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Stable isotope dilution analysis of salicylic acid and hydroquinone in human skin samples by gas chromatography with mass spectrometric detection

Anja Judefeind [a], Peet Jansen van Rensburg [b,*], Stephan Langelaar [c], Jeanetta du Plessis [a]

[a] *School of Pharmacy, North-West University, Potchefstroom Campus, Potchefstroom 2520, South Africa*
[b] *School of Biochemistry, North-West University, Potchefstroom Campus, Potchefstroom 2520, South Africa*
[c] *Uniqema, Gouda, The Netherlands*

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Abstract

A sensitive and accurate gas chromatographic–mass spectrometric (GC–MS) method has been developed for the quantitative determination of salicylic acid (SA) and hydroquinone (HQ) from human skin samples and cosmetic emulsions. Deuterium labeled SA-d_6 and HQ-d_6 were used as internal standards (IS). The samples were extracted with methanol, dried under nitrogen and derivatized with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS). Quantification was performed in SIM mode with a limit of quantification (LOQ) of 50 ng ml^{-1} for SA and 10 ng ml^{-1} for HQ. The inter-day variation (R.S.D.) was less than 5% and the accuracy was better than 13.3% for both compounds. The recoveries from the different matrices ranged between 93.1 and 103.3% for SA, and 97.3 and 100.8% for HQ.

Keywords: Hydroquinone; Salicylic acid; GC–MS; BSTFA; Skin penetration; Emulsion

1. Introduction

Salicylic acid (SA) is widely used in pharmaceutical, topical preparations as a keratolytic for the treatment of acne, psoriasis and warts as well as an antimicrobial agent. Hydroquinone (HQ) is a known skin depigmenting agent. It is available in cosmetic skin-toning products for skin lightening and in pharmaceutical preparations for the treatment of melasma and hyperpigmentation.

Both compounds are often employed in skin penetration studies to determine penetration properties, to test vehicle effects and for risk assessment [SA: 1–3, HQ: 4, 5]. Various analytical methods for quantitative determination of SA and HQ in skin samples from different species and in topical formulations exist. These include high-performance liquid chromatography (HPLC) [SA: 2, 6–9, HQ: 10, 11], capillary electrochromatography (CEC) [12], micellar electrokinetic chromatrography (MEKC) [13], spectrophotometry [11], biocatalytic sensor [14] and liquid scintillation spectrometry [SA: 2, HQ: 5]. Many of these methods require purification procedures of the samples to avoid interferences with matrix components. Common used separation procedures are liquid–liquid extraction, solid phase extraction [9] and micro-dialysis [8,10]. These methods can be time-consuming, inefficient (e.g. liquid–liquid extraction from emulsions) and might require high quantities of solvents.

The aim of this work was to develop a gas chromatographic–mass spectrometric (GC–MS) method for the quantitative determination of SA and HQ, respectively, in human skin samples and in cosmetic emulsions of various viscosities. By using the mass spectrometer (MS) in selected ion monitoring (SIM) mode, the analytes can be detected specifically in the presence of matrix components and therefore labor intensive purification procedures are circumvented. Moreover, the same method can be used for samples of various matrices (e.g. different emulsions, tape stripping samples, epidermis/dermis samples). An additional benefit is the sensitivity of the MS detection operated in SIM mode. Therefore, a GC–MS method can be utilized for the assay of low concentrations of SA and HQ.

There exists, to the best of our knowledge, no analytical method in literature for the assay of SA and HQ in human skin

* Corresponding author. Tel.: +27 18 299 2305; fax: +27 18 293 5303.
E-mail address: Peet.Jansenvanrensburg@nwu.ac.za (P.J. van Rensburg).

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samples and various cosmetic emulsions employing GC–MS. However, GC–MS methods have been developed for determination of SA in plant materials [15–18], olive oil [19], urine [20], plasma [21,22] and biological samples (rat plasma and rat liver homogenate) [23]. Different derivatization procedures are described including silylation with *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) [15,19,23], BSTFA + 1% trimethylchlorosilane (TMCS) [22], *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) [18], benzylation [21] as well as methylation with diazomethane [16,17] and methyl iodide [20]. Even fewer GC–MS methods for the determination of HQ are described in literature, e.g. the assay of HQ in urine [24,25] and water [26,27]. Procedures employed for the derivatization of HQ are silylation with BSTFA [25], hexamethyldisilazane (HMDS) [24], *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) and trimethyliodosilane (TMIS) [27] as well as alkylation with *n*-hexyl chloroformate in water [26].

The analytical procedure described here was utilized in a study to investigate influences of vehicle (various emulsions) effects on skin uptake and skin permeation. The method was specific and therefore SA and HQ could be detected from samples of various matrices. Furthermore, the analytical procedure was precise and accurate enough to establish small differences in the effects of the various emulsions on skin uptake and skin permeation. The data of the permeation study will be published elsewhere.

