Talanta 81 (2010) 530–536

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

A gas chromatography–mass spectrometric method to determine skin-whitening agents in cosmetic products

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ARTICLE INFO

Article history:
Received 21 August 2009
Received in revised form
15 December 2009
Accepted 21 December 2009
Available online 4 January 2010

Keywords:
Cosmetic
Fraud detection
Gas chromatography–mass spectrometry
Skin-bleaching agent
Skin-whitening agent

ABSTRACT

An analytical method is proposed here to determine three allowed (kojic acid, azelaic acid and arbutin) and two forbidden (resorcinol and hydroquinone) skin-whitening agents in cosmetics. The method is based on gas chromatography coupled with mass spectrometric detection, which allows the two prohibited target compounds to be identified unequivocally by means of their mass spectra acquired in full-scan mode at the retention time of these compounds. Owing to their low volatility, target analytes are derivatized using N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) (containing 1% trimethylchlorosylane) prior to injection into the chromatographic system. The accuracy and precision of the proposed method were evaluated by analysing a laboratory-made skin-whitening cosmetic sample containing known amounts of the five target compounds. The method can be easily applied by the manufacturers in the quality control of their own products, and also by safety authorities to prevent fraudulent operations and thus look after consumers' health. The proposed method was successfully applied to the analysis of nine commercial skin-whitening cosmetic products, showing that fraud was committed in four of the analyzed samples.

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1. Introduction

Currently, brown skin-spots or staining represent one of the most important aesthetic problems in humans [1]. This skin disorder, a consequence of melanin excess produced by hyperactivity of melanocytes, could have different causes, such as overexposure to solar radiation, ageing, hormonal dysfunctions during pregnancy or taking certain medicines like contraceptives, among others [2–4].

Although these spots are not normally harmful to health, many people are affected by them and seek specialist advice to try to remove these anti-aesthetic marks from their skin. Thus, apart from the most serious cases, which could require medical treatment, in most cases a cosmetic treatment can be employed, based on skin-whitening (also referred to as skin-bleaching) products. These cosmetic products contain so-called skin-whitening agents, which act by inhibiting melanin biosynthesis via different mechanisms [5–7].

Different compounds have been used as skin-whitening agents in cosmetics [8]. Among them, hydroquinone (HQ) (see Fig. 1) was one the most commonly used due to its well-known effectiveness [9–10]. Its use as a whitening agent in cosmetics (up to 2%) was first allowed in the European Union in 1984 by the Commission Directive 84/415/EEC [11]. However, its use was restricted to hair-dye products and artificial nail systems sixteen years later by Commission Directive 2000/6/EC [12], since different safety problems were attributed to its use in skin-whitening cosmetics, such as ochronosis, irritation, allergy [10,13–14], and particularly due to its carcinogenic properties over long-term use [15]. Furthermore, there is evidence of its percutaneous absorption, and it has been found to be present in the body fluids of users of skin-whitening cosmetics containing HQ [16–17], which makes its toxic potential more serious. Nevertheless, it can be used in specialised pharmaceutical products, which are not considered cosmetics, thus being regulated by the legislation concerning pharmaceutical products. Likewise, in the United States framework, HQ was allowed at concentrations up to 1.5–2% in skin-whitening products [18] (higher concentrations are only permitted in prescription drugs), which are considered over-the-counter drugs, but it has recently been banned in this type of product by the United States Food and Drug Administration [19].

Whitening properties have also been attributed to resorcinol (RS), an isomer of HQ (see Fig. 1) [20–21], but its use as skin-whitening agent in cosmetics is not permitted in either the European Union or United States.

Arbutin (ARB), which is a glucosylic ether of HQ (see Fig. 1), has been proposed as a good alternative to HQ. Although it is less effective, it does not present the side-effects attributed to the use of HQ. Other skin-whitening agents used extensively are kojic acid

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doi:10.1016/j.talanta.2009.12.037

