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Guaiazulene in health care products: Determination by GC–MS and HPLC-DAD and photostability test

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ABSTRACT

A liquid chromatographic method with UV detection (HPLC-DAD) and a gas chromatographic method coupled with mass spectrometry (GC–MS) have been developed for the determination of guaiazulene (GA) in complex matrices such as creams and toothpastes. A solid phase extraction (SPE) sample pre-treatment on a polymeric sorbent (Strata-X polymer) was applied before the HPLC analyses, which were performed on a XTerra™ C_8 stationary phase, using a mobile phase consisting of acetonitrile–water 50:50 (v/v). For GC–MS analyses, solid–liquid extraction (creams) and SPE (toothpastes) were applied. The proposed methods, based on techniques with different selectivity, were validated and both proved to be suitable to obtain an unambiguous identification and reliable determination of GA in commercial health care products (creams and toothpastes), giving concordant results. Moreover, the described methods can offer a useful analytical support for photostability studies of GA, a photolabile natural compound, in creams.

1. Introduction

Azulene and its alkyl derivative guaiazulene (GA) (1,4-dimethyl-7-isopropylazulene, structures in Fig. 1) were isolated from essential oils of medicinal herbs, and, because of their brilliant blue color, their photochemical properties were studied for many years by the chemists.

Guaiazulene is a natural product (bicyclic sesquiterpene) [1–3] derived from *Guaiac wood oil*, present in the distilled oil from *Callitris Intratropica Blue*, and is an active component of *Matricaria chamomilla* L. Moreover, guaiazulene and related sesquiterpenoids are widely distributed among marine octocorals of the order of *gorgonacea.*

In addition to its aroma, this oil displays anti-inflammatory, anti-spasmodic, antimicrobial activities [4,5] and has been used as self-medication for gastritis or canker sores for many years [6]. It was also found that guaiazulene can significantly inhibit lipid peroxidation and can scavenge hydroxyl radicals [7].

In recent years guaiazulene has become a popular ingredient in beauty, cosmetic, skin and body care products; it functions as a skin conditioning agent in cosmetic formulations, including hair dyes. Toxicological studies on azulene and its derivatives, including mutagenicity tests and clinical assessment, have been reported [4,5,8]. Azulene and its derivatives exhibit photochemical reactivity [9,10], and recently, both azulene and guaiazulene have been reported to be photomutagenic in *Salmonella typhimurium* bacteria strains (TA 102) [11].

Therefore, the use of guaiazulene in cosmetics, associated to its photochemical properties and some evidences of phototoxic effects, make of interest the development of analytical methods suitable for the detection and determination of guaiazulene in commercial health care products for quality control purposes.

Gas chromatography–mass spectrometry (GC–MS) [12,13], high-performance liquid chromatography (HPLC–UV) [14], capillary electrophoresis (CE) [13] as well as thin layer chromatography (TLC) [15] have been applied to the analysis of natural products containing guaiazulene, but few and poorly validated methods have been proposed for the determination of guaiazulene in simple pharmaceutical preparations (solutions) [16,17] and health care products [18]. Thus, this study was aimed to develop sensitive and selective GC–MS and HPLC methods suitable for the determination of guaiazulene in commercial emollient creams and toothpaste samples of complex composition. Both GC–MS and HPLC approaches have been considered in order to offer useful analytical methods for differently equipped quality control laboratories. In GC–MS the guaiazulene identity was confirmed by its mass spectrum, while in HPLC the use of a diode array detector (DAD) allowed to obtain a characteristic UV-spectrum useful to confirm its identity. The work involved the development of a new solid phase extraction (SPE) method for the selective extraction of guaiazulene from

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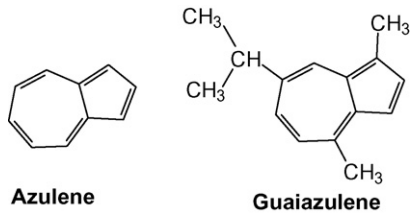

Fig. 1. Chemical structures of azulene and guaiazulene.

complex matrices and also included investigations on the photostability of guaiazulene in reconstituted creams.

2. Experimental

2.1. Materials

Guaiazulene and musk ketone (used as internal standard, IS) were obtained from Fluka (Sigma–Aldrich, CH). Emollient creams (products A and B) and the toothpastes (products C and D) were commercially available. The reconstituted creams and toothpastes, containing a known concentration of GA, were prepared and furnished by BeC s.r.l. (Forlì, Italy).

