradients from 90 to 95% eluent B in 5 min and then the gradient mentioned earlier (95 to 100% eluent B) for 20 min. The positive mode APCI ionization allowed reliable detection of the target CERs with a high precision. The APCI conditions such as ionization temperature were also adjusted to improve the peak intensity of the target CERs. A higher degree of ionization was obtained at APCI vaporizer temperature of 450 °C and capillary temperature of 275 °C.

The method development was first started with LCQ ion trap instrument. Under the collision conditions of a 3D-ion trap mass analyzer (with resonance activation and helium used as a collision gas), the quasi-molecular ions of the predominant d18:1$^{\Delta 8}$-based oat CERs were not effectively involved in fragmentation. Therefore, due to very low abundances of corresponding product ions, MS/MS-based quantification in a selected reaction monitoring (SRM) mode is hardly applicable to this type of CERs [19, 21]. On the other hand, d18:2$^{\Delta 4,8}$/t18:1$^{\Delta 8}$-based CER ions are readily fragmented under CID conditions resulting in intense signals of product ions. At the later stage of method development and validation, we shifted to another instrument with triple quadruple mass analyzer (due to instrumental failure). To be consistent with the already

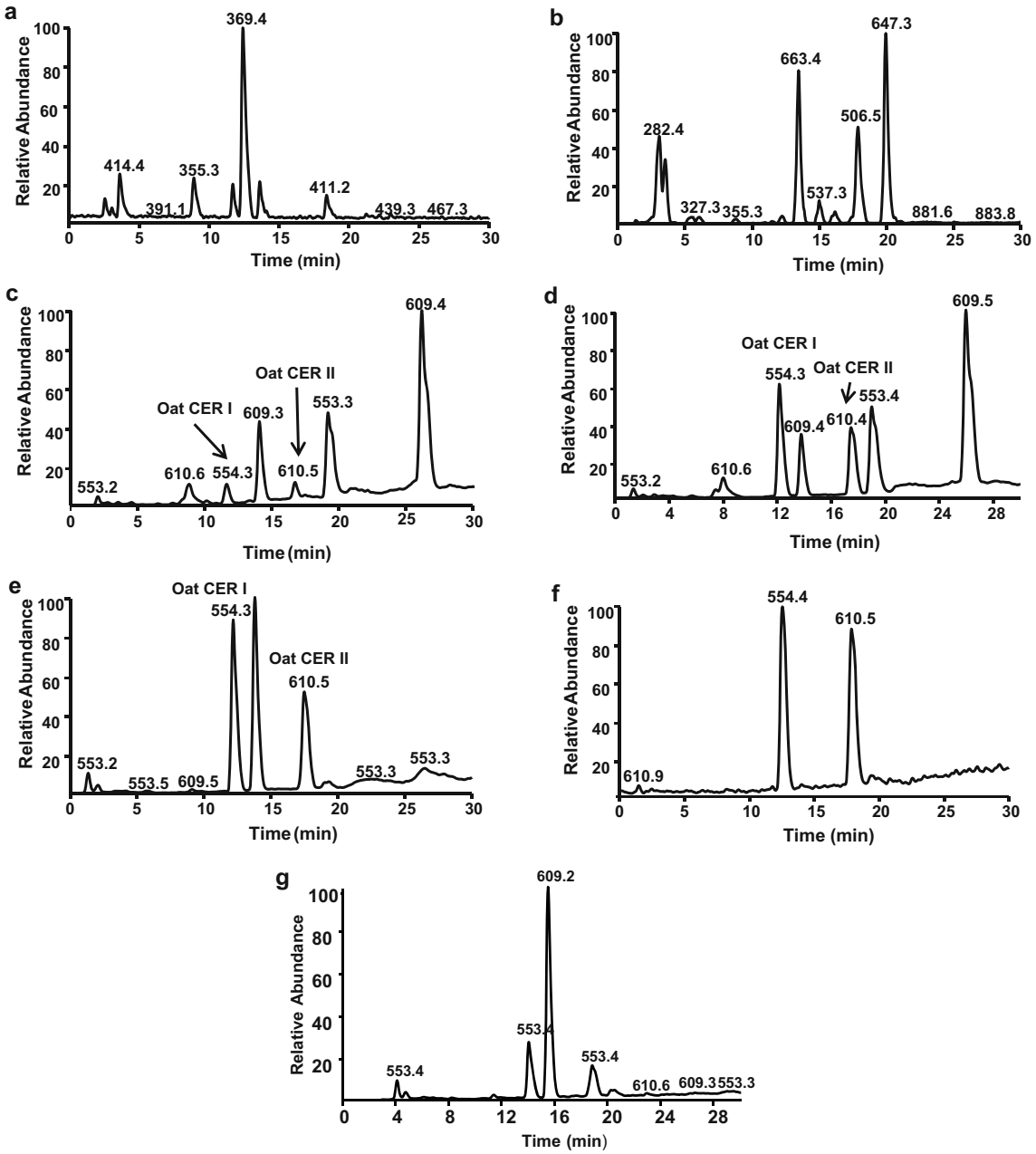

Fig. 1 Base peak chromatograms of SC (**a**) and whole skin (**b**) extracts obtained in Q1 full scan mode. The LC-MS chromatograms of SC extract spiked with oat CERs (30 ng/mL, **c**) and 900 ng/mL (**d**, **e** for two different SC extracts) acquired in SIM mode (*m/z* 554 and *m/z* 610). While (**f**) depicts the LC-MS chromatogram of oat CERs (30 ng/mL) in MeOH, (**g**) shows the chromatogram of SC extract obtained in SIM mode (*m/z* 554 and *m/z* 610)

optimized method and to get comparable data, the method validation was conducted in the later instrument under SIM scanning mode. The method specificity has also been assured by the triple quadruple instrument (see section 'Selectivity and specificity').

Method validation

In order to validate the method developed and demonstrate its reliability for the quantification of oat CERs in the skin layers, the system was challenged with skin lipid extracts obtained from different individuals. The lipid extracts of the SC, deeper skin layers (epidermal and dermal skin slices), and full-thickness skin were also used as matrix.