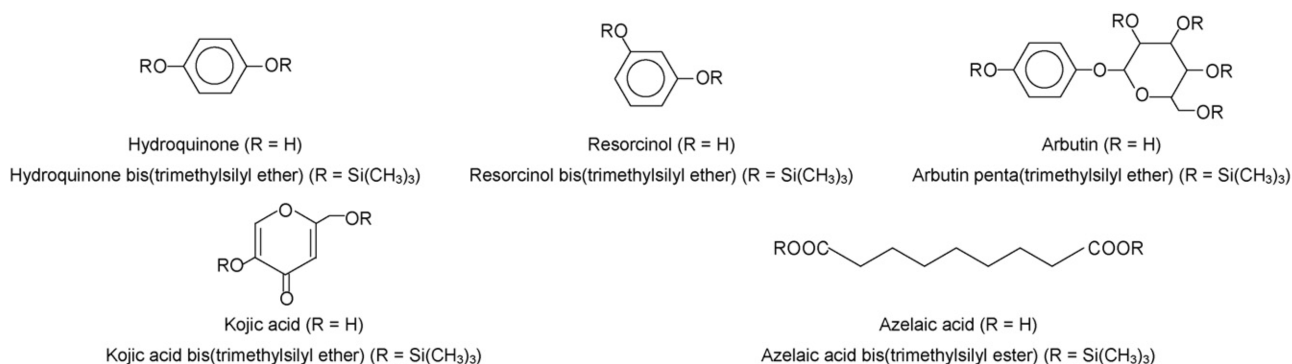

Fig. 1. Molecular structure of the skin-whitening agents being studied (R=H) and their corresponding silylated derivatives (R=$Si(CH_3)_3$).

(KA) and azelaic acid (AZA) (see Fig. 1), although they are also much less effective than HQ. Ascorbic acid and some of its derivatives have also been used at length as whitening agents, although their effectiveness is also lower than HQ.

Taking all this into account, skin-whitening products should be controlled for various reasons: on one hand, by the manufacturers themselves, in order to assure that they contain the correct amount of whitening agent, i.e. to assure the efficacy of these products; and, on the other hand, by safety authorities, to prevent fraudulent use of both HQ and RS in this type of product, i.e. to assure user safety.

However, no official analytical methods have been found in the literature, except those proposed by the EU Commission [22] and the United States Pharmacopeia [23]. In the former, HQ (and some of its ethers, but not including ARB) can be identified by means of thin-layer chromatography (TLC), followed by their quantitative determination using liquid chromatography (LC) with ultraviolet/visible detection. In the latter, HQ can be quantified by means of titration with cerium sulphate using diphenylamine as indicator.

Therefore, the development and validation of analytical methods to control skin-whitening cosmetic products is of great interest, as they will assure the efficacy and safety of this type of cosmetic product. A bibliographic search revealed more than 30 publications focusing on the determination of skin-whitening agents in cosmetics, which were recently critically reviewed [8]. Analytical techniques where determination is carried out in the liquid phase are preferred over others dealing with the vapour phase, owing to the low volatility of these compounds, with LC being the technique of choice. There is only one published article where skin-whitening cosmetics are analyzed by using gas chromatography (GC) [24], in which the determination of HQ is carried out.

The aim of this paper focuses on developing a reliable analytical method to determine three of the most commonly used skin-whitening agents (ARB, KA and AZA), which are often combined in modern skin-whitening cosmetics, as well as two prohibited skin-whitening agents (HQ, RS) using the potential GC offers. The method couples GC with a mass spectrometric (MS) detector, enabling forbidden skin-whitening agents to be detected at low concentrations, as well as reliably identifying them by means of their mass spectra.

Owing to the low volatility of the target compounds for GC analysis, a derivatization step was carried out by using a silylation agent to convert them to more volatile derivatives (see Fig. 1).

As was recently reviewed [8] and bibliographically search updated, there are no published papers reporting RS determination in skin-whitening cosmetics, and neither has a wide-ranging analytical method been published to determine HQ, ARB, KA and AZA together.

2. Experimental

2.1. Apparatus

A Focus GC gas chromatograph, equipped with an AI 3000 autosampler and coupled to a DSQ II mass spectrometric detector, from Thermo Fisher Scientific (Austin, TX, USA) was employed.

An ultrasonic water bath from Selecta (Valencia, Spain) was used to improve sample solving process in the cases of difficulty in solubilization.

2.2. Reagents and samples

Resorcinol (RS) >99% from Fluka (Buchs, Switzerland), arbutin (ARB) >99% from Bioland Ltd. (Chungnam, Korea), and kojic acid (KA) 99.2%, hydroquinone (HQ) >99%, and azelaic acid (AZA) >99% all from Guinama S.L. (Valencia, Spain) were used as standards. Pentachlorobenzene 99.8% from Riedel-de Haën (Seelze, Germany) was used as internal standard (IS) to minimize possible deviations occurred during the GC injection process.

LC-grade N,N-dimethylformamide (DMF) from Scharlau Chemie (Barcelona, Spain) was used as solvent to prepare standard and sample solutions.

N,O-bis-(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosylane (BSTFA) from Sigma–Aldrich (Steinheim, Germany) was used as derivatization reagent for GC analysis.

