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UV gradient combined with principal component analysis: Highly sensitive and specific high performance liquid chromatography analysis of cosmetic creams

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ABSTRACT

HPLC has been employed to develop a method for the analysis of cosmetic creams, in particular the compounds hydroquinone, phenol and six preservatives have been studied. UV tuning was optimized as a gradient to achieve lower limits of detection compared to those of a previously validated method. In addition the chromatograms were then exported, aligned and visualized in a principal component analysis (PCA) model. The results were the highly efficient separation of the eight studied compounds. All the compounds showed good linear correlation coefficients (≥0.9997), the detection limit was found to be in the range of 15–200 ng/mL, a 10-fold improvement for the preservatives on previous methodology and the average recovery was within limits between 83% and 117% with a relative standard deviation (RSD) less than 3.6% ($n = 6$). The PCA plot was constructed from the UV optimized cosmetic samples chromatograms from real samples, real samples that were spiked and quality controls. Quality controls contained the eight compounds and showed complete clustering in the PCA and three spiked samples containing six to seven toxic components clustered in the same quadrant. The method is highly sensitive and its potential use as a method that could be employed in the control of cosmetics, particularly those containing banned or suspected toxic additives, has been demonstrated.

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1. Introduction

The cosmetic creams industry generates billions worldwide, while at the same time being a largely self-regulated industry. Existing laws on ingredient safety are proposed by the cosmetics industry but organisations like the US Food and Drug Administration (FDA) cannot by law require safety assessments to this very day. Consequently, several products that have been shown to be toxic are not being recalled by any safety product organization [1]. The law is currently being revised and some cosmetic creams, such as skin whitening agents, have been made illegal in the UK, mainly those that contain mercury [2]. In recent years a number of other potentially harmful compounds utilised in skin whitening creams have been identified, including several glucocorticoids, which do not comply with European regulations [3]. Concerns over the toxicity and carcinogenicity of hydroquinone, frequently found in skin whitening agents, have been reported, although these findings are controversial and largely based on *in vivo* and animal studies [4,5]. The use and safety of phenols in topical treatments, again is of concern. In addition, we consider the analysis of a number of preservatives used as antimicrobial agents, including benzoic acid, sorbic acid and parabens. The presence of such additives is regulated by the European Economic Community law and covers the list of allowed preservatives and their maximum allowed concentrations. Concentrations of benzoic acid must be less than 0.5% (w/w) for leave-on products, and less than 0.4% (w/w) or 0.8% (w/w) for parabens depending on whether one or two are present, respectively, but there is little data reported on the biosafety of these additives [6,7]. Especially concerning is the absorption and accumulation of metabolites in the body since in the case of cosmetic cream products these are often applied daily or repetitively to what is in essence our biggest organ. Main health concerns relating to parabens are based on animal studies, these have shown that exposure to certain parabens can cause adverse health effects including effects on development of the male reproductive system [8,9].

There are a limited number of methods which report the sensitive quantification of potentially toxic agents in cosmetic creams, but with increasing efforts devoted to the biosafety of such formulations. With widespread concerns on public safety, the need for sensitive, quantitative analytical techniques to determine the

Abbreviations: HQ, hydroquinone; PO, phenol; BA, benzoicacid; SA, sorbicacid; MP, methylparaben; EP, ethylparaben; PP, propylparaben; BP, butylparaben.

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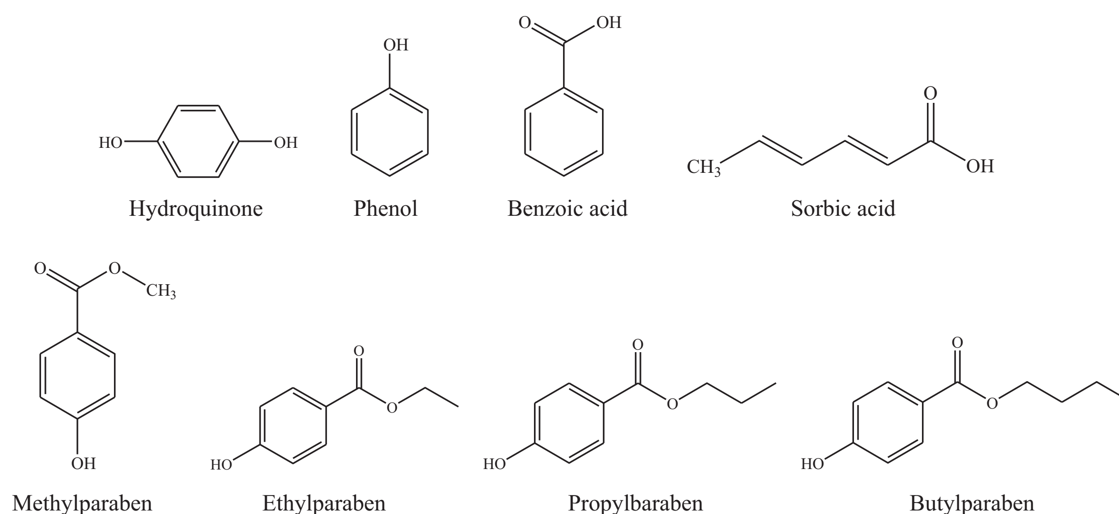