Selectivity and specificity

As stated earlier, the method is mainly designed for the quantification of oat CERs permeated into the skin following the application of various topical formulations and skin-care

products. One of the limitations in investigating CER penetration into the skin is the lack of sensitive analytical method differentiating exogenous CERs from endogenous CERs and other lipids. Therefore, a new method was needed to separate the skin components from the target oat CERs. The potential interference of the components of each skin lipid extract with the two oat CER peaks was individually analyzed. The base peak chromatograms of SC and full thickness skin lipid extracts are shown in Fig. 1a, b, respectively. Representative LC-MS chromatograms of SC extract spiked with low and high concentrations of oat CERs are depicted in Fig. 1c–e. The target oat CERs (*m*/*z* 554.3 at retention time of 11.9 min and *m*/*z* 610.5 at retention time of 17.0 min) are baseline-separated from the potential interferences (such as *m*/*z* 553.3 and *m*/*z* 609.4). Figure 1f shows the chromatogram of oat CERs in MeOH. Figure 1g depicts the LC-MS chromatogram of SC extract obtained in SIM mode (*m*/*z* 554 and *m*/*z* 610). As can be seen from Fig. 1g, no other interfering peaks have been detected within the retention time windows of both target CERs. Therefore, the method effectively differentiated the oat CERs from the endogenous components of all the extracts including CERs.

Among the epidermal CERs, the sphingosine-based CERs containing hydroxypalmitic acid and hydroxy arachidic acid (CER [AS]-C16 and CER [AS]-C20) are isobaric or isomeric with oat CERs, the difference being the position of the double bond on the SB. Unlike d18:1$^{\Delta 8}$-based oat CERs, d18:1$^{\Delta 4}$-based epidermal CERs readily loses water moiety in the ion source under the APCI conditions. As a result, the most abundant ions were represented by the corresponding neutral losses $[MH\text{-}H_2O]^+$ and, hence, parent CERs $[MH]^+$ were detected at relatively low intensities (particularly in SC extracts spiked with low concentrations of oat CERs) during SIM mode scanning (*m*/*z* 554 and *m*/*z* 610).