2. Experimental

2.1. Chemicals

Analytical grade hydroquinone and salicylic acid were purchased from Fluka (Buchs, Switzerland) and Sigma Aldrich (Dorset, UK), respectively. The stable isotopes hydroquinone-d_6 (HQ IS) (98.5%-d_6, >99% chemical purity) and 2-hydroxybenzoic acid-d_6 (SA IS) (98.7% d_6, >99% chemical purity) were obtained from Dr. Ehrenstorfer (Augsburg, Germany) and were used as internal standards. *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA, 99% purity) with 1% trimethylchlorosilane (TMCS, 99.6% purity) was obtained from SUPELCO (Bellefonte, Pennsylvania, USA). Dried pyridine (max. 0.0075% H_2O, ≥99.5% purity) was purchased from Riedel de Haën (Seelze, Germany). Methanol (Hypersolv^{TM}, HPLC grade) was obtained from BDH (Dorset, UK) and absolute ethanol (Chromasolv^{TM}, HPLC grade) was purchased from Sigma Aldrich (Johannesburg, South Africa). Disodium dihydrogen ethylenediaminetetraacetate (disodium EDTA) was obtained from BASF (Cheshire, UK) and sodium disulfite was purchased from Merck (Darmstadt, Germany). Deionized water from a Millipore Milli-Q purification system was employed.

2.2. Standard preparation

Stock solutions of SA and SA IS with a concentration of 1 mg ml^{-1} were prepared in methanol. In the case of HQ, stabilizers were required to prevent HQ oxidation. Therefore, stock solutions of HQ and HQ IS with a concentration of 1 and 5 mg ml^{-1}, respectively, were prepared using an aqueous stabilization solution of 0.02% (w/v) disodium EDTA and 0.015% (w/v) sodium disulfite (200 mg disodium EDTA + 150 mg sodium disulfite in 1 l deionized water). Disodium EDTA (for chelating metal ions) and sodium disulfite (as antioxidant) were used for the stabilization of HQ as the various emulsions, applied during the penetration studies, contained these substances to prevent oxidation of HQ. Additionally, the exposure of HQ to light and air (not in solution) were minimized during the experiments, sample preparation and storage. The stock solutions of SA and HQ were diluted to 500 and 50 ng ml^{-1} with methanol. The stock solutions of the internal standards were diluted to a concentration of 500 ng ml^{-1} for SA IS and 250 ng ml^{-1} for HQ IS with methanol. Standard solutions were stored at 4 °C. The stability of SA, SA IS, HQ and HQ IS in methanol and stabilization solution was determined and the various stock solutions were stable for at least 1 month at 4 °C.

For the preparation of calibration curves different volumes of SA standard solutions (25, 50 and 100 μl of 50 ng ml^{-1} and 25, 50 and 100 μl of 500 ng ml^{-1}) and HQ standard solutions (10, 25 and 50 μl of 50 ng ml^{-1} and 10, 25 and 40 μl of 500 ng ml^{-1}) were pipetted into micro-inserts (100 μl pulled point glass, Agilent®) of autosampler vials (1.5 ml glass screw capped vial, Agilent®) to obtain concentrations of 25, 50, 100, 250, 500 and 1000 ng ml^{-1} of SA and 10, 25, 50, 100, 250 and 400 ng ml^{-1} of HQ after adding 50 μl of derivatization mixture. Additionally, 20 μl of 500 ng ml^{-1} of SA IS standard was added to the SA calibration samples and 20 μl of 250 ng ml^{-1} of HQ IS standard was added to the HQ calibration samples. The final concentrations of the internal standards in the calibration samples were 200 ng ml^{-1} SA IS and 100 ng ml^{-1} HQ IS, respectively, in 50 μl of the derivatization mixture. After addition of standard solutions into micro-inserts of autosampler vials the samples were carefully dried under a stream of nitrogen and derivatized with 25 μl BSTFA containing 1% TMCS and 25 μl pyridine. Derivatization proceeded for 30 min at 40 °C.

2.3. Skin sample preparation

The various samples for analysis comprised receptor fluid samples (Re samples), stratum corneum samples (SC samples), residual skin samples consisting of viable epidermis and dermis (rest skin samples) and donor phase samples (Do samples) which were obtained during skin penetration studies with human female abdominal skin (method will be published elsewhere).

