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Microwave-assisted extraction of oxygen heterocyclic compounds from *Citrus*-scented creams using a deep eutectic solvent and their determination by HPLC-PDA

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ABSTRACT

This study aimed to investigate the content of oxygen heterocyclic compounds, especially coumarin and furocoumarins, in cosmetics products scented with *Citrus* essential oils. A minor component of the non-volatile fraction of *Citrus* essences is composed by oxygen heterocyclic compounds, commonly known as coumarins, furocoumarins, and polymethoxyflavones. These molecules show several biological effects on human health. On the other hand, coumarin has the potential to induce skin sensitization and furocoumarins show phototoxic activity. For this reason, several regulation and opinions have been issued concerning the maximum amount of these compounds in cosmetics. Given the regulatory framework, an analytical workflow for extraction and quantification of OHCs from cosmetics is necessary.

This research aims to address this challenge by exploring, for the first time, a deep eutectic solvent as eco-friendly alternative for microwave-assisted extraction of these molecules from *Citrus*-scented creams. A deep eutectic solvent made by chloride and urea, at a molar ratio 1:2, and 20 % of water in weight was selected. Due to the complexity of the sample, a SPE clean-up was mandatory to obtain an extract devoid of interferents. Finally, an HPLC-PDA analytical method was employed allowing the simultaneous separation and quantification of 35 oxygen heterocyclic compounds in less than ten minutes. The analytical workflow here proposed was developed, optimized and validated demonstrating the potentials of deep eutectic solvents and microwaves in this field.

1. Introduction

The term "cosmetics" is used to describe a large range of products that are applied to the human body for various purposes, including cleaning, perfuming, protecting, beautifying, enhancing attractiveness, and improving appearance [1]. Among them, the skin and sun care products represent a significant portion of the overall consumer goods market [2–3]. The current array of skincare products encompasses a vast range of formulations, catering to a multitude of beauty concerns. These include body washes, shampoos, gels, exfoliants, toners, moisturisers and creams.

Creams are semi-solid emulsions consisting of water, oil, emulsifiers, excipients and active ingredients [4]. Emulsifiers and excipients are added to the emulsion to improve stability, texture, and sensory aspects of the cream formulation. Therapeutic effects like antioxidant, anti-inflammatory, or anti-aging characteristics, are provided by active substances. In this context, a diverse range of plant-derived ingredients, such as roots, herbs, flowers, fruits, and essential oils, can be employed.

Essential oils (EOs) are regarded as one of the most appreciated ingredients for meeting the specifications required for product design

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[5–7]. Among these, *Citrus* EOs are frequently employed due to their pleasant aroma [8]. The olfactory characteristics of *Citrus* EOs are primarily attributed to their volatile fraction, whereas the non-volatile fraction gives persistence to the *Citrus* aroma. A minor component of this fraction is constituted by oxygen heterocyclic compounds (OHCs), more commonly known as coumarins (Cs), furocoumarins (FCs) and polymethoxyflavones (PMFs) [9]. These molecules are distinctive to each *Citrus* species and are therefore crucial for quality control, while coumarin is employed as a fixative or fragrance agent in cosmetics. On the other hand, coumarin has the potential to induce cutaneous sensitization and eczema via dermal administration [10]. FCs showed phototoxic activity and, when exposed to UV-A radiation, can cause erythematous, hyperpigmentation, rash, blisters and sunburn. Furthermore, the long-term use of FCs has been associated with an elevated risk of cutaneous melanoma [11].

In light of these adverse effects, several opinions and regulations have been issued over the years concerning the maximum amount of coumarin and FCs in cosmetic products [12]. Coumarin is listed in the European Regulation (EC). No 1223/2009 as one of the 26 fragrance allergens for which labelling is required at levels above 0.001 % and 0.01 % (or 10 and 100 ppm) in leave-on and rinse-off cosmetic products, respectively [1]. For lower levels of coumarin, "perfume" or "aroma" may be listed generically in the list of ingredients. Furthermore, the concentration of FCs in sunscreen and bronzing products must be below 1 mg kg^{-1} [1]. The International Fragrance Association (IFRA) selected 6 analytes (bergapten, bergamottin, byacangelicol, epoxybergamottin, isopimpinellin and oxypeucedanin) to be used as FCs markers based on their overall occurrence and concentrations in *Citrus* EOs [13]. In the 49th and 50th IFRA Amendments, the use of coumarin and FCs was restricted according to the type of cosmetic product considered. Body, face and hand creams are classified in categories 5A, 5B and 5C, respectively, as leave-on products applied to the face and body with the hands (palms). For these categories, IFRA has proposed a maximum level of 0.0015 % of 5-methoxypsoralen/bergapten, selected as the FCs marker. The coumarin concentration should be less than 0.38, 0.11 and 0.16 % for categories 5A, 5B and 5C, respectively [14,15]. Therefore, it is necessary to quantify these molecules in cosmetics, even at low concentration levels, to protect human health.

Over the years, many analytical methods have been developed for the characterization of OHCs in various sample matrices. The analytical technique mainly used for their determination is high performance liquid chromatography (HPLC) coupled to different detectors [16]. Spectrophotometric detectors have been the most widely used due to the presence of chromophores in the chemical structure of OHCs and the low cost and ease of use of the detectors [17–23]. However, mass spectrometry detectors were also used to characterize OHCs in trace amounts [24–28].