Fig. 1. Chemical structures of the compounds considered in this study.

level of dangerous agents in creams is required, particularly if regulations will soon be employed to restrict the use of such agents. Here we consider eight compounds some commonly found in cosmetic creams, which we believe to be of importance in health and safety, namely hydroquinone (HQ), phenol (PO), benzoic acid (BA), sorbic acid (SA), methylparaben (MP), ethylparaben (EP), propylparaben (PP) and butylparaben (BP) (see Fig. 1). Previously we have reported the validation of a HPLC-DAD method to separate these eight compounds of interest [10]. Since some of these components, such as hydroquinone, are not well suited to mass spectrometric detection due to poor ionisation, this study aims to increase sensitivity of the previously reported HPLC-DAD method by tuning the UV in a gradient for these particular components. This optimized, highly sensitive method allows for the simultaneous detection and quantification of the eight components and was applied to the analysis of 10 different cream samples for different cosmetic purposes. A collection of quality control samples, spiked and real samples were subsequently analyzed and visualized using a PCA model to understand the variability and test the model.

2. Experimental

2.1. Instrumentation

Chromatography was performed using on an Agilent 1100 HPLC system (Agilent, USA) consisting of a quaternary pump, an autosampler, a vacuum degasser, and a column compartment, coupled to a diode array detector. An SFE 590/1 ultrasonicator (Ultrawave Limited, Cardiff, UK) and 5415C centrifuge (Eppendorf, Germany) were used in the experiments.

2.2. Chemicals

All chemicals employed with the exception of ammonium formate were obtained from Sigma–Aldrich (St. Louis, MO, USA). Hydroquinone (HQ), phenol (PO), sorbic acid (SA), benzoic acid (BA), methylparaben (MP), ethylparaben (EP), propylparaben (PP), butylparaben (BP) were 99% purity. Methanol and acetonitrile were HPLC grade. Ammonium formate (98.2% purity) was obtained from Prolabo (VWR, UK). The water used in these experiments was purified using a Synergy UV Water Purification System (Millipore, UK) and was used to prepare all solutions for the HPLC method. The 10 cosmetic creams were purchased from UK, US, China, Spain, Sudan and Thailand and ranged from creams and lotions for skin whitening, hydration and anti-aging.

2.3. Chromatographic conditions

The chromatographic column used was a Zorbax Bonus-RP column, 100 mm × 2.1 mm I.D. with 3.5 μm particle diameter (Agilent, USA). The mobile phase consisted of a mixture of methanol and 0.05 mol/L ammonium formate solution (pH = 3.0), and the gradient elution details were as follows: 0 min, 45% methanol; 2 min, 45% methanol; 5 min, 70% methanol and maintained to a max. 20 min. The flow rate was set at 200 μL/min and the injection volume was 5 μL. All analyses were performed at room temperature. The detection wavelength conditions are shown in Table 1.

2.4. Standard solutions for quantification

Standards were prepared in 60% methanol and 40% water (v/v). Stock solutions of each standard at a concentration of 1000 mg/L were prepared. A mixture solution of the components comprised of 1.0 mL HQ, 2 mL PO, 0.2 mL SA, 1.0 mL BA, 0.5 mL MP, 0.5 mL EP, 0.5 mL PP, 0.5 mL BP, respectively, and fixed volume in a 25 mL volumetric flask in 60/40 methanol/water (v/v). Suitable working solutions with concentration in the range of 0.01–200 mg/L were also prepared as standard calibration solutions. The calibration curves were plots of area *versus* concentration and errors were calculated in OriginLab (Northampton, USA). The LODs were established at a signal-to-noise ratio (S/N) of 3.