The Re samples consisted of a water/ethanol mixture 1:1 (v/v) for SA and an aqueous stabilization solution/ethanol mixture 1:1 (v/v) for HQ. SC samples were prepared by tape stripping the surface of the skin (3 M Scotch® Magic^{TM} Tape) followed by extraction of the active ingredient with methanol (SA samples) and aqueous stabilization solution (HQ samples), respectively. The Do samples, consisting of various applied cosmetic emulsions, and the rest skin samples were also extracted using the same solvents as for the SC samples. A specified amount of internal standard was immediately added to all the skin sam-

ples. The amount of required internal standard was calculated to obtain the same concentrations as in the calibration samples after appropriate dilution, evaporation of solvent and adding 50 μl of the derivatization mixture. The samples were extracted over a period of at least 24 h whilst maintained at 4 °C until analysis. Prior to analysis, the samples were brought to room temperature for equilibration, vortex mixed for 1 min and diluted with the appropriate extraction fluid as required. A specified volume was then pipetted into micro-inserts of autosampler vials. The samples were dried under a stream of nitrogen and derivatized with 25 μl BSTFA containing 1% TMCS and 25 μl pyridine. Derivatization proceeded for 30 min at 40 °C.

2.4. Sample preparation for recovery experiments

An investigation was conducted to determine the extent of derivatization as well as extraction of the actives from skin samples. Concentrations of 750 ng ml^{-1} SA and 200 ng ml^{-1} SA IS were prepared in methanol and in the different skin samples, respectively. The skin samples were obtained from a diffusion experiment performed under the same condition as the actual penetration experiments (method will be published elsewhere), however utilizing blank emulsions containing no actives. Since different emulsions were used in the penetration studies, a mixture of the various blank emulsions was added for the recovery experiments. After completion of the experiments, the skin samples were divided in two aliquots and one of the aliquots was spiked with SA and SA IS as described above and the other served as a blank skin sample to test for interferences. Lower dilutions of the skin samples (compared to the skin samples in the penetration studies later) were prepared to ensure inclusion of the highest amount of impurities as they also consume BSTFA for derivatization. The sample prepared in methanol (without impurities) was utilized as the control sample.

The same experiments were carried out with HQ. A mixture of aqueous stabilization solution and methanol 1:1 (v/v) was substituted for pure methanol. Furthermore, HQ solutions were prepared at different concentrations of HQ (500 ng ml^{-1}) and HQ IS (100 ng ml^{-1}).

The samples were stored for 24 h at 4 °C and prepared for derivatization on the next day as described for the skin samples. The derivatized samples were subjected to GC–MS analysis in SIM mode (as described in Section 2.5) and recovery was determined by comparing the spiked skin sample with the control sample.

2.5. GC–MS analysis

The trimethylsilyl (TMS) derivatives of HQ and SA (structural formula shown in Fig. 1) were analyzed by gas chromatography using an Agilent® 6890 Plus GC equipped with an Agilent® 5973 Mass Selective (MS) Detector in electron-impact (EI) mode (70 eV, dwell time of 100) and a split/splitless injector with a single-taper splitless glass inlet liner (900 μl) with deactivated glass wool. All injections (injection volume of 1 μl) were performed with an Agilent® 7673 auto sampler. The injector was maintained at 270 °C and operated in the splitless mode at 0.8

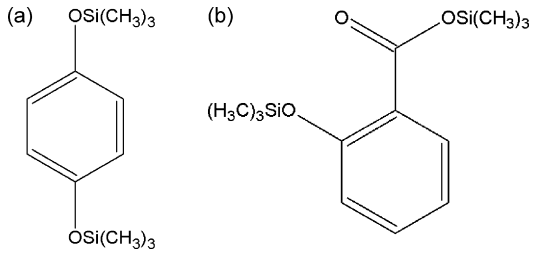

Fig. 1. Structural formula of (a) HQ-TMS (MW: 254) and (b) SA-TMS (MW: 282).

bars, with a purge flow-rate of 21.3 ml min^{-1} after 1.00 min. A 30 m length × 250 μm and 0.25 μm film thickness DB-5 column (Agilent®) was used for all analyses. Ultra high purity helium was utilized as carrier gas and the column flow was maintained at 1.2 ml min^{-1} throughout the run. The initial oven temperature was set at 80 °C with a 2.0 min hold period and was then ramped to 265 °C at 20 °C min^{-1} and finally ramped from 265 to 300 °C at a rate of 30 °C min^{-1}. The oven was maintained at 300 °C for 2.0 min for a total run time of 14.42 min. For analysis of SA, the oven was regulated at 300 °C for 10.0 min. The transfer line was set at 280 °C and the source temperature was 230 °C. The MS was operated in the selected ion monitoring (SIM) mode. Ions monitored for the derivatized HQ samples were *m/z* 254 (HQ) and 258 (HQ IS). Ions monitored for the derivatized SA samples were *m/z* 268 (SA) and 272 (SA IS).

2.6. Quantitative analysis

The ratio of the peak areas of the active ingredient to the internal standard from the various calibration standards were plotted versus the concentrations of the active in these calibration standards. The mass of the active in the various skin samples could be calculated with the calibration curves and the known dilution factor. Plots, regressions and calculations were performed and constructed with Excel® (Microsoft Corp. Seattle, USA).