HPLC grade methanol and acetonitrile were from Sigma–Aldrich (Steinheim, Germany).

A Milli-Q® (Millipore, France) water purification system was used to obtain the purified water for photostability studies and HPLC analysis.

The sample pre-treatment was performed on SPE column Strata-X Polymer (33 μm; 100 mg), obtained from Phenomenex (Italy).

2.2. Apparatus and chromatographic conditions

All the analyses were performed using an HPLC-DAD apparatus and a GC–MS system.

HPLC analyses were carried out using a Hewlett Packard Ti series 1050 liquid chromatograph, equipped with a Rheodyne Model 7125 injector and connected to a photodiode array detector (DAD, HP Ti series 1050). Chromatographic separations were performed on a Waters XTerra™ C_8, 3.5 μm (100 mm × 2.1 mm i.d.) column, using a mobile phase consisting of acetonitrile–water 50:50 (100 mm × 2.1 mm (v/v)), at a flow rate of 0.3 mL/min. The injection volume was 20 μL and UV detection was at 285 nm.

GC–MS analyses were performed on a TRACE GC2000 Series (ThermoQuest CE Instruments, Austin TX, USA) gas chromatograph equipped with a split-splitless injector (split ratio 1:50) and interfaced with GCQ Plus (ThermoQuest) mass detector with an ion trap analyzer, operating in EI mode (70 eV). The GC column was a Phenomenex ZB-5 fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness), consisting of Crossbond (5% diphenyl, 95% dimethyl polysiloxane). Helium (He) was the carrier gas at a flow rate of 1.5 mL/min. A linear temperature program was adopted to separate the different health care product components: initial 80 °C (hold time: 1 min), then ramped by 10 °C/min to 250 °C (hold time: 10 min). The temperatures of injector base, transfer line and ionization source were maintained at 280, 275 and 200 °C, respectively.

The mass spectra were recorded in full scan (50–650 amu) to collect the total ion current (TIC) chromatograms; single ion monitoring (SIM) chromatograms, for the quantitative analysis, were reconstructed at the base peak corresponding to the main fragment of the studied analyte (183 amu for guaiazulene and 279 amu for the IS, musk ketone).

A volume of 1 μL of sample and standard methanol solutions, containing musk ketone as internal standard (IS), was injected.

Tests on the photochemical stability of guaiazulene in reconstituted emollient creams were carried out using a xenon arc source to simulate the natural sunlight exposure. A 150-W xenon arc lamp (solar simulator, model 68805, Oriel Corporation, Stratford, CT, USA) was used, with a dichroic mirror (Oriel, model 81405) to block visible and IR radiation in order to minimize sample heating. An air-mass filter 1.5 (Oriel, model 81090) was used to simulate solar conditions and a UV-B-C blocking filter was employed to attenuate the UV-B component. The output beam was directed downward by a "beam-turning assembly", containing the dichroic mirror. The UV dose ($\mu W/cm^2$) from the xenon arc lamp was measured using an Oriel Goldilux radiometer (model 70127), fitted with external interchangeable probes for UV-A (dose: 82.2 $\mu W/cm^2\ min^{-1}$) and UV-B (dose: 9.1 $\mu W/cm^2\ min^{-1}$).

2.3. Assay procedure

2.3.1. Standard solutions

A stock solution of guaiazulene was prepared in methanol at the concentration of 1 mg/mL. For HPLC analyses this solution was diluted 1:200 with a mixture of methanol/water 80:20 (v/v) to the concentration of 5 μg/mL.

To validate the SPE procedure, standard solutions were injected without any treatment and after solid phase extraction on a Strata-X column (SPE procedure in Section 2.3.2). These solutions (pre- and post-SPE) were then diluted 1:2 with methanol/water 55:45 (v/v) just before HPLC injection, because of the slight solubility of guaiazulene in water.

For GC–MS analyses, the methanol stock solution was diluted 1:200 (final concentration 5 μg/mL) with methanol; 600 μL of this solution were added by 40 μL of IS solution (musk ketone 50 μg/mL in methanol) before the injection.