A laboratory-made skin-whitening cream containing different amounts of the target skin-whitening agents (0.60%, 1.01%, 1.07%, 2.17% and 2.31%, for RS, HQ, KA, AZA and ARB, respectively) was prepared in our laboratory by following a common procedure employed in the cosmetic industry. These formulations contained different usual cosmetic ingredients employed in this type of cosmetics (emollients, surfactants, smoothing agents, UV filters, moisturizing agents, preservatives), which were of cosmetic-grade and were purchased from Guinama S.L.

Nine commercial skin-whitening cosmetic creams (samples labelled from A to I) were analyzed. They were from different laboratories, and for reasons of confidentiality, the names will not be shown.

2.3. Proposed method

2.3.1. Sample and standard solutions preparation

A stock solution containing 50 $\mu g\,mL^{-1}$ of pentachlorobenzene was prepared in DMF.

On one hand, a multi-component stock standard solution containing 5 $\mu g\,mL^{-1}$ of both RS and HQ, 15 $\mu g\,mL^{-1}$ of both KA and AZA, and 10 $\mu g\,mL^{-1}$ of ARB was freshly prepared daily in DMF. Aliquots of 40, 80, 120, 160 and 200 μL were transferred to five

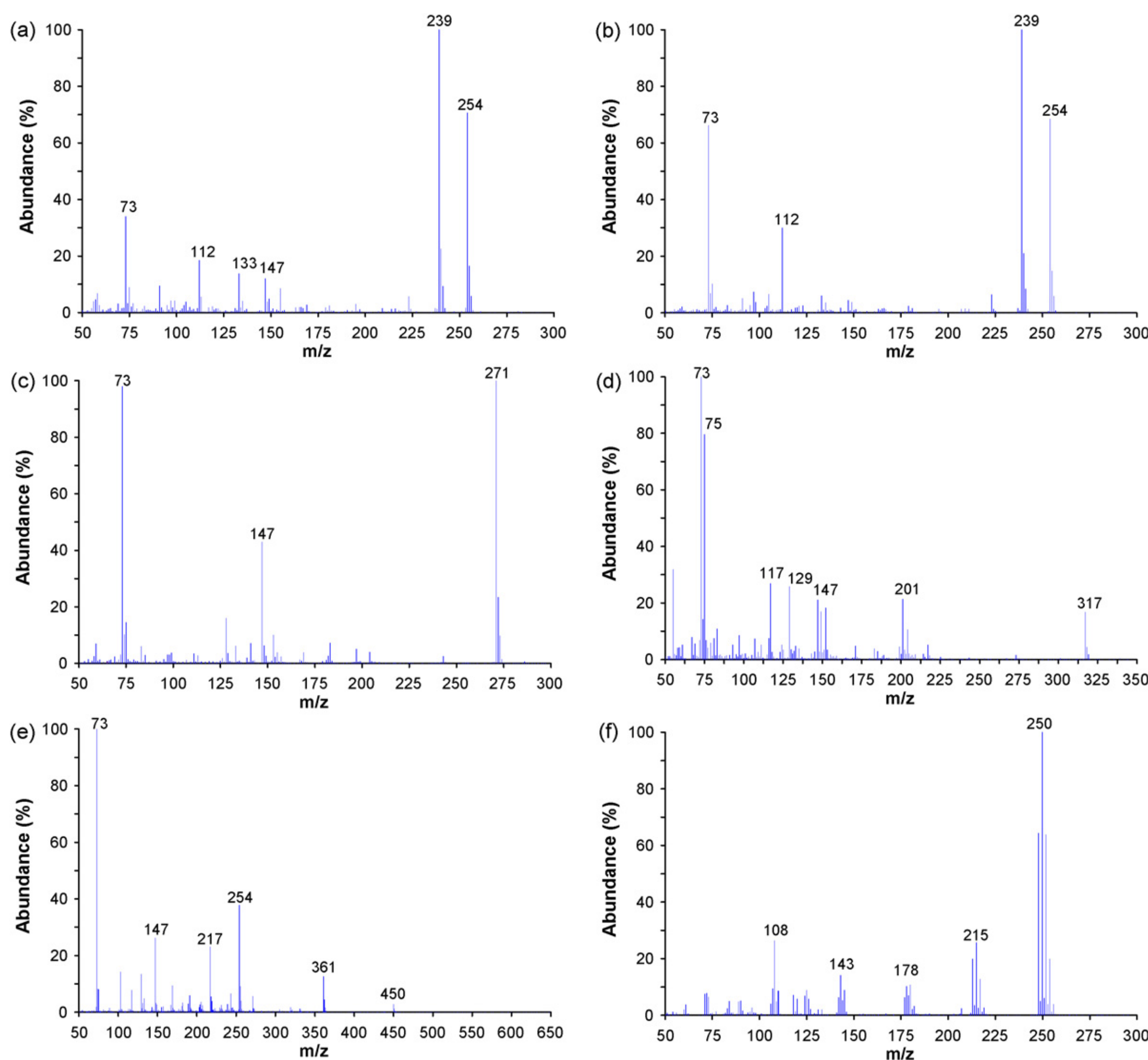

Fig. 2. Mass spectra of resorcinol bis(trimethylsilyl ether) (a), hydroquinone bis(trimethylsilyl ether) (b), kojic acid bis(trimethylsilyl ether) (c), azelaic acid bis(trimethylsilyl ester) (d), arbutine penta(trimethylsilyl ether) (e) and pentachlorobenzene (f).