2.5. Sample extraction

0.2 g of the cosmetic cream was accurately weighed in a glass tube. After this 5 mL of extraction solvent (methanol:water = 60:40,

Table 1
The program table for a gradient in detection wavelength.

Time	Wavelength channel	Detection wavelength	Reference wavelength
0.0	B	290	360
2.5	B	272	360
3.4	B	256	360
4.0	B	232	360
4.5	B	256	360

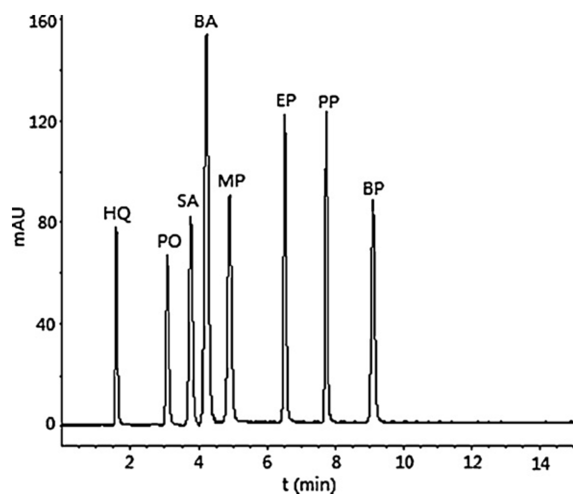

Fig. 2. Chromatogram of components under the optimum chromatographic conditions and employed as QC mixtures for the PCA model. The injection volume: 2 μL of the standard mixture solution including 40 μg/mL for HQ and SA, 80 μg/mL for PO, 8 μg/mL for SA, 20 μg/mL for four parabens. In order of elution HQ, PO, SA, BA, MP, EP, PP, BP.

v/v) were added, followed by sonication for 30 min. The extract was centrifuged for 10 min at 5000 rpm. The extract was then filtered with a 0.20 μm membrane filter from Triple Red (Buckinghamshire, UK). The filtrate was then injected into the micro-HPLC instrument.

2.6. Standard solutions for PCA

Eight quality controls (*QC*) were prepared in 60% methanol and 40% water (v/v) in the following manner: different cosmetic creams not containing the studies compounds (in lotion, cream, gel format) were spiked with the 8 compounds and extracted. QC mixtures solutions were made to contain concentrations of 40 μg/mL for HQ and SA, 80 μg/mL for PO, 8 μg/mL for SA, 20 μg/mL for four parabens (as depicted in Fig. 2).

Real samples: Real cream samples which by quantitative analysis contained four contaminants (four samples), two contaminants (five samples) and one contaminant (one sample) were employed to be spiked and be included in the model to attain samples with one to seven compounds as follows.

Real samples spiked with HQ, PO, SA and BA: Four compounds were not detected in the cohort of analyzed samples. To circumvent this and test the PCA model, we spiked two samples with end concentrations of 10 μg/mL, with HQ, PO, SA, two samples with HQ, PO and three with HQ.

Final number of compounds per sample: one sample 1 compound, two samples 2 compounds, two samples 3, one sample 4, one sample 5, one sample 6, and two samples 7 with compounds.

2.7. Chemometrics

To construct the PCA we followed a method employed in detecting toxic compounds from herbal preparations which uses QC and spiked samples together with real samples [12,13].

Gradient UV chromatograms were integrated and exported to a CVS data file format, and checked for baseline and peak alignment using in-house developed algorithms using Microsoft Excel 2003. A matrix containing retention times and absorbance as *y* and *x*-axis was then exported to create a PCA model using SIMCA-P+11.5 (Umetrics, Umeå).

3. Results

The basic optimization of extraction and chromatographic conditions were investigated previously in a validation paper. The same instrumental setup and column were chosen as the starting point to work on optimizing the detection limits. The efficiency was recalculated at the beginning of the study and found to be in the order of 88,000 plates per meter for a peak eluting at 11.5 min under isocratic conditions (column specifications showed efficiencies not higher than 70,000 plates per meter for the same retention time). The efficiency was deemed slightly higher than usual for this column, however this did not impact the higher sensitivity observed with the UV gradient as the modifications in the capillary HPLC instrument where the extra-column volumes of connections and the detector cell had been minimized had been reproduced.