3. Results and discussion

3.1. Derivatization

In view of the fact that the underivatized SA and HQ are not volatile enough (with boiling points of 211 and 286 °C, respectively) for gas chromatographic analysis and because of their polarity, derivatization was required to convert them to more volatile and less polar derivatives. Trimethylsilylation of hydroxyl and carboxyl groups utilizing BSTFA containing 1% TMCS was chosen for this study as it is a commonly used derivatization procedure to replace active hydrogen with a trimethylsilyl group. TMCS as a silylation catalyst was used to facilitate the reaction and pyridine was added to the BSTFA/1% TMCS mixture to capture the released protons. The TMS derivative of each compound did no longer contain polar groups and could be eluted as a narrow peak with good peak symmetry.

In literature various temperatures and durations of derivatization can be found for SA and HQ [15,19,22,23,25]. In our

study, several temperatures and durations (18 h at 20 °C, 15 and 30 min at 40 °C, 15 min at 60 °C) as well as two different ratios of BSTFA with 1% TMCS/pyridine mixture (1:1 and 7:3 (v/v)) were tested. No significant differences occurred between the various temperature procedures. The variation in peak area after incubation at 20 and 60 °C was 3.6% for SA and 1.2% for HQ. Insignificant differences, as also seen for double injections of the same sample, might have resulted from different injection volumes, small changes in column temperature or injector. Furthermore, slight differences between different samples could be explained by experimental error during sample preparation (pipetting small volumes). Therefore, it is necessary to incorporate an appropriate internal standard to obtain more reproducible results (i.e. lower R.S.D. in double injections). Lower peak areas were integrated with the BSTFA containing 1% TMCS/pyridine ratio of 7:3 (v/v) compared to the ratio of 1:1 (v/v) for both compounds, SA and HQ. The increase in peak area from the ratio 7:3 (v/v) to the ratio 1:1 (v/v) was 12% for SA and 7.5% for HQ.

Identical experiments were performed in SCAN mode with 1 and 0.5 μg ml^{-1} concentrations of SA and HQ, respectively. The results confirmed the previous data as no differences could be established between the various temperature procedures. Moreover, no secondary peaks, indicating potential degradation product(s) of SA-TMS or HQ-TMS, occurred in the total ion chromatograms.

These results indicate that room temperature (20 °C) was sufficient to complete trimethylsilylation of SA and HQ as no increase in peak area occurred at higher temperatures. In subsequent experiments the derivatization procedure of 30 min at 40 °C with a BSTFA containing 1% TMCS/pyridine ratio of 1:1 (v/v) was used to accelerate the trimethylsilylation reaction. The BSTFA with 1% TMCS/pyridine ratio of 1:1 (v/v) was selected as it yielded higher peak areas.

Additionally, tests were performed to monitor the stability of the SA-TMS and HQ-TMS, respectively, as well as their trimethylsilylated internal standards in the derivatization mixture. Therefore, the same sample was injected several times over a certain time period. It was found that SA-TMS + SA IS-TMS were stable for at least 20 h and HQ-TMS + HQ IS-TMS for at least 24 h. This suggested a single day loading of samples during the analyses.

3.2. Chromatography

The mass spectra of SA-TMS and SA IS-TMS are shown in Fig. 2. The molecular ions of SA-TMS (*m/z* 282) and SA IS-TMS (m/z 286) were unobservable or very weak. The mass spectra of HQ-TMS and HQ IS-TMS are given in Fig. 3. The mass ions show that both functional groups were derivatized for all compounds. The mass fragment pattern of SA-TMS and HQ-TMS were confirmed by computer library (Nist 98) matching. The 4-*m/z* shift in the observed ions of the internal standard compared to the ions of the standard is due to the substitution of four hydrogen atoms by deuterium atoms and was observed for both actives, SA and HQ.

The quantitative determination of SA and HQ was performed in SIM mode for higher sensitivity and specificity. SIM (*m/z*

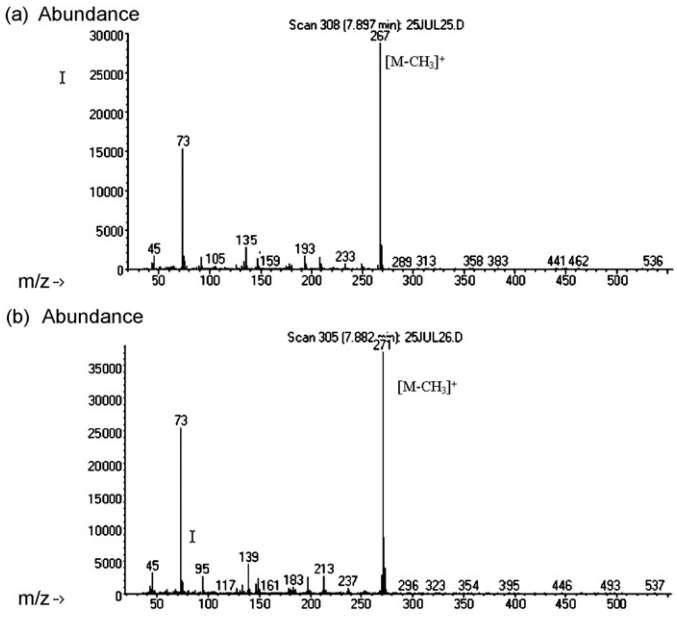

Fig. 2. Mass spectra of standard (a) SA (1 μg ml^{-1}) and (b) SA IS (μg ml^{-1}) as their TMS derivatives.