All the mentioned techniques allow direct analysis of EOs, with high OHCs concentrations, or simple matrices, such as hydroalcoholic fragrances [29,30]. In the case of complex cosmetic matrices with low levels of EOs in their formulation, it is mandatory to undertake a sample preparation step in order to obtain an extract devoid of interferents and enriched in OHCs. To the best of authors knowledge, few studies have addressed this issue [17,24–27,31,32]. Of these, only three investigated the OHCs content specifically in creams [24,25,31]. In these works, organic solvents were used for ultrasound-assisted extraction of coumarin derivatives. Solid phase extraction (SPE) was then used for cleanup and/or enrichment. Due to the complexity of cream matrices, the choice of extraction solvent is challenging. The most commonly used organic solvents for extraction of OHCs are methanol and ethyl acetate. Nevertheless, the growing interest in green and environmentally friendly analytical methods has led to the increased use of deep eutectic solvents (DESs) as greener alternatives to conventional solvents and ionic liquids [33]. In cosmetic field, DESs have only been used to extract parabens and fluorescent whitening agents from creams [34,35].

Therefore, the aim of this study is to propose an eco-friendlier method for the extraction of OHCs from *Citrus*-scented creams. For this purpose, a DES was used for the first time in this field. Moreover, to increase the matrix-solvent interaction, microwave-assisted extraction (MAE) was performed. The new analytical workflow proposed here is based on MAE, using a DES, followed by SPE clean-up in combination with HPLC-PDA detection.

2. Materials and methods

2.1. Chemicals

Solvents, standard materials and reactive were acquired from Merck Life Science (Merck KGaA, Darmstadt, Germany), except when otherwise specified.

Choline chloride (purity ≥ 99.9 %) and urea (purity 99.0–100.5 %) were used for DES preparation.

Water, methanol (MeOH, HPLC grade, purity ≥ 99.9 %), ethanol (EtOH, gradient grade for HPLC, purity ≥ 99.9 %) ethyl acetate (EtOAc, HPLC Plus, purity ≥ 99.9 %), and tetrahydrofuran (THF, HPLC grade, purity ≥ 99.9 %) were used for sample preparation and/or HPLC–PDA analyses. Acetonitrile (UHPLC-MS, purity ≥ 99.9 %) was used to prepare alkyl aryl ketone stock solutions and mixtures.

Thirty-five analytical standards among OHCs were employed for HPLC-PDA analyses. Angelicin, aurapten, bergamottin, bergapten, byakangelicin, byakangelicol, citropten, cnidicin, cnidilin, coumarin, epoxyaurapten, epoxybergamottin, gardenin A, gardenin B, heraclenin, herniarin, imperatorin, isobergapten, isoimperatorin, isomeranzin, isopimpinellin, meranzin, meranzin hydrate, nobiletin, oxypeucedanin, oxypeucedanin hydrate, phellopterin, psoralen, sinensetin, tangeretin, tetra-O-methylscutellarein, 5-geranyloxy-7-methoxycoumarin, 5-O-demethylnobiletin, 8-geranyloxypsoralen, 8-methoxypsoralen were obtained from Merck Life Science (Merck KGaA, Darmstadt, Germany). Epoxyaurapten standard material was purchased from Labochem (Labochem Science S.r.l, Catania, Italy). A stock multi-analyte solution containing the 35 compounds at 100 mg L^{-1} were prepared in EtOH. For the construction of calibration curves, solutions of the standard materials were obtained by diluting the stock mixture in the range from 0.05 to 50 mg L^{-1}.

Six alkyl aryl ketones were employed as homolog series for the LRI calculation. A mixture of acetophenone (C8), propiophenone (C9), butyrophenone (C10), valerophenone (C11), hexanophenone (C12), and heptanophenone (C13) was prepared at a final concentration of 10 mg L^{-1} each in acetonitrile.

All the solutions were stored at − 4°C and sonicated for 10 min before injection.

2.2. Samples and sample preparation

2.2.1. Samples

Eight body creams were purchased from local markets in Gembloux, Belgium. As reported in Table S1, seven cream samples were selected for the presence of *Citrus* EO or extract as ingredient (C1-C7); one was fragrance-free (C0). This sample was analyzed in triplicate to confirm the absence of *Citrus* fragrance and used as a blank.

Two lemon EOs, one cold-pressed and one distilled, were purchased from a local market in Messina, Italy. The two oils were used to develop and validate the extraction procedure.

2.2.2. Deep eutectic solvent preparation

DES was prepared by mixing, heating and stirring choline chloride (HBA), urea (HBD) and water [36]. Specifically, choline chloride and urea, at a molar ratio 1:2, were placed in a round-bottom flask and stirred in a thermostatic bath at 70°C for about 40–50 min. When a clear and homogeneous liquid was obtained, the bath temperature was lowered to 50°C and 20 % of water in weight was added. The mixture was kept under medium stirring for 10–15 min. Finally, the liquid was cooled

to room temperature and stored under a hood until use.

2.2.3. Microwave-assisted extraction

1 g of cream sample was weighed into a 15 mL falcon tube, 3 mL of DES and 100 μL of ethyl acetate were added as extraction solvents. The mixture was vortexed for 30 sec and subjected to MAE using a Synthwave system (Milestone Srl, Bergamo, Italy). The samples were heated at 70°C, testing different ramping programs. The optimal microwave conditions were as follows: temperature ramp reaching 70°C in 2 min and hold for 1 min. The mixture was centrifuged in a Frontier Benchtop Centrifuge (OHAUS Europe GmbH, Nänikon, Switzerland) at 10000 rcf for 5 min. The supernatant was collected and all the procedure was reapeted one more time. Finally, the two aliquots were combined an subjected to SPE clean-up.