2.3.2. Sample preparation

For HPLC analysis, accurately weighted aliquots of creams or toothpaste (200 mg) were dissolved in 20 mL of a mixture of methanol/water 80:20 (v/v) and sonicated at 40 °C for about 10 min; after cooling, the solutions were centrifuged for 5 min at 3000 rpm. An aliquot (3 mL) of supernatant was subjected to the SPE procedure as follows.

The SPE column (Strata-X 33) was first conditioned with methanol (3 mL) and equilibrated with water (3 mL), then 3 mL of sample or standard solutions in methanol/water 80:20 (v/v) were loaded and the column was washed with 1 mL of water. The analyte GA was eluted with 4 mL of methanol and the volume was adjusted to 5 mL. The resulting solution was diluted 1:2 with methanol/water 55:45 (v/v) before the HPLC injection. GC–MS analysis of toothpaste were performed on the eluted methanolic solution, after the addition of the IS as described below.

For GC–MS analysis of emollient creams, 200 mg were dissolved in 20 mL of methanol and sonicated at 40 °C for about 10 min; after cooling, the solution was centrifuged and 40 μL of IS solution (50 μg/mL) were added to 600 μL of supernatant. Toothpastes have been analysed by injecting the methanolic sample solutions obtained after SPE procedure and IS addition.

2.3.3. Method validation

In order to quantify the guaiazulene content in health care products, standard solutions of guaiazulene were prepared at 5 levels of concentration over the range of 1.40–11.2 μg/mL (pre-SPE) and 0.84–6.72 μg/mL (post-SPE). These solutions were analysed by HPLC-DAD and calibration graph was constructed by plotting the peak area *versus* the corresponding drug concentrations. The accuracy of the method was assessed by recovery experiments,

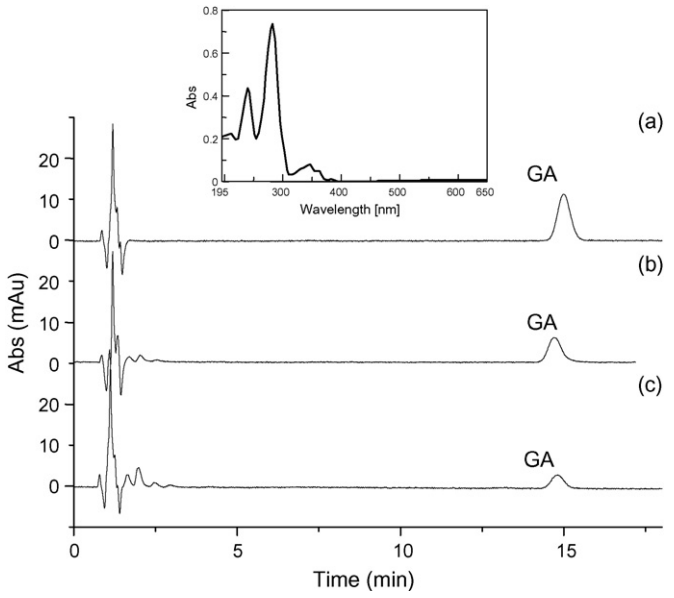

Fig. 2. HPLC chromatograms of GA standard solution (a), representative cream (b) and toothpaste (c) sample solutions post-SPE. Chromatographic conditions: column Waters XTerra™ C_8, 3.5 μm (100 mm × 2.1 mm i.d.), mobile phase consisting of acetonitrile–water 50:50 (v/v), flow rate of 0.3 mL/min, detection at 285 nm. Insert: on-line UV-spectrum of GA.

by analysing the reconstituted samples (prepared in triplicate), obtained by incorporating GA in blank creams at three concentration levels (0.007–0.015–0.030% w/w, theoretical final concentration of guaiazulene post-SPE: 0.44–0.90–1.78 μg/mL). The recovery on the toothpaste was evaluated by analyzing the reconstituted products (guaiazulene concentration: 0.010–0.012–0.015%, w/w) in triplicate.

GC–MS calibration graph was obtained by plotting the ratio of the reconstructed peak areas (SIM) of the analytes (guaiazulene to IS) *versus* the corresponding guaiazulene concentration ($n = 5$, range 0.47–9.37 μg/mL). Recovery experiments were performed at three concentrations (ranges mentioned before) for guaiazulene in reconstituted creams and toothpaste and the accuracy was calculated.

