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Advanced HPLC Method with Diode Array Detection Using a Phenyl-Bonded Column for Simultaneous Quantitation of Three Sunscreen Filters in a Moisturizing Sunscreen Cream for Acne-Prone Skin

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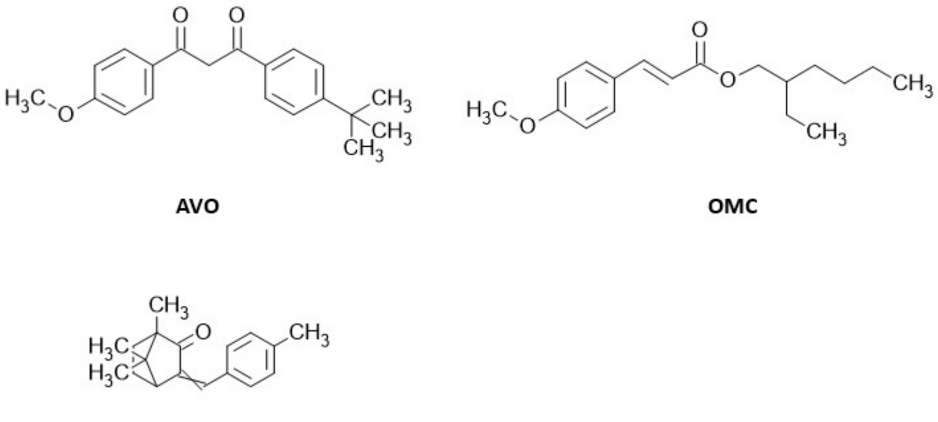
Abstract: This study introduces a novel, robust, and efficient method for the simultaneous quantitative determination of three sunscreen filters namely 4-methylbenzylidene camphor, octyl methoxycinnamate, and avobenzone, in a moisturizing sunscreen cream specifically designed for acne-prone skin. The method employs high performance liquid chromatography with photodiode-array detection, providing reliable separation of the analytes. Chromatographic separation was achieved using a Fortis Phenyl analytical column (150.0 × 2.1 mm, 5 μm), with isocratic elution at a flow rate of 0.4 mL/min. The mobile phase was composed of a 57/43 (v/v) mixture of acetonitrile/45 mM aqueous ammonium formate solution, ensuring sufficient resolution and peak symmetry for the target compounds. The method was validated comprehensively for critical performance parameters, including linearity, precision, accuracy, and robustness. Linearity was established across a suitable range for all three analytes, with high correlation coefficients. Precision was confirmed with intra-run and total precision coefficients of variation of ≤ 4.6 %, while accuracy assessments yielded a percent recovery between 98.6 and 100.4, for all quality control levels. Additionally, the method was able to effectively separate the sunscreen filters from other cosmetic ingredients, such as [β-(1.3), (1.6)-D-glucan], Low Molecular Weight (LMW) hyaluronic acid and plant extracts ensuring specificity in complex formulations. This straightforward and time efficient sample preparation process, involving methanol extraction followed by serial dilution, makes the method suitable for routine quality control in cosmetic laboratories. The method was successfully applied to the analysis of two different lots of a commercial sunscreen cream, achieving excellent recovery for all filters, ranging between 94.6% and 99.8%, thus demonstrating its reliability and applicability for the quality control of cosmetics.

Keywords: 4-methylbenzylidene camphor; enzacamene; octyl methoxycinnamate; 2-ethylhexyl-4-methoxycinnamate; avobenzone; butyl methoxydibenzoylmethane; cosmetics; sunscreen; HPLC; quality control

1. Introduction

The protective role of cosmetics containing UV filtering agents against the harmful effects of ultraviolet (UV) radiation is well documented in the literature [1,2]. With growing awareness of UV radiation's damaging impact on the skin, the global market for UV filters in cosmetics is expanding rapidly. In 2023, it is estimated to be valued at 0.31 billion USD, with a projected compound annual growth rate (CAGR) of 5.4% (3). However, adverse health effects, including (photo)allergic contact dermatitis, carcinogenic, estrogen-disrupting activity, neurotoxicity and reproductive toxicity/embryotoxicity have been described for several UV filtering agents [2,4-10]. Additionally, systematic absorption following topical application has been demonstrated for several of these agents [11]. Due to these significant safety concerns and their widespread use as the primary strategy for skin protection, the levels of UV filters in cosmetics are subject to strict regulation. For example, the European Union, Australian, Canadian and US regulations regulate the permissible concentrations of specific UV filters in cosmetic products [12-15].