injection vials (1.5 mL volume), respectively, to which DMF was added up to 200 μL, to prepare a set of standard solutions ranging from 1 to 5 μg mL^{-1} for both RS and HQ, 3 to 15 μg mL^{-1} for both KA and AZA, and 2 to 10 μg mL^{-1} for ARB. Next, 50 μL of pentachlorobenzene solution and 100 μL of BSTFA were added. Then, the vials were sealed and shaken to form the derivatives.

On the other hand, each sample was homogenized and, in triplicate, an appropriate amount (ca. 0.01–0.2 g) depending on the concentration of the target analytes, was weighed into a 10 mL volumetric flask, and dissolved in approximately 5 mL of DMF (at this step, an ultrasonic water bath was used to facilitate sample solving process). Next, the mixture was diluted to the mark with the same solvent. At this point, an additional dilution with DMF can be performed for samples with high content of any of the target analytes. Finally the solution was filtered through a 0.45 μm nylon membrane filter (if necessary). An aliquot ranging from 50 to 200 μL, depending on the concentration of the target analytes, of each sample solution were placed in 1.5 mL injection vials, to which, just like standard preparation, DMF was added up to 200 μL. Afterwards, 50 μL of pentachlorobenzene solution and 100 μL of BSTFA were added to the each vial, which were sealed and shaken to form the derivatives.

2.3.2. GC–MS conditions

Sample and standard solutions (1 μL) were injected in split mode (1:10) into the GC–MS system. A Trace TR-5MS (95% dimethyl–5% phenylpolysiloxane, 30 m, 0.25 mm i.d., 0.25 μm film thickness) analytical column from Thermo Fisher Scientific (Austin, TX, USA) was used. High-purity helium (99.9999%) from Carburos Metálicos S.A. (Paterna, España) was used as carrier gas at 1 mL min^{-1} flow rate. The oven temperature program was: from 120 °C (1 min) at 20 °C min^{-1} to 280 °C (2 min). Both injector and transfer line temperatures were set at 280 °C.

The MS detector operated in electron impact ionization mode with an ionization energy of 70 eV. The source temperature was set at 225 °C. The measurements were carried out in full-scan mode ranging from m/z 50 to m/z 500, with a solvent delay of 3 min. Extracted ion chromatograms at m/z 239 for RS and HQ, m/z 271 for KA, m/z 317 for AZA, m/z 254 for ARB and m/z 250 for pentachlorobenzene were used.

Internal standard calibration (using pentachlorobenzene as internal standard) was used. For this purpose, the ratio of the peak area of each target analyte to pentachlorobenzene (by using the corresponding extracted ion chromatogram) was plotted versus analyte concentration.

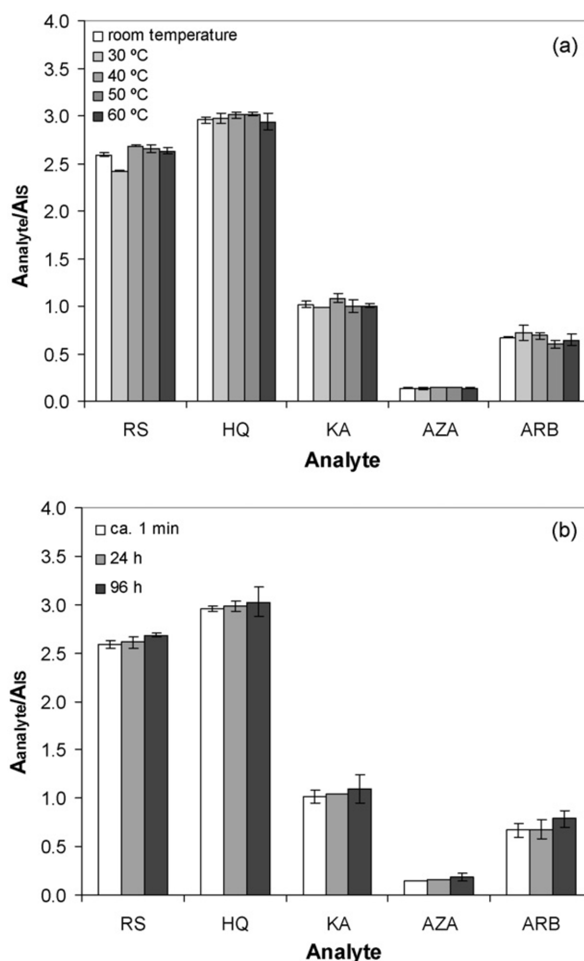

Fig. 3. Effect of temperature (a) and reaction time (b) on the derivatization reaction. The *Y*-axis shows the ratio of the extracted ion chromatogram peak area of each target analyte ($A_{analyte}$) to IS (A_{IS}) (mean of two replicates, where the error charts represent the standard deviation). For experimental details see Section 3.1.2.