268 and 272) chromatograms for SA-TMS and SA IS-TMS, respectively, and SIM (*m/z* 254 and 258) chromatograms for HQ-TMS and HQ IS-TMS are shown in Fig. 4. All chromatograms show a symmetrical, narrow peak for each analyte and respective internal standard. The retention times for SA-TMS (*m/z* 268) and SA IS-TMS (*m/z* 272) are identical at about 7.9 min. The same occurred with HQ-TMS (*m/z* 254) and HQ IS-TMS (*m/z* 258) with retention times of 7.1 min.

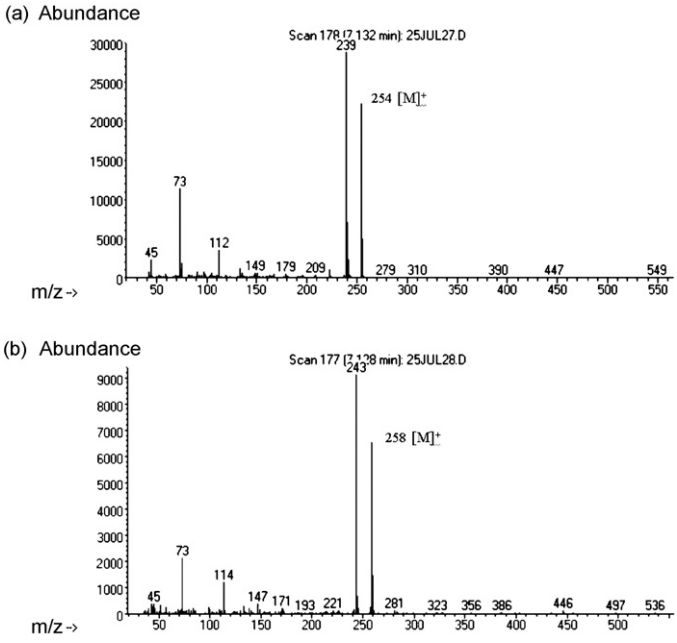

Fig. 3. Mass spectra of standard (a) HQ (0.5 μg ml^{-1}) and (b) HQ IS (0.5 μg ml^{-1}) as their TMS derivatives.