To ensure a sufficient specificity of the method, SC extracts spiked with oat CERs were additionally analyzed by LC-MS/MS in a product ion scanning mode. To illustrate applicability of this strategy, we complemented a Q1 full scan (which is less specific than SIM) with product ion scanning at *m*/*z* 554 and 610 at the collision energies of 20–50 eV. The resulted QqQ-MS/MS fragmentation patterns are presented in Fig. 2a, b. The tandem mass spectra dominated with characteristic losses of water ($[MH\text{-}H_2O]^+$), whereas intensities of the signals representing $[MH\text{-}FA]^+$, $[MH\text{-}FA\text{-}H_2O]^+$, and $[MH\text{-}FA\text{-}2H_2O]^+$ were much lower. Therefore, the interference of target analytes detection with isobaric compounds present in the skin samples is avoided due to a high selectivity of chromatographic separation. The proposed mechanism of CER fragmentation is presented in Fig. 2c.

Matrix effect and carry-over

During the method development, emphasis was given to avoid the possible interferences and matrix effects of the components of the SC extracts. As can be seen from Table 1, the mean MF values were found to be around 100% at 90 ng/mL spiking level indicating a negligible

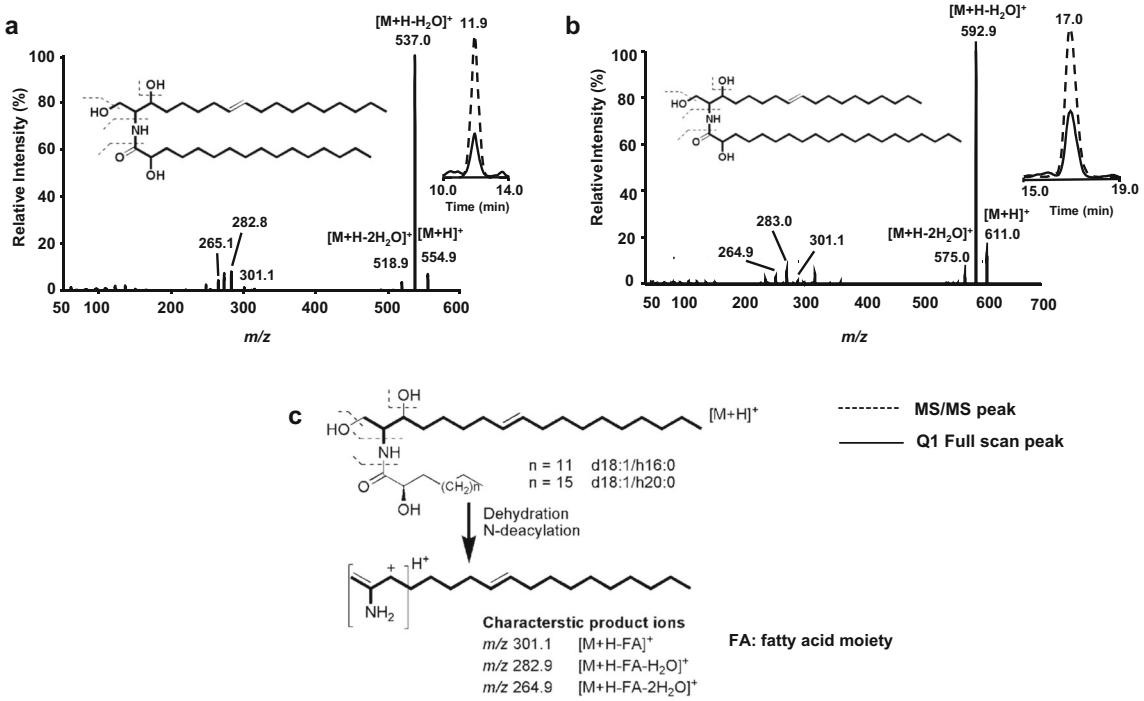

Fig. 2 MS/MS fragmentation of oat CERs (**a**, **b**) at CID 20 eV and suggested fragmentation pattern (**c**) [27].