3. Results and discussion

3.1. Studies on the derivatization reaction

As mentioned before, owing to the low volatility of the target analytes for GC analysis, a derivatization step was necessary to convert them to more volatile derivatives. Different derivatization strategies, such as silylation, alkylation, esterification or acylation [25–27], have been extensively used for GC analysis, and a huge number of applications in analytical chemistry can be found elsewhere. Moreover, derivatization has the added advantage in that the derivatives have the molecular weight increased, and therefore the sensitivity in mass spectrometry is also improved, since the total ion current is increased. Among derivatization strategies, silylation is by far the major derivatization method used for compounds with labile hydrogens, since the derivatization process can be easily achieved and moreover there are a large number of silylation reagents available. As is well known, when a silylation reagent is used, labile hydrogen of the compound is replaced by an alkylsilyl moiety, usually trimethylsilyl. Thus, the OH moieties are turned into trimethylsilyl ethers, and the COOH moieties into trimethylsilyl esters, these derivatives being more volatile than the parent compounds.

BSTFA (containing 1% trimethylchlorosylane) in DMF was selected to carry out this work. On one hand, the use of trimethylchlorosylane as catalyst, and on the other hand, the use of DMF as acceptor of the releasing protons from reaction, makes easy the derivatization process, which took place rapidly at room temperature as was checked experimentally (see Section 3.1.2 below).

3.1.1. Proposed structures for silylated target analytes and interpretation of the mass spectra

The chemical structures for the target analytes, after silylation, are shown in Fig. 1. These structures are according to the obtained mass spectra (see Fig. 2), which were matched against those found in the NIST Mass Spectral Library. As can be seen, in general the derivatized target analytes exhibit, at different extent, mass fragments at m/z 147 ($[(CH_3)_3Si–O–Si(CH_3)_2]^+$) and at m/z 73 ($[Si(CH_3)_3]^+$), which are characteristic of trimethylsilylated compounds.

The mass spectra of silylated RS and HQ are rather identical. The most intense fragments are located at m/z 254 ($[M]^+$) and at m/z 239 ($[M-CH_3]^+$). Silylated KA exhibits a fragment at m/z 271 ($[M-CH_3]^+$), besides of the abovementioned fragments at m/z 147 and at m/z 73. With respect to AZA, different fragments at m/z 317 ($[M-CH_3]^+$), at m/z 201 $(M-CH_3-CO_2-(CH_3)_2Si(CH_2))]^+$, at m/z 129 (unknown), at m/z 117 ($[CO_2Si(CH_3)]^+$) and at m/z 75 ($Si(CH_3)_2OH]^+$) are observed, besides of those at m/z 147 and at m/z 73. With regard to ARB, fragments at m/z 450 ($[M-HO–Ph–O–Si(CH_3)_3]^+$), at m/z 361 ($[M-HO–Ph–O–Si(CH_3)_3-O–Si(CH_3)_3]^+$), at m/z 254 (unknown) and at m/z 217 ($[(CH_3–O–CH_2–CH{=}CH–O–Si(CH_3)]^+$ are found, besides of those at 147 m/z and at 73 m/z. However, it should be

Table 1
Analytical features of the proposed chromatographic method.

Analyte	RSD in area quotient (%) [a]			Working range [b] (μg mL^{-1})	Intercept	slope (mL μg^{-1})	R^2	LOD [c] (μg mL^{-1})	LOQ [c] (μg mL^{-1})
	Repeatability	Intra-day variation	Inter-day variation						
RS	1.8	4.1	5.1	1–5	−0.003 ± 0.004	0.259 ± 0.001	0.9998	0.1	0.3
HQ	1.9	4.6	4.3	1–5	0.02 ± 0.01	0.295 ± 0.003	0.9997	0.2	0.6
KA	2.2	5.7	6.1	3–15	−0.01 ± 0.01	0.102 ± 0.002	0.9991	0.5	1.5
AZA	4.1	6.8	8.9	3–15	−0.001 ± 0.002	0.014 ± 0.001	0.9990	0.6	1.8
ARB	5.8	7.8	9.0	2–10	0.004 ± 0.009	0.067 ± 0.002	0.9990	0.5	1.5

[a] Referred to a solution containing 5 μg mL^{-1} of RS, HQ, KA, AZA and ARB (five experiments).
[b] In all the cases, linear range reaches up to 50 μg mL^{-1} at least.
[c] LOD = limit of detection. LOQ = limit of quantification.

pointed out that despite its molecular weight is 632 g mol^{-1}, no fragments above 450 *m*/*z* are observed.