2.2.4. Solid-phase extraction

The SPE procedure was carried out on Oasis HLB Vac Cartridges (3cc, 60 mg, 30 μm, Waters, Milford, MA, USA). SPE columns were conditioned with 2 mL of methanol and equilibrated with 2 mL of 40 % methanol in water (*v/v*). The sample extract (6 mL) was loaded into the cartridge. Next, the cartridge was washed with 1.5 mL of 5 % methanol in water (*v/v*) and dried under vacuum for 1 min. Finally, the analytes were eluted with two aliquots of 0.75 mL of ethanol and injected into the HPLC-PDA instrument.

2.3. HPLC-PDA instrumentation and method

The analyses were carried out on a LC-2040C 3D Nexera-i PDA integrated UHPLC system (Shimadzu, Duisburg, Germany).

According to method previously developed by Arigò and co-workers [22], the separation was achieved on an Ascentis Express C18 column (50 × 4.6 mm, 2.7 μm; Merck Life Science) using water/methanol/THF (85:10:5, *v/v*) as solvent A and methanol/ THF (95:5, *v/v*) as solvent B at a flow rate of 2 mL min^{-1}. The gradient was as follows: 0–4.5 min, 15–28 % B, 7.0 min, 60 % B, 11 min, 85 % B, hold for 3 min. The injection volume was 2 μL, and the oven temperature was 40°C. The PDA parameters were: 4.1667 Hz, time constant 0.480 s, wavelength range 190–370 nm.

Labsolutions 5.85 (Shimadzu) was used for data collection and handling. Peaks identification was achieved by using a dual-filter: an internal UV/Vis library (spectral similarity > 75 %) and Linear Retention Index (LRI) approach (LRI tolerance of ± 4 LRI units).

Quantification of OHCs was performed using the calibration curves previously validated in our laboratory [22]. Briefly, calibration curves have been constructed in EtOH for all the target analytes in the range 0.05–100 mg L^{-1} (0.05, 0.1, 1, 5, 10, 25, 50, 100 mg L^{-1}, five replicates for each concentration) (Table S2). Method validation was carried out according to the method previously developed and validated by Arigò and coworkers [22].

2.4. Extraction method validation

A lemon cold-pressed EO was analysed by HPLC-PDA to determine the quali-quantitative composition of OHCs. A lemon distilled EO was analysed to confirm the absence of target analytes. In order to determine extraction recovery and matrix effect, mixtures of the two EOs at three different ratios were spiked into the sample blank (C0). Specifically, 500 μL, 1 mL and 2 mL of cold-pressed EO were added to 10 mL flasks and diluted with distilled oil. 100 μL of each mixture (low, medium and high concentration) were then spiked into the blank sample, before the extraction procedure to assess recovery and after extraction to assess any matrix effect.

Extraction recovery was evaluated as R % by comparing the peak areas of the target analytes present in the mixture of lemon cold-pressed and distilled EOs with those obtained for the spiked sample at low, medium and high concentrations (five replicates for each level).

To determine the matrix effect, the spiked sample was analysed at low, medium and high concentrations (five replicates for each level) and regression lines were constructed for all target analytes. A t-test was performed to compare the slopes obtained from oils and OHCs spiked sample, indicating no significant matrix effect for $p > 0.05$.

3. Results and discussion

3.1. Optimization of extraction procedure

To optimize the extraction procedure, a fragrance-free cream was selected as sample blank. A lemon cold-pressed EO was chosen to be spiked into the sample blank due its richer OHCs quali-quantitative profile compared to other *Citrus* EOs. The selected oil was diluted 1:20 *v/v* with ethanol and analysed in triplicate by HPLC-PDA. The chromatogram reported in Fig. 1 shows the OHCs qualitative profile.

Thirteen OHCs were detected and quantified (Table 1), showing the typical profile of a genuine lemon cold-pressed EO [37]. Bergamottin, 5-geranyloxy-7-methoxycoumarin and 8-geranyloxypsoralen resulted the main compounds among the OHCs investigated.

Once the quali-quantitative composition of the lemon EO had been determined, it was used as a reference material to be spiked into the fragrance-free cream. The choice not to use a certified standard mix was due both to the high cost of the standards and to better mimic the real formulation of a fragrant cream where EO is used. So, 100 μL of lemon EO was spiked in 1 g of cream fragrance-free to optimize the OHCs extraction procedure.

Starting from literature data [24,25], 1 g of spiked cream was mixed with 4 mL of methanol, vortexed and sonicated for 20 min. Then, the mixture was centrifuged at 15000 rcf for 5 min in order to obtain a well-defined phase separation. The extract was collected and subjected to SPE clean-up. Nevertheless, in the previous research, different cosmetic categories were examined (creams, pomades, shower gels, shampoos, perfumes, deodorants), and in some instances, the formation of emulsions during the extraction step was observed [25]. Indeed, in our case, following this extraction procedure, the sample was not properly solubilized. Due to that, other solvents, such as ethanol and ethyl acetate, were tested in the same conditions. In the case of ethanol, the sample was completely dissolved in the solvent, exhibiting no phase separation even after centrifugation. Using ethyl acetate, both good solubilization and phase separation were reached. However, the supernatant was not clear even after SPE purification, therefore it was decided to abandon the use of this solvent directly. Ethyl acetate exhibits an intermediate polarity, thus it can be both lipophilic and hydrophilic, therefore it can solubilize not only OHCs but also apolar compounds such as fats and oils, as well as medium polar compounds such as aldehydes, ketones and carboxylic acids comprise in the cream formulation.