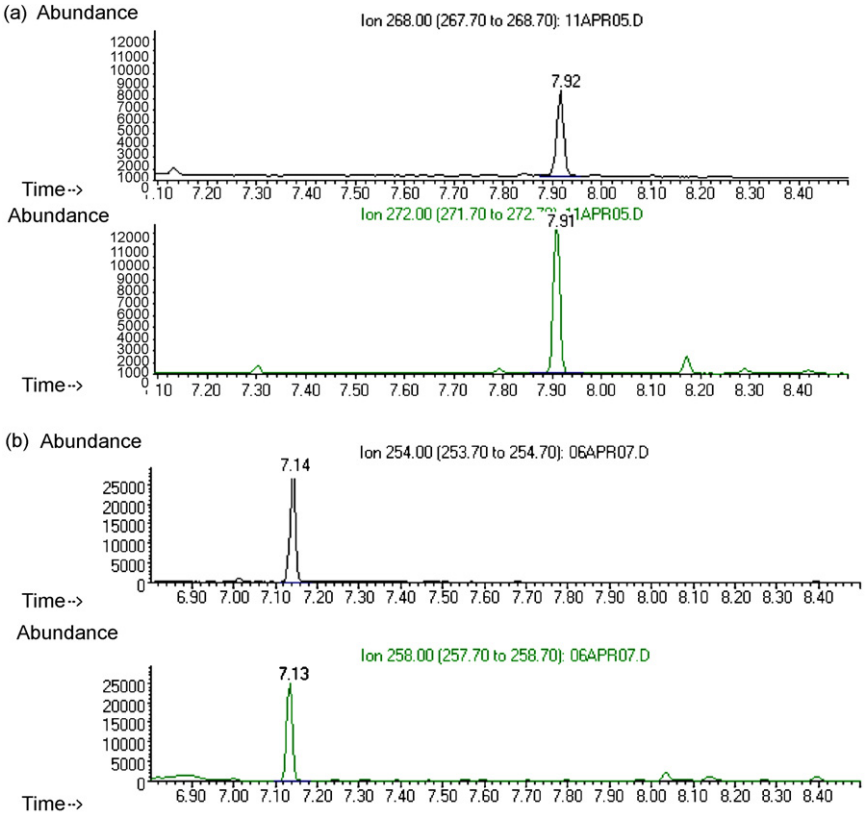

Fig. 4. (a) SIM chromatogram (*m/z* 268 and 272) of standard SA sample (100 ng ml^{-1}) with SA IS (200 ng ml^{-1}) after derivatization and GC-MS analysis as described in text. (b) SIM chromatogram (*m/z* 254 and 258) of standard HQ sample (100 ng ml^{-1}) with HQ IS (100 ng ml^{-1}) after derivatization and GC-MS analysis as described in text.

3.3. Monitored ions in SIM mode

Various ions for the different compounds were tested in SIM mode and investigated for peaks at the actives' retention times to be able to choose the ion for each analyte. The investigated ions and results are shown in Table 1. The ions were tested for sensitivity as well as for interference with skin samples. An ion was found to be sufficiently sensitive when a concentration of 100 ng ml^{-1} of analyte or 200 ng ml^{-1} of IS could be detected with symmetric peak shape and with a R.S.D. value (double injection) lower than 2%. Re samples were used for the interference test as they might contain extracted skin lipids, proteins, amino acids, etc. According to the guideline for bioanalytical method development from the US Food and Drug Administration (FDA) [28], a minimum of six different skin donors should be employed to establish the specificity of the method. The availability of human skin is limited and therefore only two skin donors were used to test for interference. However, during diffusion studies a blank Re sample was collected from each skin donor used in the experiments and analyzed for interference.

For SA the highest sensitivity was seen with the $[M–CH_3]^+$ ion at *m/z* 267 (Table 1a), although interference with the skin samples occurred. On average, slightly less interference was detected with the ion at *m/z* 268 as no peak was obtained with

Table 1a
Peak areas of skin, standard SA and SA IS samples at analytes' retention time obtained in SIM mode of various ions

m/z	Skin donor 1	Skin donor 2	SA 100 ng ml^{-1}	SA IS 200 ng ml^{-1}
SA				
209	–	–	1633	n.a.
267	288	266	18987	532
268[a]	–	51	5257	498
269	–	–	1956	n.a.
SA IS				
271	–	n.a.	–	40995
272[a]	–	n.a.	–	8364

Derivatization and GC–MS analysis as described in text; n.a.: not analyzed.
[a] Selected ions for analysis.

Table 1b
Peak areas of skin, standard HQ and HQ IS samples at analytes' retention time obtained in SIM mode of various ions

m/z	Skin donor 1	Skin donor 2	HQ 100 ng ml^{-1}	HQ IS 200 ng ml^{-1}
HQ				
239	450	496	27467	n.a.
240	–	–	6022	n.a.
254[a]	–	173	20534	1133
255	2672	1427	4956	n.a.
HQ IS				
258[a]	–	n.a.	–	41188

Derivatization and GC–MS analysis as described in text; n.a.: not analyzed.
[a] Selected ions for analysis.

skin donor 1 and a small interference peak with skin donor 2 which resulted in the same percent interference as with the ion at *m/z* 267. Although the abundance of the ion at *m/z* 267 was approximately four times higher, the ion 268 was selected for further analysis. The lower sensitivity was sufficient to analyze the skin samples and the employment of the ion 267 would have necessitated in higher dilutions of some skin samples. However, it should be emphasized that the sensitivity of the method for SA can be increased by using the SIM mode at *m/z* 267 and therefore quantitative determination of SA concentration below 50 ng ml^{-1} (LOQ) is achievable. The SA sample did not exhibit a peak at *m/z* 271 and 272 which were the tested ions for SA IS. Similar observations were made for the skin samples at *m/z* 271 and 272. Consequently, the SA sample and skin samples did not interfere with the SA IS ions. For SA IS the ion 272 was chosen for SIM mode, although the intensity was lower than for 271. The reason for this decision was that for the same concentration of SA and SA IS, respectively, the abundance of the SA peak at *m/z* 268 and the SA IS peak at *m/z* 272 were similar. Contrary to the SA sample, the SA IS sample showed a peak at the selected SA ion (*m/z* 268). This interference might be attributed to an impurity in the stable isotope standard (impurity with SA or another deuterated SA compound) or to an ion fragment of SA IS-TMS in its mass spectrum.