Fig. 2 also shows the mass spectrum for pentachlorobenzene, where different clusters typical for chlorinated compounds are observed (*m*/*z* 250 [M]$^+$, *m*/*z* 215 [M − Cl]$^+$, *m*/*z* 178 [M − 2Cl]$^+$, *m*/*z* 143 [M − 3Cl]$^+$ and *m*/*z* 108 [M − 4Cl]$^+$).

3.1.2. Effect of temperature and reaction time

The effect of the temperature and reaction time on the silylating reaction was studied.

First of all, 200 μL of a multi-component standard solution (10 μg mL^{-1} of each target analyte in DMF) was mixed with 50 μL of pentachlorobenzene (50 μg mL^{-1}) in a 1.5 mL injection vial, and 100 μL of BSTFA were added. Immediately, the vial was sealed and immersed in a termostatized water bath at the selected temperature during 20 min. Next, the vial was removed and immersed in a water bath at room temperature during 5 min to reach room temperature, and finally it was injected into the GC–MS system. This experiment was done in duplicate at temperatures ranging from room temperature (ca. 27 °C) to 60 °C. Results, which are shown in Fig. 3(a), show that temperature has no effect in the reaction yield.

Finally, in similar way, the reaction time was studied from the minimum possible (ca. 1 min) to 96 h, at room temperature. Results, which are shown in Fig. 3(b), also show that reaction time has no effect in the reaction yield.

Both studies prove that reaction takes place immediately and it is not kinetically dependant for the target analytes. Moreover, results from Fig. 3(b) also show that the derivatives are stable during 4 days at least.

3.1.3. Amount of necessary silylating reagent

The amount of necessary BSTFA was also studied. For this purpose, and taking into account that samples can contain different ingredients with labile hydrogen, a relatively high amount of a model sample was weighed (0.2 g) in a 10 mL volumetric flask. An aliquot of the multi-component standard solution was added in order to have, at least, 50 μg mL^{-1} of the target analytes, and then was diluted to the mark with DMF. 200 μL of this solution was mixed with 50 μL of pentachlorobenzene (50 μg mL^{-1}) in a 1.5 mL injection vial. Volumes ranging from 25 to 200 μL of BSTFA were added at room temperature to different vials, and DMF was also added to reach to an equal volume of 450 μL in all the assayed vials. The resulting solutions were injected into the GC–MS system. Results (not shown) revealed that it was necessary to add at least 75 μL of BSTFA. Nevertheless, 100 μL was preferred in order to ensure the total derivatization of the analytes in case a greater amount of sample should be needed.

3.2. Study of mass-spectrometry acquisition mode

In a first attempt, full-scan acquisition mode was employed in order to achieve the monitoring of the GC chromatograms. Although different peaks coming from silylation by-products could appear, these do not interfere with peaks of the target analytes. However, peaks coming from column bleed could interfere if overlap with target analytes. Moreover, a relatively high number of potentially interfering peaks appeared when samples were analyzed, since samples contain other ingredients that might be silylated as well as the target analytes. These potentially interfering compounds were different depending on the analyzed sample. As example, Fig. 4 shows a total ion chromatogram for a blank (a), for a multi-component standard solution (5 μg mL^{-1}) (c) and for a laboratory-made skin-whitening cosmetic sample (e). Nevertheless, in order to avoid potential interfering peaks (and thus, to increase selectively), and to increase the signal-to-noise ratio, full-scan mode with extracted ion chromatograms were preferred. Fig. 4 shows an extracted ion chromatogram for a blank (b), for a multi-component standard solution (5 μg mL^{-1}) (d) and for a laboratory-made skin-whitening cosmetic sample (f), which are cleaner than the corresponding total ion chromatogram.

3.3. Analytical figures of merit of the proposed method

The repeatability of the area quotient measurements was tested by injecting a derivatized standard solution containing 5 μg mL^{-1} of all the target analytes five times. Results are shown in Table 1, displaying that high precision was achieved.

The reproducibility was evaluated by preparing and injecting a derivatized standard solution containing 5 μg mL^{-1} of all target analytes five times, within the same working session (intra-day variation) or at different working sessions (inter-day variation), respectively. Results (Table 1) show that both intra-day and inter-day variations were less than 10%.