3.1. Optimum detection wavelength

In order to obtain the higher sensitivity for the eight studied components considered in this study, the detection wavelength was changed during the sample run. The detection wavelength conditions are shown in Table 1. A UV detection gradient was employed choosing the highest absorbance as shown in the DAD for standard compounds at the time of the eluting peaks. Fig. 2 depicts a chromatogram of the eight studied components under these optimal conditions.

3.2. Linearity and limits of detection

As shown in Table 2, good correlation coefficients were observed for each component ($r^2 \geq 0.9997$). As expected, enhanced sensitivity was obtained owing to the optimal tuning of the UV detector for each component. Limits of detection for HQ, PO, SA, BA and the four parabens were 0.15, 0.2, 0.015, 0.2 and 0.02 μg/mL, respectively (Table 2). This approach permits improved limits of detections for each of the eight components, with particular improvement for the four parabens illustrating limits of detection up to 10 times greater than those of our previously validated method [10] (in Table 2 previous LODs can be seen). In addition, these LODs were also lower

Table 2
Linear equation, correlation coefficient and detection limit of the studied components.

Components	Linear equation	Correlation coefficient	Linear range (μg/mL)	Detection limit (μg/mL)	Detection limit[a] (μg/mL)
HQ	$y = 21.75x$ (0.0) + 0.68 (4.9)	1.0000	0.3–200	0.15	0.2
PO	$y = 12.88x$ (0.0) – 2.77 (2.6)	1.0000	0.5–200	0.2	0.5
SA	$y = 189.50x$ (0.9) + 5.70 (32.3)	0.9999	0.03–150	0.015	0.05
BA	$y = 72.07x$ (0.5) – 12.96 (21.3)	0.9998	0.5–150	0.2	1.0
MP	$y = 84.09x$ (0.4) + 8.59 (30.1)	0.9999	0.05–200	0.02	0.2
EP	$y = 83.67x$ (0.4) + 13.70 (27.6)	0.9999	0.05–200	0.02	0.2
PP	$y = 81.73x$ (0.4) + 2.21 (32.0)	0.9998	0.05–200	0.02	0.2
BP	$y = 75.91x$ (0.5) + 3.37 (39.8)	0.9997	0.05–200	0.02	0.2

[a] LODs acquired for the same method and instrumental setup with no UV gradient as published here [10].

Table 3
The recovery and precision of the studied component standard solution added to the cosmetics ($n = 6$).

Components	Amount added levels (μg)		Average recoveries (%)	RSD (%)
	Added	Found		
HQ	10	10.08–10.72	100.8–107.2	2.1–2.8
	50	50.50–54.25	101.0–108.5	0.9–1.5
	250	263.0–266.2	105.2–106.5	1.2–2.6
PO	10	10.38–11.48	103.8–114.8	2.2–2.9
	100	113.1–116.7	113.1–116.7	1.0–2.1
	250	288.5–293.0	115.4–117.2	0.7–1.6
SA	1	0.96–1.07	96.6–107.5	2.4–3.2
	10	8.76–9.82	87.6–98.2	1.9–2.6
	100	96.1–100.3	96.1–100.3	2.1–2.7
BA	10	9.31–10.53	93.1–105.3	2.1–3.6
	100	83.0–86.2	83.0–86.2	1.5–2.1
	250	223.0–247.2	89.2–98.9	1.6–2.5
MP	1	0.92–1.06	92.6–106.7	2.1–2.7
	10	10.44–11.00	104.4–110.0	1.7–2.7
	100	109.6–115.1	109.6–115.1	2.0–2.5
EP	1	0.98–1.13	98.2–113.5	2.6–3.1
	10	9.34–10.08	93.4–100.8	1.3–2.6
	100	99.2–101.9	99.2–101.9	1.1–1.8
PP	1	0.91–1.04	91.1–104.2	1.9–2.7
	10	0.92–0.96	92.2–96.9	1.0–2.4
	100	102.0–103.9	102.0–103.9	0.6–1.5
BP	1	1.060–1.134	106.0–113.4	2.1–2.7
	10	9.45–10.60	94.5–106.0	1.8–2.5
	100	103.4–108.3	103.4–108.3	1.3–1.8

than previous reports where amounts were quantified for single component extraction, such as that reported by Lopez-Garcia et al. for hydroquinone in gel and cream, with LOD and LOQ of 0.08 and 0.26 μg/mL, respectively [11].

3.3. Recovery and precision

The recovery and precision of the method were tested by adding the cosmetics with high, medium and low concentration levels for all studied components. The results were shown in Table 3, the recovery and relative standard deviation (RSD%) values ranged from of 83.0–117.2% and 0.6–3.6%, respectively. Phenol showed recoveries that were higher than the other compounds, although within regulations, this was also observed in the previous validation study [10].