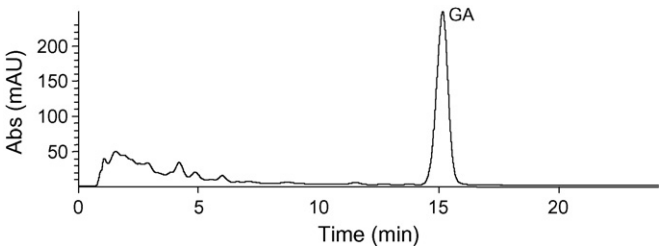

Fig. 3. HPLC-DAD chromatogram of a degradated GA standard solution, exposed to UV-A radiation (30 min). Chromatographic conditions as in Fig. 2.

The intra-day and inter-day precision of the assay method was evaluated by replicated analyses (intra-day: $n = 6$, inter-day: $n = 18$ in three different days) of the commercial emollient cream and toothpaste.

2.3.4. Photostability test

Thin layers of reconstituted emollient cream (0.030%, w/w of GA) were exposed to UV-A and UV-B radiation (xenon lamp) for increasing time (0 min–3 h) to study the photostability of guaiazulene; as dark control a thin layer of the same cream was exposed to air (0 min–3 h), protecting from light (dark control). At selected times of irradiation, an amount of sample (about 200 mg) was treated as described in Section 2.3.2 and analyzed by GC–MS and HPLC-UV. To minimize the error due to the evaporation of guaiazulene under photoexposure, the content of GA in the cream (dark control and irradiated sample) was calculated referring to a poorly volatile component (hexadecanol) present in the cream. The ratio of the reconstructed peak area (SIM) of GA to that of hexadecanol (55 amu) was used to monitor the decrease of the GA content in the cream sample under UVA and UVB photoexposure, through a comparison with the dark control.

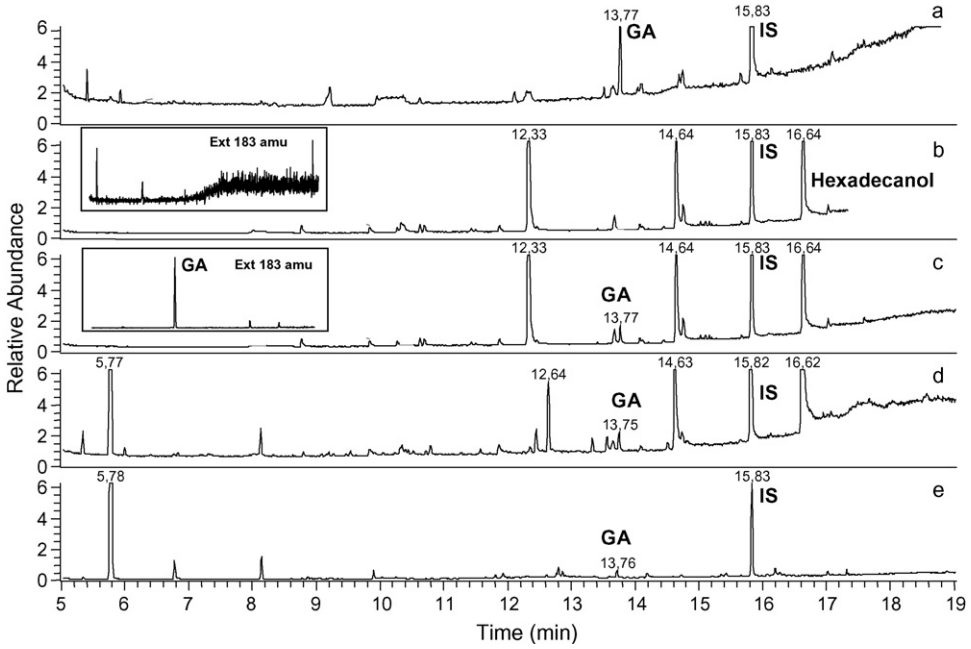

Fig. 4. TIC GC–MS chromatograms of guaiazulene standard solution (a), sample solutions from blank reconstituted cream (b) and GA-containing reconstituted cream (c), commercial creams A (d), and B (e). Insert: 183 amu extract ion chromatogram. Experimental conditions in Section 2.2.