The calibration parameters and limits of detection (LOD) and quantification (LOQ) of the studied compounds are also shown in Table 1. LOD and LOQ were estimated as $3.s_{y/x}/b$ and $10.s_{y/x}/b$ [28],

Table 2
Results obtained on analysing a laboratory-made skin-whitening sample using the proposed method.

Analyte	Real content (% w/w)	Found content ± s[a] (% w/w)	Error (%)
RS	0.60	0.62 ± 0.03	3.3
HQ	1.01	1.03 ± 0.03	2.0
KA	1.07	1.09 ± 0.02	1.9
AZA	2.17	2.12 ± 0.04	−2.3
ARB	2.31	2.34 ± 0.03	1.3

[a] Average values of three determinations ± standard deviation (s).

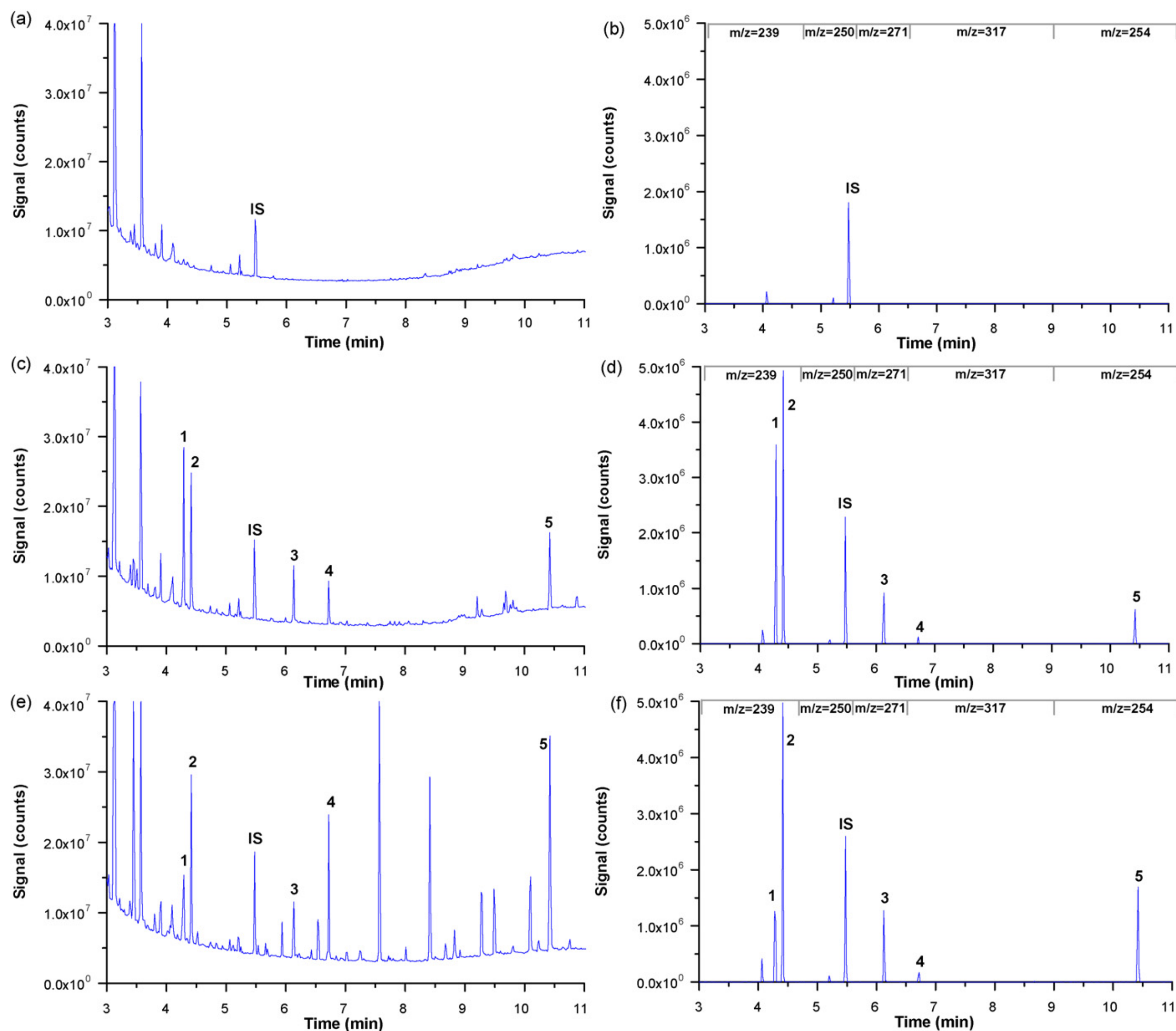

Fig. 4. Total ion chromatogram and extracted ion chromatogram, respectively, for a blank (a and b), for a multi-component standard solution (5 μg mL^{-1}) (c and d) and for a laboratory-made skin-whitening cosmetic sample (e and f) obtained by using the GC–MS proposed method. For experimental conditions see text. Peaks: 1 = resorcinol bis(trimethylsilyl ether), 2 = hydroquinone bis(trimethylsilyl ether), 3 = kojic acid bis(trimethylsilyl ether), 4 = azelaic acid bis(trimethylsilyl ester), 5 = arbutine penta(trimethylsilyl ether), IS = internal standard (pentachlorobenzene).

respectively, where $s_{y/x}$ and b are the residual standard deviation and the slope of the calibration line, respectively.

To estimate the accuracy of this method, a laboratory-made skin-whitening cosmetic product was analyzed (see chromatogram in Fig. 4), which contained known amounts of the analytes. The results are shown in Table 2. The obtained RSD in concentration values ranged from 1.3% to 4.8%, showing precise results. The method has a low relative error (<5%), which demonstrates the good accuracy of the results.

3.4. Application of the proposed method to the analysis of commercial skin-whitening cosmetic products

The method was applied to determine the target skin-whitening agents in 9 commercial skin-whitening cosmetic products (data given in Table 3). As can be seen, there were discrepancies between the results obtained and the label claims. On one hand, samples F and H should have contained KA and AZA according to the label, but

Table 3
Results obtained on analysing commercial skin-whitening cosmetic products using the proposed method.

Sample	Found content ± s[a,b,c] (% w/w)				
	RS	HQ	KA	AZA	ARB
A	ND	ND	2.54 ± 0.04	4.42 ± 0.03	ND
B	ND	ND	0.89 ± 0.02	ND	1.67 ± 0.05
C	ND	ND	ND	ND	1.99 ± 0.08
D	ND	ND	1.43 ± 0.05	ND	ND
E	ND	8.6 ± 0.5	8.5 ± 0.4	ND	ND
F	ND	9.0 ± 0.1	9.3 ± 0.4	ND	ND
G	0.060 ± 0.004	ND	4.7 ± 0.3	ND	ND
H	0.208 ± 0.005	ND	4.9 ± 0.1	ND	ND
I	ND	ND	1.15 ± 0.05	3.85 ± 0.04	ND

[a] Average values of three determinations ± standard deviation (s).