Table 1 Validation of the RP-UHPLC-APCI-MS method for quantification of oat CERs in the skin

	Retention time (min)	S/N	LOD (ng/mL)	LOQ (ng/mL)	Percentage recovery	Mean of MF (%)[a]	RSD of MF (%)[a]
CER I (*m/z* 554.3)	11.9	8.1	10.0	30.8	103.7	101.1	5.2
CER II (*m/z* 610.5)	17.0	8.0	9.3	29.5	94.9	102.5	4.2

[a] Skin extracts were spiked with 90 ng/mL of oat CERs

effect of sample matrix on ionization of the analytes. The mean MF values of spiking at high concentration level (900 ng/mL) were also close to 100% (data not shown). The RSD values observed for MFs also indicate that the ionization process was not significantly affected by the presence of matrix. Therefore, the matrix effects in the current analytical system can be considered as insignificant.

The carry-over effect of the method was assessed by injecting high concentration of oat CERs (1050 ng/mL) followed by blank samples (MeOH and SC extracts). No peak was detected in the retention windows of the oat CERs indicating that the method is free of carry-over effect.

Sensitivity

The estimated LOD and LOQ of the method are shown in Table 1. The spike level (25 ng/mL) was in the recommended range, i.e., LOD < spike level < 10 × LOD with reasonable percentage recoveries (103.7 and 94.9% for oat CER I and CER II, respectively). Accordingly, the method's LOD and LOQ were estimated to be around 10 and 30 ng/mL for each oat CER, respectively. The method was found to be sensitive enough to detect and quantify the trace amounts of oat CERs in the deeper layers of the skin, as supported by the results from the skin permeability studies described below.

Linearity

As stated earlier, the method was optimized to avoid the matrix effect (mean MF values around 100%) and, hence, it was not needed to use matrix-matched calibration. Thus, calibration curves of oat CERs were constructed in MeOH. The LC-MS method showed linear responses over a concentration range from 30 to 1050 ng/mL for both CERs. The slope and intercept of the linear equations were found to be as follows: slope 4582.5 ± 358.0 and intercept 2658.1 ± 216.7 for oat CER I with R^2 of 0.9997 ± 0.0001 and slope 3538.7 ± 285.1 and intercept 38,379.6 ± 3293.2 for oat CER II with R^2 of 0.9991 ± 0.0001. The mean accuracy and the RSD values of the back-calculated concentrations of the calibration standards are shown in Table 2. The RSD values of the back-calculated concentrations are within the acceptable limits (± 15% of the nominal value).

Precision and accuracy

The results of the method's precision and accuracy are presented in Table 3. The RSD of within-run and between-run precision ranged from 1.2 to 7.3 and 1.2 to 7.8%, respectively. On the other hand, the recovery (%) ranged from 94.1 to 109.2 and 92.5 to 109.4% for within-run and between-run experiments, respectively. The results suggest that the method is accurate and precise for the quantification of oat CERs in the skin layers.

Table 2 Mean accuracy of back-calculated concentrations of calibration standards for the RP-UHPLC-APCI-MS method for quantification of oat CERs in the skin ($n = 3$)

Nominal concentration (ng/mL)	Back-calculated concentration (ng/mL)		Mean accuracy (%)		RSD (%)	
	CER I	CER II	CER I	CER II	CER I	CER II
30	29.5	28.5	98.4	95.1	8.2	6.0
50	49.8	50.8	99.6	101.6	2.3	4.1
100	101.3	98.5	101.3	98.5	2.5	3.6
200	186.6	183.0	93.3	91.5	7.6	7.8
400	416.0	403.0	104.0	100.7	8.9	8.8
500	515.7	520.7	103.1	104.1	8.1	8.7
900	912.3	915.6	101.4	101.7	4.3	5.8
1050	1048.1	1053.6	99.8	100.3	1.1	2.0