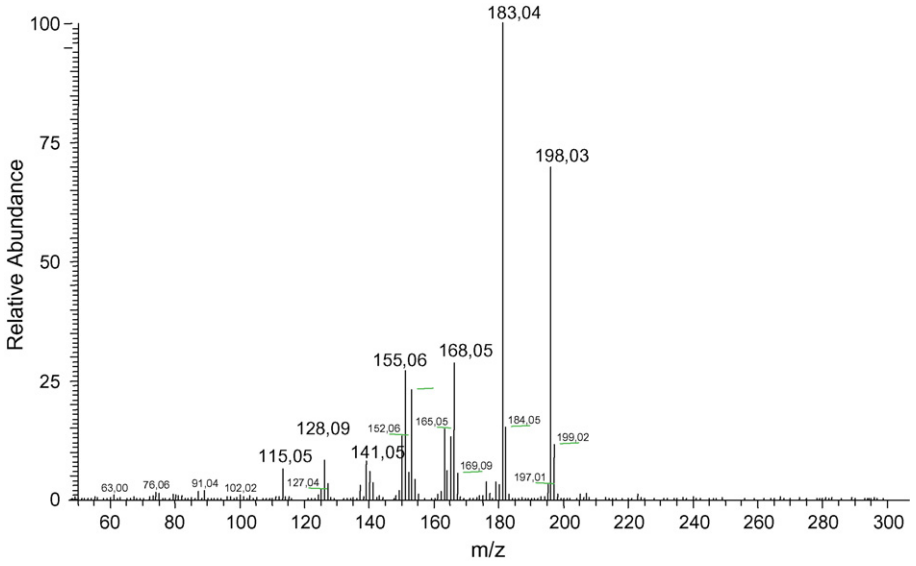

Fig. 5. MS spectrum of guaiazulene ([M⁺] at *m*/*z* 198).

3. Results and discussion

The present study included the following steps: (a) Development of suitable HPLC-DAD and GC–MS chromatographic conditions, (b) Setting a practical SPE method for the selective extraction of GA from matrices of complex composition, (c) Application of the validated method to the analysis of commercial products and (d) Photostability testing of a reconstituted cream containing guaiazulene.

3.1. Chromatography

3.1.1. HPLC method

Several attempts were made to develop an HPLC-DAD method, under reversed-phase conditions, for the determination of guaiazulene at low concentration in health care products. Different chromatographic columns were evaluated; C_{18} columns were found to give a strong retention owing to the hydrophobic character of guaiazulene (log *P* = 5.74, predicted value) [13] with bad peak shape. The use of a C_8 stationary phase with a mobile phase consisting of acetonitrile–water, in combination with UV detection at 285 nm, resulted in a simple HPLC method with adequate selectivity. Guaiazulene in samples was identified by comparing the UV spectrum (insert in Fig. 2) and retention time (R_t = 14.5 min) to those of the standard.

Representative chromatograms from guaiazulene standard and from cream and toothpaste samples, subjected to SPE procedure, are shown in Fig. 2. The absence of interfering peaks noticeable in the baseline confirms the high selectivity of the method. The relatively long GA retention time was chosen in order to avoid possible interferences by peaks at lower R_t due to GA photodegradation products (chromatogram in Fig. 3). The peak purity of GA (99.8%) is assured by the software elaboration (HP Chemstation, Hewelett Packard, USA).

3.1.2. GC–MS method

In Fig. 4a representative TIC chromatogram of guaiazulene standard solution is shown. Fig. 4b and c shows GC–MS and extract ion (183 amu; insert) chromatograms obtained from blank (Fig. 4b) and reconstituted cream (Fig. 4c), respectively, while Fig. 4d and e illustrates representative chromatograms from two different commercial creams. The identification of guaiazulene in the products was based on the comparison of its retention time and mass spectrum in samples with those of the pure standard and with the library mass spectra (General Purpose, Terpene "ThermoQuest" and NIST libraries). Mass spectrum of GA is shown in Fig. 5; the signal at *m*/*z* 198 corresponds to the molecular ion [M]⁺ of GA, *m*/*z* 183, 168 and 155 correspond to the loss of one, two methyl groups and the isopropyl group, respectively; the peak at *m*/*z* 128 represents the molecular ion [M]⁺ of azulene.

Although the cream sample was simply subjected to treatment with methanol and centrifugation, the matrix components did not interfere with the analysis, providing good selectivity.