It was thus decided to follow an alternative route and to evaluate the use of DES for this specific application. To choose the DES suitable for OHCs extraction, the selection criteria were based on the polarity of DESs, referring to their E_T (30) values (if available) compared to methanol [38]. DES made from choline chloride and urea (molar ratio 1:2) have an E_T (30) values similar to those of methanol (55.4 kcal/mol) [39]. However, the usefulness of this type of DES as an extraction solvent has been questioned due to its viscosity. To obtain a less viscous and more manageable solvent, 20 % water in weight was added [40]. Therefore, the ternary mixture of choline chloride, urea and water was selected and tested as extraction solvent. According to the extraction conditions used previously, 1 g of spiked cream and 4 mL of DES were vortexed and sonicated for 20 min. Again, the sample did not dissolve completely in the solvent. However, a homogeneous solution was observed when the mixture of cream and DES was heated for a few seconds at temperatures above 60°C. Thus, a microwave system was chosen to develop an extraction method exploiting both the high microwave absorption ability of DES and the matrix disaggregation

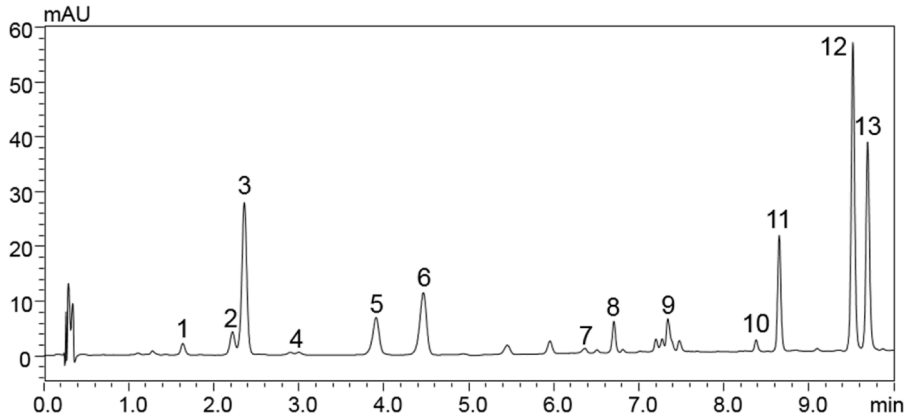

Fig. 1. HPLC-PDA chromatogram of a lemon essential oil extracted at 315 nm. The sample was diluted 1:20 *v/v* with ethanol before the injection into the LC system. For peak identification see Table 1.

Table 1
Concentration (mg L^{-1} ± standard deviation) of OHCs in lemon cold-pressed EO.

N°	Compound	Class	Concentration
1	Byakangelicin	FC	68.6 ± 0.3
2	Oxypeucedanin hydrate	FC	125.1 ± 1.0
3	Citropten	C	599.8 ± 4.2
4	Bergapten	FC	11.9 ± 0.7
5	Byakangelicol	FC	319.5 ± 1.4
6	Oxypeucedanin	FC	461.8 ± 3.1
7	Imperatorin	FC	40.0 ± 5.9
8	Phellopterin	FC	132.0 ± 2.7
9	Isoimperatorin	FC	141.6 ± 1.9
10	Cnidicin	FC	39.6 ± 1.2
11	8-geranyloxypsoralen	FC	739.6 ± 4.0
12	Bergamottin	FC	1391.3 ± 5.1
13	5-geranyloxy-7-methoxycoumarin	C	912.6 ± 4.1

obtained thanks to the motion generated in the sample by the oscillations of the electromagnetic field.

To optimize the MAE procedure, the quantity of sample was kept fixed at 1 g, while the variables considered were extraction temperature (60−80°C range), extraction time (2−6 min range), and volume of DES (2–4 mL range). Peak area of the thirteen OHCs present in the spiked sample were used as indicators to determine extraction efficiency compared to the areas obtained from the injection of the same concentration of lemon EO directly in the HPLC system (Fig. 2).

DES are very good microwave absorber, thus they heat-up very quickly and allow for a good solubilization of a broad range of compounds. The extraction temperature impacted mainly the extraction of the less polar OHC, starting from #7 to #13. Nevertheless, temperature over 80°C caused a slight significant decrease of compounds #11, 12 and 13. Therefore, it was decided to continue with 70°C and evaluate the impact of time. A clear increase was observed when extracting with overall 3 min compared to 2 min, while extending to 6 min did not provide any further improvement. Interestingly a difference was observed if the final temperature was hold for 1 min ramping up in two minutes, or the other way around. This may be due to a better disaggregation of the cream matrix by ramping more softly the temperature.

Considering the wide range of polarity of the target analytes, the optimal compromise for the MAE conditions among those tested were 1 g sample, 3 mL DES, temperature ramp to 70°C in 2 min and hold for 1 min. However, the use of the selected DES, being highly polar, allowed good extraction yields for more polar OHCs but not for less polar analytes, such as bergamottin and 5-geranyloxy-7-methoxycoumarin. Therefore, 100 μL of ethyl acetate were added to the 3 mL of DES before the MAE, increasing by 14 % the extraction efficiency of less polar compounds at the expense of a small loss of more polar compounds (Fig. 2 d). The optimized MAE procedure was repeated twice after confirming that the third extraction did not contribute any further to the overall recovery. The two aliquots of extract were combined and subjected to SPE clean-up.