Table 3 Within-run and between-run precision and accuracy of RP-UHPLC-APCI-MS method for quantification of oat CERs in the skin ($n = 5$)

Nominal concentration (ng/mL)	Oat CERs	Calculated concentration (ng/mL) ($n = 5$) Within-run	Recovery (%)			RSD (%) ($n = 5$)	
			Between-run	Within-run	Between-run	Within-run	Between-run
30	CER I	28.8	29.3	95.9	97.6	7.3	3.5
	CER II	32.8	32.8	109.2	109.4	3.7	4.8
90	CER I	84.7	83.2	94.1	92.5	2.6	3.5
	CER II	95.1	97.3	107.5	108.1	1.2	5.5
400	CER I	421.2	429.1	105.3	107.3	2.4	7.8
	CER II	425.3	430.3	106.3	107.6	4.0	6.0
800	CER I	829.4	830.8	103.7	103.9	2.2	1.8
	CER II	836.0	872.8	104.5	109.1	1.5	1.2

Application of LC/APCI-MS method in ex vivo permeation studies

The skin permeation of exogenous CERs such as PhytoCERs from the various topical dosage forms needs to be investigated to demonstrate their potential application in improving the barrier function of diseased, aged, and/or affected skin. Even though the CERs are intended for the SC delivery, depending upon the delivery systems used and the components of the formulations, the CERs can possibly penetrate into the deeper layers of skin. The CER skin permeation studies, therefore, require sensitive and selective analytical methods for quantifying the exogenous CERs in the SC, viable EP, and DR of the skin. Against this background, the applicability of the current LC-MS method for the investigation of oat CERs permeation across the different layers of skin was studied. It has been shown that CERs have poor penetration across the skin from conventional formulations such as ointments and creams [28] and, hence, the oat CERs were incorporated into amphiphilic cream (0.25%) in attempt to detect and quantify the small quantities of CERs distributed across the skin layers using the validated LC-MS method.

The extraction of oat CERs from the skin samples was carried out by using *n*-hexane/ethanol (2:1, *v*/*v*) as this solvent mixture was shown to be an effective solvent for extraction of CERs from the skin samples [17, 18, 28]. For exhaustive extraction of oat CERs, the treatment with organic solvents was carried out overnight after sonication for 30 min at 40 °C. The actual concentrations of oat CERs measured in the skin slices ranged from 49 to 675 ng/mL. The extraction recoveries were found to be 83.4, 87.9 and 83.2% for the incubation periods of 30, 100, and 300 min, respectively. Despite the fact that over 85% of the oat CERs remained in the cream after the three incubation periods, the method quantified the minute quantities of oat CERs that penetrated into and distributed across the skin layers (Table S2 in the ESM, and Fig. 3a, b).

The main barrier (rate-limiting step) in CER transport across the skin is the SC. The small portion of oat CERs which