Table 1
Linearity data for guaiazulene in HPLC-DAD and GC–MS analyses of standard solutions (ST) (*n* = 5)

Method sample	Slope (±S.D.)	*y*-Intercept (±S.D.)	r^2
HPLC			
ST pre-SPE	295,600 (±3985)	29.03 (±27.16)	0.9986
ST post-SPE	246,400 (±11,500)	−1.613 (±12.30)	0.9887
GC–MS			
ST	31.78 (±0.8879)	−0.00287 (±0.01142)	0.9915

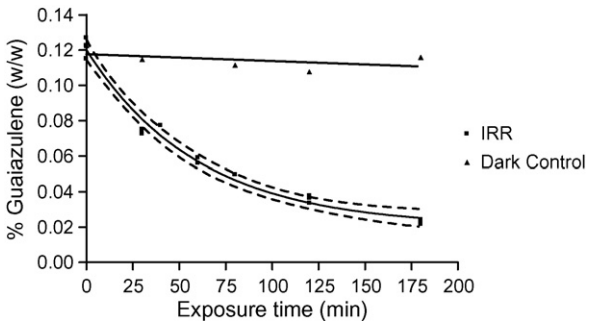

Fig. 6. Photodegradation profile of guaiazulene in reconstituted cream (GA concentration = 0.030%, w/w) under exposure to UV-A and UV-B radiation (IRR) and in dark control. Data obtained by GC–MS method.

Table 2
Accuracy data from recovery experiments (3 levels concentration; see Section 2.3.3)

Method sample	Theoretical % recovery (±CV%)			Cochran's test values (C_{table} = 0.684)	Mean t-test values (t_{table} = 2.145)
HPLC					
ST post-SPE	**1.40** μg/mL[a]	**5.60** μg/mL[a]	**11.2** μg/mL[a]		
	98.48 (±0.72)	97.33(±3.41)	100.89 (±1.27)	0.331	1.629
Rec. cream	**0.007**%[b]	**0.015**%[b]	**0.030**%[b]		
	102.25 (±7.75)	101.56 (±4.25)	97.42 (±1.71)	0.150	2.114
Rec. toothpaste	**0.010**%[b]	**0.012**%[b]	**0.015**%[b]		
	93.23 (±5.29)	94.23 (±5.06)	99.54 (±2.31)	0.238	2.133
GC–MS					
Rec. cream	**0.007**%[b]	**0.015**%[b]	**0.030**%[b]		
	96.44 (±3.61)	97.09 (±3.12)	97.74 (±7.17)	0.184	0.650
Rec. toothpaste	**0.010**%[b]	**0.012**%[b]	**0.015**%[b]		
	97.13 (±2.56)	96.25 (±4.83)	98.64 (±3.94)	0.221	1.965

Rec: reconstituted.
[a] Concentration of GA in standard solutions subjected to SPE.
[b] Theoretical GA concentrations (% w/w) in reconstituted creams and toothpaste.

3.2. Sample preparation

Guaiazulene is present in cosmetics of complex composition at low concentrations, therefore selective and effective extraction procedures of the analyte have to be applied. To this end, SPE was chosen. Cartridges containing C-18 and CN sorbents were tested, but easy saturation by the samples was observed and the recovery of the analyte was not satisfactory.

A successful SPE method was developed using a Strata-X polimeric sorbent; these cartridges were suitable for the extraction of guaiazulene from the matrix (cream and toothpaste) giving high recovery values (98.38% for standard solutions, 100.41% and 95.66% for reconstituted products, containing known quantities of GA). The sample solutions from SPE were then subjected to HPLC-DAD and GC–MS (toothpaste) analyses. For GC–MS analysis of creams, a simple solid–liquid extraction (under ultrasonication) with methanol, followed by centrifugation, was applied.

3.3. Method validation

3.3.1. Linearity and sensitivity

The linearity of the response in HPLC was evaluated for pre- and post-SPE guaiazulene standard solutions, while in GC–MS only methanolic standard solutions were analysed. The obtained regression equations (n = 5) are reported in Table 1.

The limit of detection (LOD) of the HPLC method was 0.0185 μg/mL, as $3\sigma/S$ (where σ is the standard deviation of the y-intercepts of regression line, and S is the slope of the calibration curve), the limit of quantitation (LOQ) was 0.0616 μg/mL, as $10\sigma/S$. Since the GA recovery was fundamentally quantitative (100.41% for creams and 95.66% for toothpaste), the found chromatographic LOQ can be considered equivalent to the working quantitation limits (0.002%, w/w). The values were confirmed by using the approach based on serial dilutions and analysis of standard solution.