SPE optimization was performed in parallel with MAE optimization when extract volume and mixture changed. Indeed, changing the volume and composition of the sample loading solution impacted the entire elution profile, requiring continuous verification. The SPE procedure was adapted from Ma et al., and Kreidl et al. [24,25]. As previously reported, polymeric reversed-phase sorbents showed good retention for coumarin derivates compounds. Therefore, an Oasis HLB cartridge (3cc, 60 mg, 30 μm) was selected. The cartridge was first washed with 2 mL of methanol and then conditioned with 2 mL of 40 % of methanol in water. Initially, methanol was replaced with ethanol, which resulted in a 30 % reduction in recovery. Therefore, the effect of washing solutions of methanol and water in different proportions was investigated. As can be seen in Fig. 3 a, the solution of 5 % of methanol in water was the best compromise to obtain good yields for all the OHCs investigated. Finally, methanol, acetonitrile and ethanol were tested as elution solvents. Comparing the peak area of the thirteen analytes present in lemon EO spiked, the best results were obtained using ethanol (Fig. 3 b). The optimized SPE workflow then consisted of loading 6.2 mL of extract, washing the cartridge with 1.5 mL of an aqueous solution of 5 % methanol and eluting the analytes with two aliquots of 0.75 mL of ethanol.

3.2. Extraction method validation

A fragrance-free cream was chosen as sample blank. To assess extraction recovery and matrix effect, mixtures of lemon cold-pressed and distilled EOs at three different ratios were spiked into the sample blank. Since the concentration of EOs in real cream samples is very low, a cold-pressed oil was diluted with a distilled one to simulate a true flavoring process. As described in section 2.4, 10 mL flasks were filled with 500 μL, 1 mL, and 2 mL of cold-pressed EO and diluted with the distilled one. The blank sample was then spiked with 100 μL of each mixture (low, medium, and high concentration) both before and after the extraction process to evaluate recovery and any potential matrix effects.

Extraction recovery was evaluated as R % by comparing the peak areas of the thirteen OHCs present in the spiked sample with those obtained from the direct injection of the mixture of lemon cold-pressed and distilled EOs at low, medium and high concentrations (Table 2). Values between 48–78 % were obtained for all the target analytes investigated. The lowest recovery values were obtained for bergamottin, in agreement with literature data [17,25]. Prosen et al. and Kreidl et al. attributed this to the strong interaction of bergamottin with the SPE sorbent. In this