The ions for HQ and HQ IS (Table 1b) were selected by a similar procedure. The largest peak areas for HQ were obtained with the ions at *m/z* 239 and 254. Due to the interferences with skin samples, the ion at *m/z* 239 was rejected and the ion at *m/z* 254 was selected for HQ. The small peak obtained with skin donor 2 could be considered insignificant as a high concentration of blank matrix skin sample (implying a high concentration of impurities) was used to test for interference. The skin samples in further analyses were more diluted and therefore impurities were insignificant. An interfering peak was considered insignificant when the peak area of interfering compounds was less than 2% of the peak area of the analyte at the limit of quantification (LOQ). For HQ IS the ion at *m/z* 258 was preferred, because no interference with skin samples appeared and the sensitivity of the HQ IS ion at *m/z* 258 was comparable to the sensitivity of the HQ ion at *m/z* 254 when the same concentrations of both compounds were used. As it was already seen with the SA and SA IS samples, no peak was obtained with the HQ sample tested for the HQ IS ion (*m/z* 258). However, the HQ IS sample showed a peak at *m/z* 254 indicating interference.

Though interference of the internal standards with their respective analytes was observed, the selected ions (SA: 268, SA IS: 272, HQ: 254 and HQ IS: 258) were used for further analyses. The advocating of these procedures was that the concentrations of the internal standards were equal in all the samples (calibration samples as well as skin samples) and therefore, the same amount of impurities or interferences will occur in the various samples.

3.4. Calibration curve

Three calibration curves, prepared in methanol, were analyzed on three different, inconsecutive days. The regression coefficients exceeded 0.999 for all calibration curves and consequently, the correlation between the ratio of peak areas (analyte/IS) and concentration of analyte was found to be linear for both compounds within the tested concentration ranges. The linear regression lines were described by the equations:

$$y = 0.00581x + 0.04881 \quad \text{for SA} \tag{1}$$

$$y = 0.01291x - 0.01232 \quad \text{for HQ} \tag{2}$$

which were the average of the three calibration curves from three different days. Another set of calibration curves were generated in various matrices (blank Re sample, blank SC sample and blank Do sample) and compared with the calibration curves prepared in methanol. No significant differences were observed between the various regression lines. As a result and the fact that no significant interference occurred from the impurities of the various matrices, further calibration samples were prepared in methanol.

3.5. Accuracy and precision

The values of the three calibrations of each analyte were used to establish accuracy and precision for each concentration level. The results are presented in Table 2. Accuracy is defined as the percent deviation of the found concentration (C_{found}) from the added concentration (C_{acced}) and precision is expressed as the relative standard deviation (R.S.D.). According to the FDA [28], accuracy and precision should be determined from a minimum of five determinations per concentration level. A test of six injections of a single sample resulted in R.S.D. values of 0.71% for SA and 0.56% for HQ. Due to the low R.S.D. values the calibration samples were only injected in duplicate.

The accuracy and precision of the analytical method is within the recommendation of the FDA with values below 15% for both. These values for precision and accuracy of the FDA were used as an indication, though they pertain to the validation in the biological matrix and not in methanol. The accuracy of 25 ng ml^{-1} of SA was with a value greater than 20% (criterion for LOQ according to FDA [28]) not acceptable. Thus, the limit of quantification was set at 50 ng ml^{-1} for SA. The LOQ of HQ was 10 ng ml^{-1}, as it could be determined accurately and precisely.

3.6. Recovery experiments

The recovery of SA from the different skin samples compared to the control was as follows: 103.3% from the Do sample, 98.1% from the SC sample, 93.1% from the rest skin sample and 96.8% from the Re sample. The impurities in the various blank skin samples (without SA and SA IS) did not interfere with the analysis of SA and SA IS as no peaks in the SIM (*m/z* 268 and 272) chromatograms occurred at the retention time of SA and SA IS, except of an insignificant peak (peak area smaller than 2% of peak area of SA at LOQ) with the blank SC sample.

The following recoveries of HQ were obtained: 100.8% from the Do sample, 99.9% from the SC sample, 97.3% from the rest skin sample and 100.3% of the Re sample. No peaks were found at the retention times of HQ and HQ IS, respectively, for the

Table 2
Accuracy and precision of the assay of SA and HQ

C_{added} (ng ml^{-1})	C_{found} (ng ml^{-1}) mean ± S.D.[a]	Accuracy (%)		Precision (R.S.D.)	
		Intra-day[b]	Inter-day[a]	Intra-day[b]	Inter-day[a]
SA					
25	33.8 ± 1.2	30.0 to 38.5	35.3	≤1.23	3.4
50	56.6 ± 0.3	12.8 to 13.9	13.3	≤0.90	0.5
100	96.2 ± 3.8	−7.0 to 0.4	−3.8	≤0.39	3.9
250	244.9 ± 5.2	−4.3 to −0.3	−2.0	≤0.39	2.1
500	484.7 ± 11.1	−5.4 to −1.0	−3.1	≤0.64	2.3
1000	1008.8 ± 22.4	−1.3 to 3.2	0.9	≤0.59	2.2
HQ					
10	11.1 ± 0.1	9.7 to 12.3	11.2	≤0.50	1.2
25	26.8 ± 0.8	5.2 to 11.2	7.4	≤0.43	3.1
50	52.9 ± 1.8	3.3 to 9.9	5.8	≤0.92	3.4
100	94.6 ± 4.1	−9.4 to −1.2	−5.4	≤0.47	4.3
250	246.4 ± 10.4	−5.7 to 2.6	−1.4	≤0.44	4.2
400	403.1 ± 20.1	−2.8 to 6.5	0.8	≤0.18	5.0

[a] $n = 3$.
[b] $n = 2$/day, values for 3 days.

monitored ions at *m*/*z* 254 and 258 after injecting the various blank skin samples, except of the blank SC sample which showed a minor peak (peak area smaller than 2% of peak area of HQ at LOQ) in the SIM (*m*/*z* 254) chromatogram.