In this context, the aim of this study is to develop a novel analytical method to ensure compliance with the labeled sun protection factor (SPF) of three sunscreen filters namely 4-methylbenzylidene camphor (4-MBC) also known as enzacamene, octyl methoxycinnamate (OMC) also known as 2-ethylhexyl-4-methoxycinnamate (EHMC) and avobenzone (AVO), in commercial sunscreen creams. The chemical structures of the targeted analytes are presented in Figure 1. Given that UV filters are part of a complex cosmetic matrix, their quantitation requires effective separation. High pressure liquid chromatography (HPLC) coupled with ultraviolet (UV), or photo-diode array (PDA) detection is the most common methodology for analyzing cosmetic ingredients after their extraction from cosmetic formulations [16, 17]. Liquid chromatography is indeed the most widely used method for separating sunscreen filters [18-26], while thin-layer chromatography is used less frequently [27]. Gas chromatography often involving derivatization procedures, is also employed [28]. Other less frequently applied analytical techniques for sunscreen filter analysis include Raman spectroscopy [29] and chemiluminescence [30].



AVO

OMC

4-MBC

Figure 1. Structures of UV filters analyzed in this study: AVO, Avobenzone; OMC, Octyl methoxycinnamate and 4-MBC, 4-methylbenzylidene camphor.

The targeted analytes, 4-MBC, OMC, and AVO, are representative of three major classes of organic UV filters used in topical sun protection formulations [31]. 4-MBC is currently regulated as a UVB filter, with a concentration limit of 4 % in Canada. However, it is not approved for use by the U.S. Food and Drug Administration (FDA). On April 3, 2024, the European Commission issued Regulation (EU) 2024/996, amending the EU Cosmetics Regulation (EC) No 1223/2009 to prohibit the use of 4-MBC in cosmetic products. From May 1, 2025, products containing 4-MBC will no longer be allowed on the EU market, and from May 1, 2026, all such products must be withdrawn [32]. OMC is one of the most common sunscreen agents, approved for use up to 10% in the EU. AVO, a globally approved UVA filter, has concentration limits ranging from 3 % in the U.S. and Canada to 5 % in Australia and Europe [12-15]. AVO is favored for its ability to absorb a broad range of UV radiation, but its susceptibility to photodegradation is a significant drawback [33]. In 2024, a study revealed that AVO and OMC were the two most frequently used UV filters, found in 12.1 % and 10.1 % of 742 cosmetic products, excluding sunscreens but including hygiene, personal care, and makeup products [34]. Another 2024 study identified AVO as the most common filter in 56 % of adult sunscreens sold in Poland [35]. Additionally, a 2021 study in Thailand found OMC to be the most frequently used UVB filter, while AVO was the most common UVA filter in sunscreens and other cosmetics [36].

As mentioned, a review of the literature reveals various analytical methods for identifying or quantifying sunscreen filters in cosmetic formulations [18-30]. However, few methods [18-26] encompass all three targeted analytes, and none addresses their analysis in the presence of glucans, low molecular weight (LMW) hyaluronic acid and plant extracts. Reversed-phase HPLC (RP-HPLC) with an octadecyl silica (C18) column remains the most used method for simultaneous quantitation of organic UV filters in cosmetics [19-25]. Recently, phenyl-bonded stationary phases have gained attention for their ability to improve separation and selectivity, and they have been applied in the analysis of various compounds [37], including the quantitation of 4-MBC, OMC, and AVO after supercritical fluid extraction [26].

This study introduces a novel RP-HPLC-PDA method using a phenyl-bonded stationary phase, with a simple sample preparation procedure involving serial dilution and filtration. This method aims to control the content of specific UV filters in cosmetic products, focusing on routine analysis and quality control in cosmetic laboratories. One key challenge in RP-C18-based chromatography is the poor resolution between OMC and AVO peaks, which often requires extensive optimization [18, 20]. AVO, in particular, poses a chromatographic challenge due to its beta-dicarbonyl structure and enolization behavior, complicating its retention and separation [23, 31, 38]. This study proposes an alternative RP-HPLC column with a phenyl-bonded stationary phase, offering efficient retention and satisfactory separation of this challenging pair (OMC and AVO), even in the presence of a third UV filter. Additionally, other ingredients such as LMW hyaluronic acid and trimethylglycine do not interfere with the analysis of the targeted analytes.