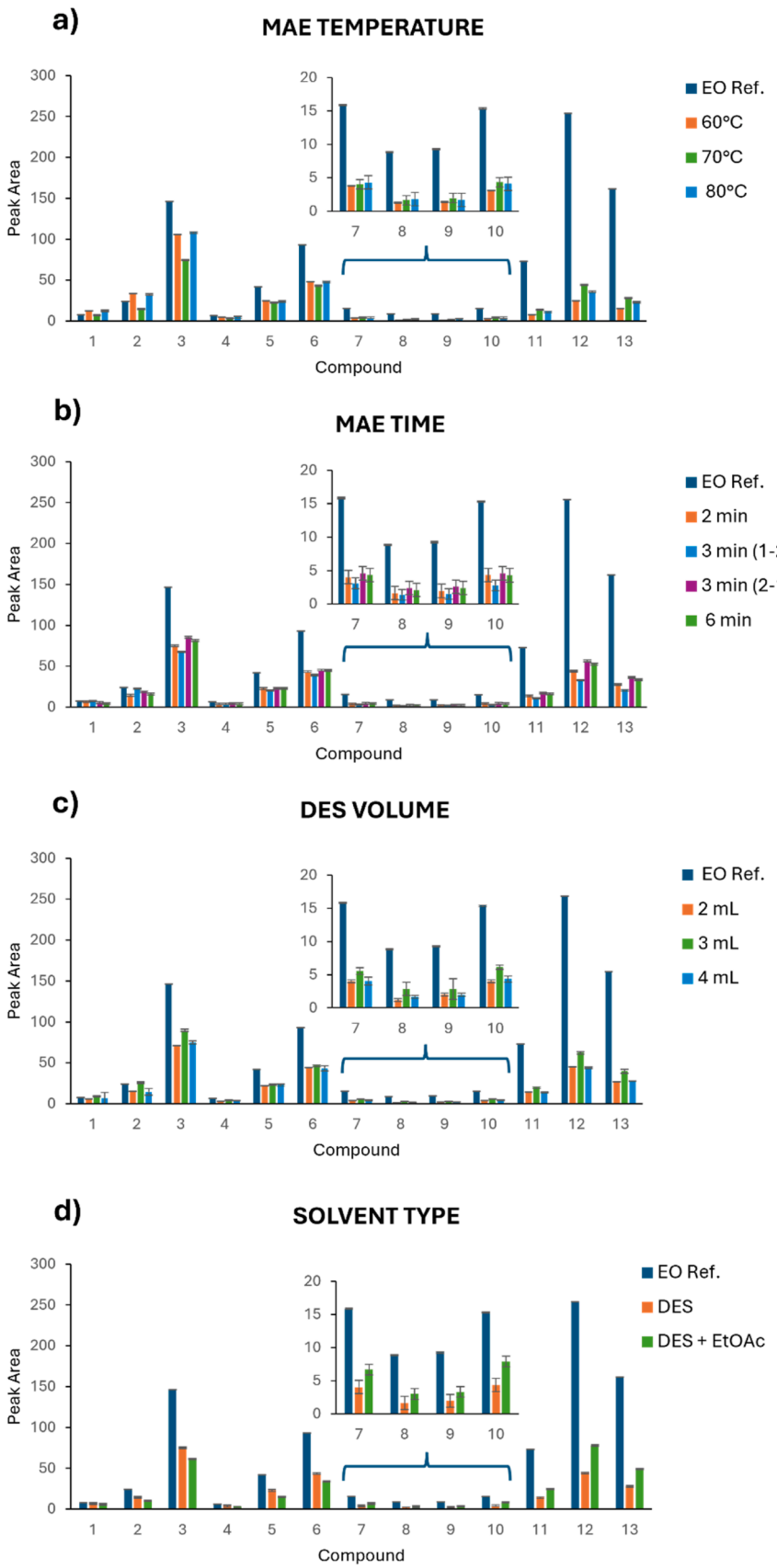

Fig. 2. Comparison of MAE extraction conditions applied to 1g of sample in function of : a) Temperature: 4 mL of DES, 1 min of temperature ramp to 60, 70 or 80°C (hold 2 min); b) Time: 4 mL of DES extracted at 70°C using 1 min of ramp and 1 or 2 min of holding, 2 min of ramp and 1 min of holding, and 5 min of ramp and 1 min of holding; c) DES volume: 2, 3, and 4 mL for 2 min of temperature ramp, 1 min at 70°C; d) Solvent type: 3 mL of DES or 3 mL of DES + 100 μL of ethyl acetate for 2 min of temperature ramp, 1 min at 70°C. For compounds identification see Table 1.

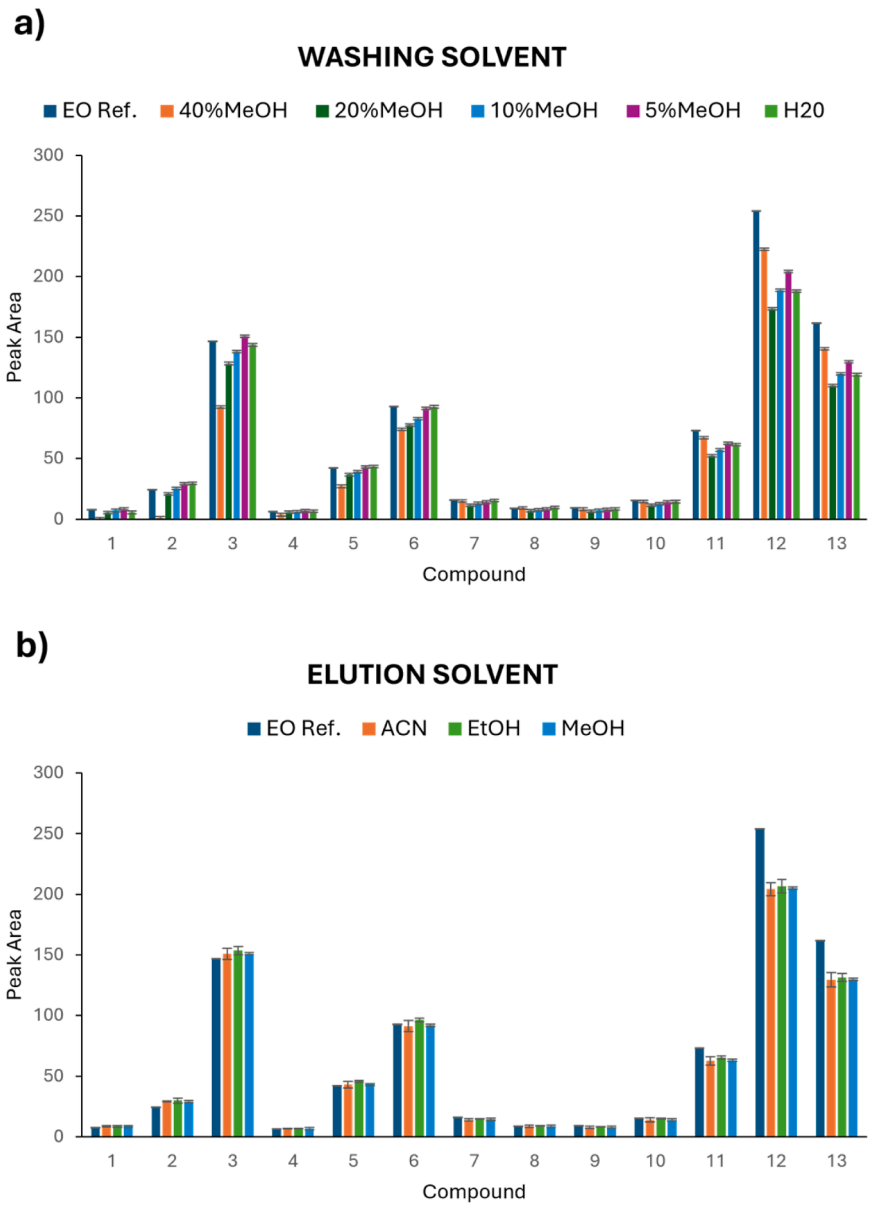

Fig. 3. Optimization of SPE parameters: a) washing solvents (40,20,10,5 % of methanol in water and water); b) elution solvents (acetonitrile, ethanol and methanol). For compounds identification see Table 1.