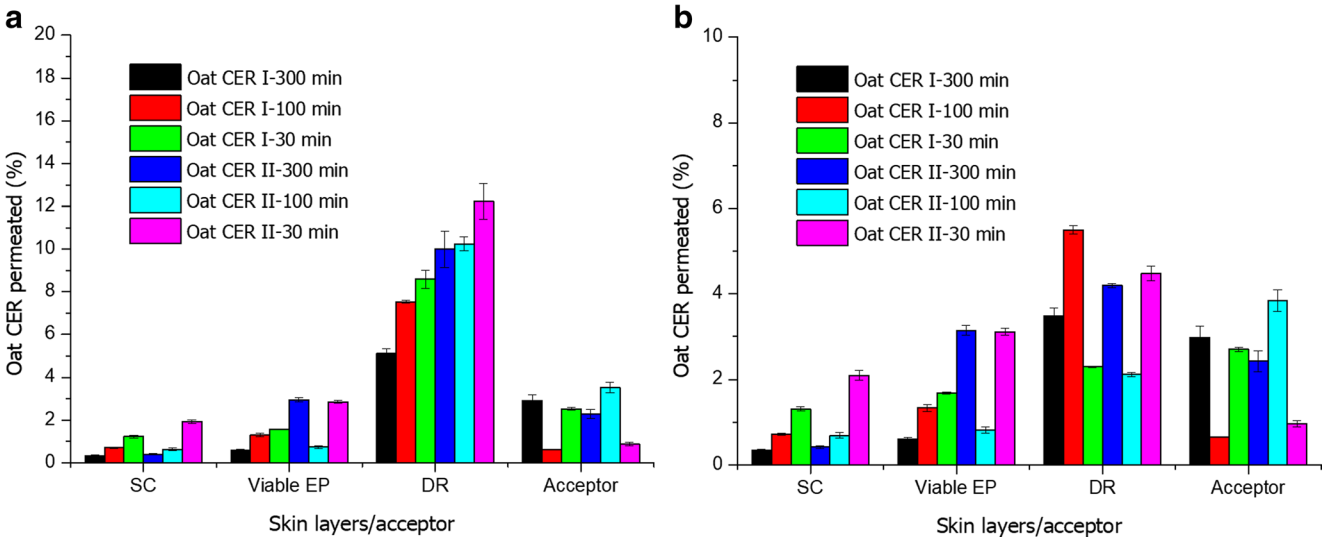

Fig. 3 Percentage of oat CERs permeated (± SD) into the various layers of the skin from an amphiphilic cream containing oat CERs: SC, viable EP (EP1 + EP2), DR (For **a**: DR1 + DR2 + DR3 + remaining skin tissue and for '**b**' without the remaining skin tissue) and acceptor (filter gauze + acceptor fluid)

has overcome the SC barrier distributes into the viable EP and DR layers as well as the acceptor. The longer the penetration time is, the higher the possibility of detecting the CERs in the acceptor fluid. Thus, oat CERs were not detected in the acceptor fluid after 30 and 100 min of incubation periods (only detected in filter gauze). The thickness of the remaining skin tissues varies in the skin samples used in the three incubation periods. The thickness variation affected the quantities of oat CERs considered as DR (Fig. 3a). Figure 3b shows the permeation profile of oat CERs without considering the remaining skin tissues (DR = DR1 + DR2 + DR3). The normalized amount of oat CERs (ng/10 μm skin slice) permeated and distributed across the various skin layers is shown in Table S3 in the ESM. The interesting thing we can see from the table is that the portion of oat CERs penetrated into the skin is mainly concentrated in the SC where the CERs are needed. Therefore, formulation strategies enhancing the penetration of oat CERs and localizing them in the SC should be designed and further investigated. The method clearly indicated the depth and extent of penetration of oat CERs into excised human skin. The method, therefore, can be used to investigate the skin distribution of oat CERs from the various dermal and transdermal formulations including nano-sized systems such as microemulsions and nanoparticles which are shown to improve the skin permeability of drugs.

Conclusions

A simple, sensitive, selective, precise, and accurate LC/APCI-MS method with no or minimal matrix effect was developed and validated for the quantification of oat-derived CERs in the skin layers. The applicability of the method to real samples was tested using an amphiphilic cream containing oat CERs. The method allowed selective quantification of oat CERs distributed across the various skin layers. Due to good performance of the proposed method, it can be used to investigate the penetration of oat CERs into the skin from the various topical dosage forms.

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Compliance with ethical standards

An ethical clearance was obtained from the Ethics Committee of the Faculty of Medicine, Martin Luther University Halle-Wittenberg for the SC lipid extraction and ex vivo permeability studies. An informed consent was also obtained from the volunteer subjects.

Conflict of interest The authors declare that they have no conflict of interest.

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