2. Materials and Methods

2.1. Reagents

Acetonitrile, water and methanol of HPLC grade, were from Merck (Darmstadt, Germany) and were employed for the extraction procedure and the development and validation of the chromatographic method. Analytical grade ammonium formate, ammonium acetate and ammonium bicarbonate were obtained from Acros Organics (New Jersey, USA). 4-methylbenzylidene camphor (4-MBC, 99.8 % pure) and avobenzone (AVO, 99.6 % pure) were purchased from Bell Flavors and Fragrances GmbH (Leipzig, Germany), while octyl methoxycinnamate (OMC, 99.9 % pure) was purchased from Roumboulakis SA, Ashland representative in Aspropyrgos, Greece.

Two different lots of a hydrating sunscreen cream for acneic, oily skin with SPF 15 were purchased from a local pharmacy. The cosmetic cream is labelled to contain 2.5% w/w 4-MBC, 7.5% w/w OMC and 2% w/w AVO, trimethylglycine, glycerol, propylene glycol together with low molecular weight (LMW) hyaluronic acid for hydration, Zea mays starch extract for oil absorption and skin soothing and schizophyllan for protecting the skin from environmental pollutants. A full list of ingredients (INCI names) is provided below: Aqua, Ethylhexyl Methoxycinnamate, 4-Methylbenzylidene Camphor, Butyl Methoxydibenzoylmethane, Glycerin, Isononyl Isononanoate, Sodium Acrylates Copolymer, Cetyl Dimethicone, Dimethicone, Tocopheryl Acetate, Zea Mays Kernel Extract, Helianthus Annuus Seed Oil, Betaine, Phospholipids, [β-(1.3), (1.6)-D-glucan], Sodium Hyaluronate, Cyclopentasiloxane, Cyclohexasiloxane, Sodium Polyacrylate, Propylene Glycol, Decylene Glycol, Hydrogenated Polydecene, Polyglyceryl-10 Stearate, Disodium EDTA, Phenoxyethanol, BHT, Imidazolidinyl Urea, Parfum, Benzyl Salicylate, Coumarin, d-Limonene, Linalool.

2.2. Equipment, Consumables and Software

The high-performance liquid chromatography (HPLC) system consisted of a Waters 1515 isocratic pump (Waters Co, Milford, MA, USA) equipped with a temperature oven, a Waters 717 plus autosampler, and a Waters 996 PDA detector. Data acquisition and analysis were performed using the Empower software (Waters, Milford, MA, USA). All substances were detected using the

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photodiode array (PDA) detector over the wavelength range 200 to 390 nm and the chromatograms were extracted at λ_{max} 300 nm for the detection of 4-MBC and OMC, and at 359 nm for the detection of AVO. The chromatographic column was a Fortis Phenyl, 150 x 2.1, 5 μm particle size, 135 Å (Fortis Technologies Ltd., Neston, Cheshire, UK). The mobile phase was degassed by vacuum filtration through nylon filters with a pore size of 0.45 μm from Gelman Sciences Ltd (Portsmouth, Hampshire, UK). The substances were dissolved in an appropriate solvent for the preparation of solutions in an Elma Transonic 460 ultrasonic bath. Regenerated Cellulose (RC) syringe filters (13 mm, pore size 0.45 μm) were obtained from RephiLe Bioscience Ltd Europe, Novalab representative (Athens, Greece) for sample filtration prior to chromatographic analysis. The ADME Boxes ver. 3.0.3 (Build 45) software from Pharma Algorithms Inc. (Toronto, Canada) was used to predict the physicochemical properties of the targeted analytes and the SPSS software ver. 22.0.2 (IBM Corporation, USA) was used for the statistical analysis of data.

2.3. Preparation of Stock Standards, Calibration Standards and Quality Control Samples

To prepare stock standard solutions of the targeted analytes at 0.50 mg/mL, accurately measured amounts of each analyte that correspond to 12.5 mg of pure analyte (4-MBC, OMC, AVO) were transferred to 25.0 mL volumetric flasks and diluted to volume with acetonitrile (ACN). Mixed stock solution A, containing 4-MBC, OMC, and AVO at 100, 300 και 80 μg/mL, respectively and mixed stock standard solution B at 10 μg/mL 4-MBC, 30 μg/mL OMC, and 8 μg/mL AVO were prepared in methanol (MeOH) by appropriate dilutions. These solutions were stored in the dark and under refrigeration at 4℃ and remained stable for two months.

Mixed calibration standards and mixed quality control (QC) samples were freshly prepared every working day in a mixture of MeOH/ACN/45 mM aqueous ammonium formate (AMF) solution at a ratio 20/40/40, v/v/v (dilution solvent). These calibration standards contained 4-MBC over the range 0.8 to 4.0 μg/mL, OMC over the range 2.4 to 12.0 μg/mL, and AVO over the range 0.64 to 3.2 μg/mL. QC samples were prepared in the same way at three concentration levels, 0.8, 2.0 and 4.0 μg/mL for 4-MBC, 2.4, 6.0 and 12.0 μg/mL for OMC and 0.64, 1.6 and 3.2 μg/mL for AVO.