[b] ND = not detected.
[c] According to the label, samples A, F, H and I contain KA and AZA, sample B contains KA and ARB, sample C contains ARB and samples D, E and G contain KA.

no AZA was found when these samples were analyzed. However, HQ was found in the place of AZA in sample F, while RS was found in sample H. Furthermore, HQ was also found in sample E, and RS in sample G, although they were not declared on the label given their use is prohibited. These four samples were produced by the same manufacturer, who is evidently committing fraud, using forbidden ingredients in their cosmetic formulations that could cause damage to human health. Moreover, it should be pointed out that HQ was present at a very high concentration (8.6% and 9.0%, for samples E and F, respectively), which increases its toxicity to an even greater extent.

It should be pointed out that the whole mass spectrum showed that no peak overlapping existed. The mass spectra, jointly with the retention time, provided unequivocal proof of the fraud.

Nevertheless, the authors of this paper have to emphasize that these are isolated cases, and may not be widespread, taking into account the responsibility of the cosmetic manufacturers. To our knowledge, these products were immediately withdrawn after our findings were reported.

4. Conclusions

A GC–MS method is proposed to determine three allowed and two forbidden skin-whitening agents in cosmetics, with good limits of detection. The mass spectra acquired in full-scan mode, jointly with the retention time, offer unequivocal proof of the identity of the target forbidden compounds.

The proposed method is accurate and precise, as can be concluded by the results obtained in the analysis of a laboratory-made skin-whitening sample.

The method can be easily applied both by the manufacturers themselves to perform the quality control of their products, and by safety authorities in order to avoid fraudulent operations and thus look after consumers' health.

Acknowledgements

The authors acknowledge the financial support of the Spanish Government (Projects CTQ2006-00296 and CTQ2009-12709), especially A. Balaguer for his predoctoral grant. The collaboration of Ana Torrens (from Corporación Dermoestética S.L., Paterna, Valencia, Spain) is deeply acknowledged. The authors are also grateful to Prof. Isabel Fernández, from Department of Organic Chemistry at University of Valencia, for her assistance in the interpretation of mass spectra.

The authors would like to make special mention of the contribution of our colleague, Prof. M.C. Pascual-Martí who passed away during this work was being completed.

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