Table 2
Extraction recovery calculated at low, medium and high concentration level, extraction recovery average ± standard deviation, limit of detection (LoD) and limit of quantification (LoQ) for each target compound.

Compound	Extraction recovery (R %)			Average R% ± sd	LoD (mg L^{-1})	LoQ (mg L^{-1})
	High	Medium	Low			
Byakangelicin	60	62	60	61 ± 1	0.074	0.228
Oxypeucedanin hydrate	65	78	69	71 ± 7	0.045	0.114
Citropten	59	61	62	61 ± 2	0.041	0.123
Bergapten	73	71	70	71 ± 2	0.018	0.065
Byakangelicol	62	66	62	63 ± 2	0.062	0.222
Oxypeucedanin	64	64	70	66 ± 3	0.158	0.530
Imperatorin	75	53	50	59 ± 14	0.083	0.297
Phellopterin	57	51	50	53 ± 4	0.204	0.677
Isoimperatorin	51	56	56	54 ± 3	0.057	0.170
Cnidicin	53	56	74	61 ± 11	0.036	0.111
8-geranyloxypsoralen	52	50	50	51 ± 1	0.031	0.124
Bergamottin	50	47	48	48 ± 2	0.058	0.165
5-geranyloxy-7-methoxycoumarin	52	50	50	51 ± 1	0.100	0.195

study the recovery obtained from the SPE purification was tested by spiking the extract with the oil mixture prior to SPE clean-up, obtaining values close to 100 % for all analytes. Therefore, the poor recovery was clearly due to a low extraction yield. Nevertheless, the LoDs and LoQs were determined at low mg L^{-1} levels, below the legal limits, thus not posing any limitation to the applicability of the method.

Matrix effects were evaluated by comparing the slopes of external and matrix-matched calibration curves using *t*-tests. No significant matrix effect ($p > 0.05$) was found between external and matrix-matched curves for all the target analytes, indicating that the calibration curve in solvent can be used for quantification purposes.

Therefore, the quantitative characterization of 35 OHCs was based on external calibration, by constructing calibration curves in EtOH for all the target analytes in the range from LoQ to 100 mg L^{-1} (LoQ values for each analyte was reported in supplementary material). LoD values were in the range 0.01–0.45 mg L^{-1} and LoQs were between 0.05 and 0.97 mg L^{-1}.

3.3. Quantification of oxygen heterocyclic compounds in samples

The proposed analytical workflow was applied for the screening of nine Cs, seven PMFs and nineteen FCs in seven *Citrus*-scented cream samples. Samples are listed in Table S1, namely from C1 to C7. Each sample was extracted and analysed in triplicate. Table 3 reported the results of the quantitative evaluation of the target OHCs without recovery correction. In Fig. 4 a chromatogram of oxygen heterocyclic compounds characterized in sample C2 was presented.

Given the regulatory framework [1,15], attention was focused on the levels of coumarin and FCs to verify compliance with the regulatory limitations.

As can be seen in Table 3, coumarin was not detected in samples C4 and C7 within the Cs class, whereas the coumarin content in samples C1, C5 and C6 was in the 0.24–3.70 mg kg^{-1} range. However, samples C2 and C3 exceeded the safe limit for coumarin set by the European Regulation (10 mg kg^{-1}) with 20.12 ± 0.72 and 59.82 ± 2.70 mg kg^{-1}, respectively. Furthermore, sample C3 did not comply with the labelling requirements as no coumarin was declared in the list of ingredients [1].

In most of the samples analysed, FCs were quantified at levels below the 15 mg kg^{-1} recommended by the IFRA 50th Amendment for body, face and hand creams [15]. In fact, total FCs were quantified in the range of 0.28–5.60 mg kg^{-1} in samples C2, C3, C4, C5 and C6. Samples C1 and C7 were notable exceptions in that the total FC content exceeded the recommended safe limits with total FCs amount of 28.00 ± 0.24 and 21.24 ± 0.01 mg kg^{-1}, respectively.

4. Conclusion

This research provided an efficient analytical workflow for the extraction and quantification of OHCs in cosmetics by MAE, SPE and subsequent HPLC-PDA analysis.

To the best of the authors' knowledge, this is the first time that a DES has been used for OHCs MAE from cosmetic samples. The extraction procedure was developed, optimized and validated demonstrating the potentials of DESs and MAE in this field. The HPLC-PDA method employed allowed the simultaneous separation and quantification of nine Cs, nineteen FCs and seven PMFs in less than ten minutes.

The method was applied to screen seven *Citrus*-scented creams. Of the samples analysed, two exceeded the recommended level for furocoumarins. In addition, one cream did not comply with labelling requirements as it did not list "perfume" or "aroma" as an ingredient, despite the presence of coumarin above the recommended limit.

Therefore, this work aims to alert producers and consumers to the risks associated with the frequent use of cold-pressed *Citrus* EOs as ingredients in cosmetic products and to provide a reliable method for quality control in cosmetics. However, this is a preliminary study and other DESs and extraction procedures will be tested to improve recovery values.

CRediT authorship contribution statement

Giovanna Cafeo: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Donatella Ferrara:** Software, Investigation. **Andrea Schincaglia:** Methodology. **Marco Beccaria:** Visualization, Methodology, Conceptualization. **Marina Russo:** Writing – review & editing, Visualization, Supervision. **Luigi Mondello:** Resources, Funding acquisition. **Paola Dugo:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Giorgia Purcaro:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Data curation, Conceptualization.