3.4. Determination of real samples

The studied compound residues in 10 cosmetics products were examined using this method. The 10 samples were found to contain at least one paraben and the identification of four preservatives was seen in four samples. The components detected in the studied samples were MP, EP, PP and BP and their concentrations were found in the range of 13.85–2762 mg/kg of cream sample, and total concentrations for parabens in the studied samples were in the range of 594.0–3830 mg/kg per cream sample. This is of particular concern since animal studies show that 4.8 mg/kg bw/day resulted in developmental toxicity [4]. This raises concerns since a study of the long-term effects of parabens in humans has not been reported. Currently in several countries the maximum allowed amount of four parabens in cosmetics is 0.4%, respectively and the maximum allowed total amount of parabens in cosmetics is 0.8%. The remaining components, HQ, PO, SA and BA were not detected in this cohort of studied cosmetic samples.

3.5. Principal component analysis (PCA)

A principal components analysis (PCA) was performed to compare the variation of the extracted samples. The 10 creams and lotions differed from country of origin, composition and general usage (whitening, hydration and anti-aging) and were spiked with the compounds that were not detected in the real samples. The model was constructed by adding to it eight samples as QC prepared with all the standard compounds (Fig. 3). By doing this mixture of known chromatograms as QCs and real samples as test group the PCA should show how far the samples were from the predetermined QC chromatograms. In particular these QC chromatograms contained all of eight toxic compounds. QC samples showed as a cluster and completely superimposed, hence the samples with a higher number of toxic compounds would show near this dense cluster, indeed this was the case and are shown as a small area in the scores plot (shadowed triangular area). This is not surprising since a PCA shows the biggest differences between scores and these samples were nearer to the standard compounds than the rest. These three samples contained six to seven compounds in the real samples, as observed in the same quadrant. The remaining cosmetic samples spread furthest from the QCs, contained between one and five extracted compounds. This preliminary model has its

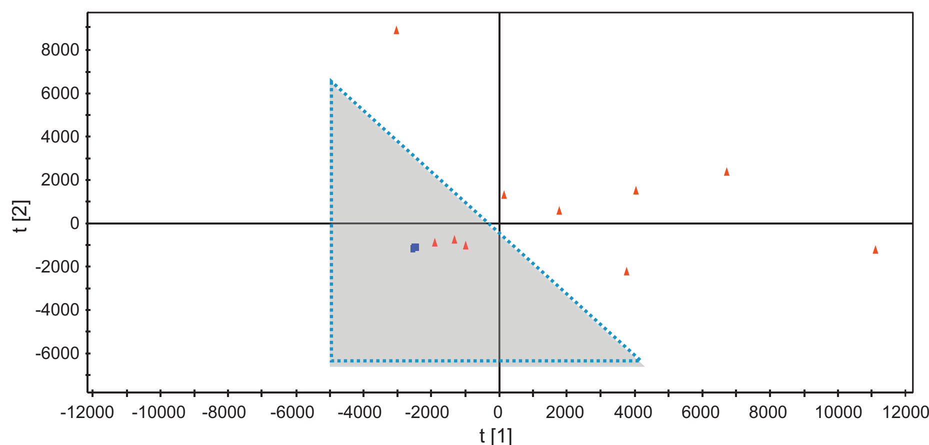

Fig. 3. (A) Representation with a PCA model of all the samples (squares are standard compounds and triangles are creams and lotions), in the scores plot the shadowed triangular area shows the superimposition of eight chromatograms produced with standard compounds, next to that three samples containing from 6 to 7 compounds in the extract are represented. The remaining cosmetic samples contain 1–5 potentially toxic compounds.

limitations, a robust model that could be applied automatically in quality control will have to include hundred of samples and sensitivity and selectivity should be also assessed. Moreover the QCs should be included in the model in a range of concentrations.

4. Conclusions

A sensitive method for the analysis of eight compounds in cosmetic creams with insufficient toxicological information showing good reproducibility and accuracy has been demonstrated. Parabens were present in all the cosmetic creams tested in this project with some showing alarmingly high concentrations illustrating their prevalence in cosmetic products. In our opinion, current safe amounts do not account for the daily usage of cosmetics and should promptly be assessed by health agencies. A PCA model was constructed to test samples and it showed that creams with a high number of these compounds clustered near prepared quality control samples.

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