The sensitivity of the GC–MS method (LOQ = 0.651 μg/mL, as $10\sigma/S$, correspondent to 0.006%, w/w) was found to be adequate for these analysis purposes.

3.3.2. Accuracy and precision

Accuracy was evaluated by analyzing reconstituted creams and toothpaste at 3 level concentrations (see Section 2.3.3; 6 replicates analysis for each concentration, n = 18). Accuracy is calculated by comparing the measured and the theoretical concentrations, taking the regression curve obtained for guaiazulene standard solutions (pre-SPE) as reference. Once it has been established that the variances were homogeneous (Cochran's test: calculated $C < C_{table}(0.05, 5, 2) = 0.684$) and that the means were valid (t-test: t calculated < $t(p = 0.05; (n - 1) = 14) = 2.145$), the mean recovery levels and their coefficient of variation (CV%) were calculated. In Table 2 the accuracy and precision data are summarized for the HPLC and GC–MS analysis of reconstituted health care products.

3.4. Applications

3.4.1. Analysis of commercial samples

The developed HPLC-DAD and GC–MS methods were applied to the determination of guaiazulene in commercial health care products. In particular, two emollient creams (products A and B) and two toothpastes (products C and D) were analysed; the guaiazulene content (0.012%) is declared only in the toothpaste C.

Replicated analyses (n = 18, in three different days) were carried out for each commercial product; the results are reported in Table 3. The two different methods to analyze the creams A and B were compared. Since the F-test [F = 1.5 (A), 2.71 (B) < F_{tab} 5.05] and the Student's t-test [t = 0.6 (A), 1.67 (B) < t_{tab} 2.228] are valid, the results obtained by SPE-HPLC and GC–MS were not significantly different. The results found (guaiazulene %, w/w) and the statistical data are presented in Table 3. Inter-day and intra-day precision

Table 3
Assay results and precision data for the determination of guaiazulene in commercially available creams (A and B) and toothpastes (B and C)

Sample	SPE–HPLC			GC–MS		
	% Found GA[a] (w/w)	Intra-day precision (CV%)	Inter-day precision (CV%)	% Found GA (w/w)	Intra-day precision (CV%)	Inter-day precision (CV%)
A	0.0150	6.39	7.61	0.0141	4.95	4.99
B	0.0027	4.64	2.91	0.0025	8.87	8.01
C	0.0114	8.25	7.67	0.0105	8.91	9.02
D	<LOQ	–	–	<LOQ	–	–

[a] Corrected on the basis of % recovery.

within the concentration ranges were all satisfactory, suggesting that the extraction procedure was reproducible.

The content of GA in toothpaste D was below the method detection limit; therefore its possible presence cannot be confirmed. The found GA concentration in toothpaste C was found to be 0.0114% (HPLC) and 0.0105% (GC–MS); value corrected on the basis of % recovery, corresponded to the 95% and 88% of the declared content (0.0120%, w/w).

3.4.2. Photodegradation studies

A reconstituted cream, containing 0.030% (w/w) of GA, was exposed to UV-A and UV-B radiations (solar simulator, xenon arc lamp) at ambient temperature. The photodegradation was monitored by GC–MS technique and HPLC-UV (chromatogram from photodegradated GA standard solution is shown in Fig. 3). In Fig. 6 the photodegradation profiles, as GA percentage decrease, of the photoexposed sample and dark control are illustrated (GC–MS data). As shown, after 3 h only about the 21% of guaiazulene was still present in the cream. These results suggest that the cream ingredients do not offer a significant photoprotection to GA and assume an interesting value due to the reported photomutagenic properties of guaiazulene [11]. The elucidation of the photodegradation process was not the aim of the present work; studies are in progress on this complex subject and will be subsequently published.

4. Conclusions

In the present study sensitive, accurate and reproducible analytical methods are proposed to identify and quantify guaiazulene in health care products. Two different HPLC-DAD and GC–MS methods were validated and applied to the analysis of GA in commercial creams and toothpastes, giving results in substantial agreement. Hence, each chromatographic method can be independently used for the determination of GA; the HPLC offers a lower limit of quantitation and a wider field of application, while the GC–MS method is more rapid for the creams analysis, because a simple solid–liquid extraction is involved. These two methodological approaches offer the opportunity of a reliable assay of GA in commercial samples and a valid support for monitoring the photostability of GA-based health care products.

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