Table 3
Concentration (mg kg^{-1} ± standard deviation) of OHCs in samples analysed.

Compound	Class	C1	C2	C3	C4	C5	C6	C7
Coumarin	C	3.70 ± 0.10	20.12 ± 0.72	59.82 ± 2.70	-	1.83 ± 0.24	0.24 ± 0.00	-
Meranzin hydrate	C	-	-	0.51 ± 0.00	0.03 ± 0.00	-	0.08 ± 0.01	-
Meranzin	C	-	-	-	12.31 ± 0.07	-	-	-
Epoxyaurapten	C	-	-	0.07 ± 0.00	-	-	0.15 ± 0.00	-
Aurapten	C	-	14.85 ± 1.81	0.14 ± 0.00	-	0.12 ± 0.00	0.11 ± 0.00	-
5-geranyloxy-7-methoxycoumarin	C	-	-	-	-	1.13 ± 0.01	-	-
Tot Cs		*3.70 ± 0.10*	*34.97 ± 2.53*	*60.54 ± 2.70*	*12.34 ± 0.07*	*3.08 ± 0.25*	*0.58 ± 0.01*	-
Bergapten	FC	-	0.27 ± 0.04	0.45 ± 0.01	-	-	0.05 ± 0.01	-
Oxypeucedanin	FC	-	-	1.91 ± 0.05	-	-	-	-
Imperatorin	FC	0.33 ± 0.10	-	-	-	-	-	-
Phellopterin	FC	20.75 ± 0.06	-	-	-	-	-	-
Isoimperatorin	FC	6.78 ± 0.05	-	-	-	-	-	-
Epoxybergamottin	FC	-	-	-	0.28 ± 0.02	3.51 ± 0.05	0.31 ± 0.01	-
8-geranyloxypsoralen	FC	-	-	0.44 ± 0.04	-	-	-	-
Bergamottin	FC	0.14 ± 0.03	0.38 ± 0.08	-	-	2.09 ± 0.04	-	21.24 ± 0.01
Tot FCs		*28.00 ± 0.24*	*0.65 ± 0.12*	*2.80 ± 0.10*	*0.28 ± 0.02*	*5.60 ± 0.09*	*0.36 ± 0.02*	*21.24 ± 0.01*
Nobiletin	PMF	-	1.83 ± 0.06	0.09 ± 0.03	0.51 ± 0.00	-	-	-
Tetra-O-methylscutellarein	PMF	-	0.32 ± 0.02	-	-	-	-	-
Tangeretin	PMF	-	0.65 ± 0.11	-	-	-	-	-
5-O-demethylnobiletin	PMF	-	0.85 ± 0.03	-	-	-	-	-
Gardenin B	PMF	-	19.64 ± 0.87	-	-	-	-	-
Tot PMFs		-	*23.29 ± 0.87*	*0.09 ± 0.03*	*0.51 ± 0.00*	-	-	-
All		*31.70 ± 0.33*	*58.92 ± 3.75*	*63.43 ± 2.83*	*13.14 ± 0.09*	*8.68 ± 0.33*	*0.95 ± 0.04*	*21.24 ± 0.01*

Herniarin, Byakangelicin, 8-methoxypsoralen, Psoralen, Angelicin, Oxypeucedanin hydrate, Citropten, Isopimpinellin, Isomeranzin, Heraclenin, Sinensetin, Isobergapten, Byakangelicol, Cnidilin, Gardenin A, Cnidicin, were under LoD values for all the samples analyzed.

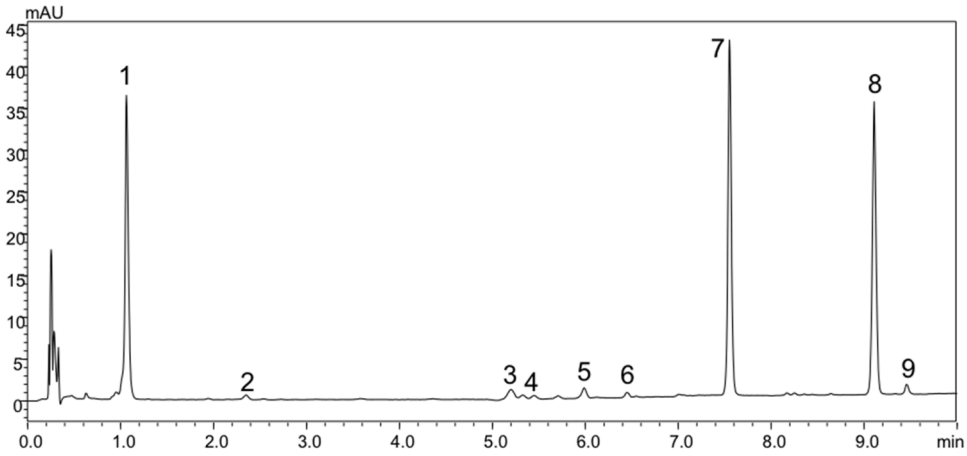

Fig. 4. HPLC-PDA chromatogram of oxygen heterocyclic compounds identified in sample C2. Peak 1. Coumarin; 2. Bergapten; 3. Nobiletin; 4. Tetra-O-methylscutellarein; 5. Tangeretin; 6. 5-O-demethylnobiletin; 7. Gardenin B; 8. Aurapten; 9. Bergamottin.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2025.466085.

Data availability

Data will be made available on request.

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