The recovery experiments demonstrated that the extraction and derivatization procedures were complete up to a concentration of 750 ng ml^{-1} SA and 500 ng ml^{-1} HQ in various skin samples.

3.7. Skin samples analysis

The method was used for the quantitative determination of approximately 300 SA and 400 HQ skin samples (data not presented). The R.S.D. of the double injection did not exceed 1.5% with few exceptions between 1.5 and 2.5%. A calibration curve was run preceding each set of analysis to control the sensitivity of the glass inlet liner and the DB-5 column. The R.S.D. of the slopes was 3.0% for SA (14 calibrations) and HQ (19 calibrations), respectively. The correlation coefficients exceeded 0.999 for all calibration curves of SA and exceeded 0.995 for the calibrations of HQ. Three concentration levels (low, medium, high) of each calibration set were selected to determine accuracy and precision during the time of analysis. The results are shown in Table 3.

Table 3
Accuracy and precision of control samples during skin sample analysis

C_{added} (ng ml^{-1})	C_{found} (ng ml^{-1}) mean ± S.D.	Accuracy (%)	Precision (R.S.D.)
SA ($n = 14$)			
50	55.1 ± 2.4	10.1	4.4
500	494.6 ± 12.9	−1.1	2.6
750	752.7 ± 30.8	0.4	4.1
HQ ($n = 19$)			
10	10.3 ± 0.3	3.2	2.7
250	250.5 ± 8.0	0.2	3.2
400	400.8 ± 20.0	0.2	5.0

An additional control was the calculation of the total recovery of the active at the end of the diffusion studies. Thus, the sum of Do sample, SC sample, rest skin sample and all Re samples of each diffusion cell was assessed and referred to the amount of the applied active which was also determined for each cell. According to Diembeck et al. [29], the total recovery of the compound is recommended to be 100 ± 15%. Thirty cells were run with SA and the total recovery was 100.8 ± 2.8% (mean ± S.D.). The recoveries of SA were between 98 and 104% with the exception of three cells (93% and two cells of 107%). Diffusion studies of HQ were carried out with thirty-six cells and a total recovery of 98.2 ± 3.2% (mean ± S.D.) was obtained. The recoveries of HQ were between 95 and 103% with the exception of three cells (88, 91 and 94%). In general, the recovery of the active from each cell was within 100 ± 5% with few exceptions which were within the recommended 100 ± 15%.

These recovery values corroborate that no loss of analyte during the experiments and sample treatment (resulting in lower recoveries) as well as no interference with impurities (resulting in higher recoveries) occurred. That indicates that the extraction from the different samples and the derivatization were complete and no binding to Franz cell surfaces, glass vials or tubing took place. Furthermore, it confirms the stability of HQ and SA during the experiments, storage and sample handling as well as the reliability of the analytical method. It has been mentioned in earlier work [30,31] that sublimation of SA might arise during solvent evaporation. The good reproducibility and high recovery values of the obtained SA data indicate that sublimation was not an issue during this assay. The slight lower recovery values for HQ might indicate a minor degradation of HQ most probably due to oxidation during the extraction time where skin discs with the applied HQ formulations were exposed to light and air.

4. Conclusions

A reliable, sensitive and selective method for the determination of SA and HQ in various human skin samples and emulsions

using GC–MC has been presented in this paper. It was shown that the quantitative determination of SA and HQ was accurate and precise in the concentration range between 50 and 750 ng ml^{-1}, and 10 and 400 ng ml^{-1}, respectively, and good recoveries of 750 ng ml^{-1} SA and 500 ng ml^{-1} HQ were obtained from the various matrices. The sample preparation and the derivatization procedures were simple and rapid. Possible interferences with occurring impurities could be circumvented by utilizing the SIM mode which additionally resulted in higher sensitivity. Accuracy, precision and recovery improved by the use of the respective deuterated compounds as internal standards.

The present method was used for the analysis of skin samples obtained during penetration studies. The consistent results over a longer time period of analysis showed the robustness of the method. The results demonstrated that the analytical method was suitable for the assay of low concentrations of SA and HQ in a higher number of skin samples and could be applied in similar studies in the future.

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