2.4. HPLC-PDA Analysis

For quantitation purposes, 4-MBC and OMC were detected at 300 nm and AVO was detected at 359 nm. The mobile phase consisted of a mixture of ACN/45 mM AMF aqueous solution in a 57/43 (v/v) ratio. Analysis protocol, including elution, was isocratic, with a flow rate set at 0.4 mL/min. The analysis was performed using a 10 μL loop volume, at room temperature (25 ^{o}C).

2.5. Sample Preparation

To assess the suitability of the proposed RP HPLC-PDA method for sunscreen analysis, a sunscreen cream was analyzed, which contained all three filters in the following proportions 2.5% w/w 4-MBC; 7.5% w/w OMC; 2.0% w/w AVO. Hence, the sunscreen cream incorporated the three filters at the same ratio as in the working solutions and quality controls. Approximately 100 mg of sunscreen cream were accurately weighed into a 50 mL beaker, and 25 mL of MeOH were added. The mixture was stirred vigorously, and the contents of the beaker were transferred to a 100 mL volumetric flask, and 25 mL of MeOH were added. The flask was placed in an ultrasonic bath for 10 min to ensure complete dissolution. The solution was then diluted to the mark with MeOH. Accurately, 800 μL of the diluted solution were transferred to a 2 mL volumetric flask and diluted to the mark with MeOH. Accurately, 200 μL of the further diluted solution were transferred to a 1 mL vial, and 400 μL of ACN and 400 μL of 45 mM AMF aqueous solution were added. The solution was then filtered through an RC filter (13 mm, pore size 0.45 μm). The filtration of 4-MBC, OMC, and AVO through the RC syringe filter resulted in very high recovery, with 99.1%, 99.6%, and 99.8% of the compound being recovered, respectively. This confirmed the suitability of RC filters for sample preparation prior to injection into the HPLC-PDA system. The filtered solution was then injected into the HPLC-PDA system for analysis. A schematic of the cream pre-treatment procedure is provided in Figure 2.

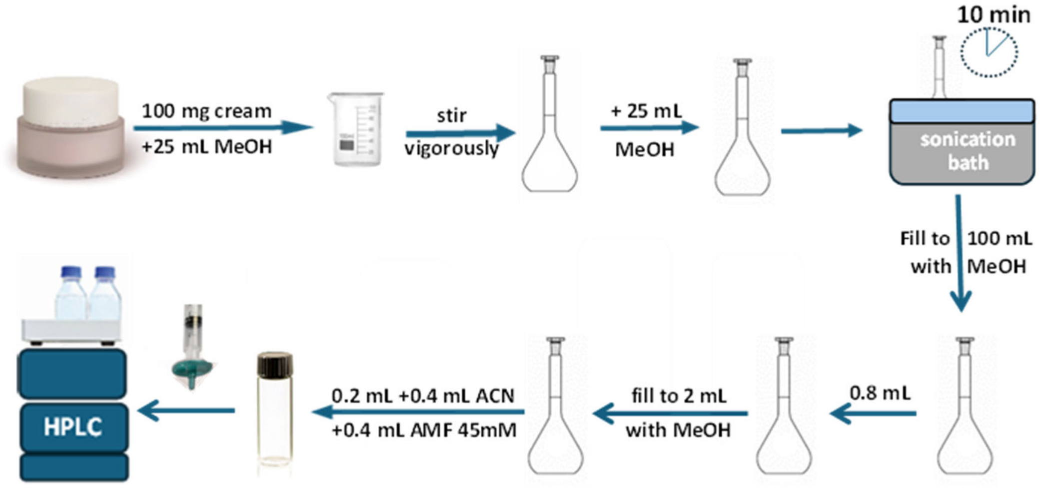

Figure 2. Schematic representation of a sunscreen cream pre-treatment procedure.

3. Results and Discussion

3.1. Chromatographic Method Development

The Fortis Phenyl analytical column, featuring a diphenyl-bonded packing material, offers unique chromatographic properties by combining π-π interactions with hydrophobic interactions. Unlike traditional alkyl-bonded phases like octadecyl silica (C18) or octyl silica (C8), the phenyl groups on the stationary phase introduce an additional π-π interaction mechanism with electron-rich aromatic rings present in the analytes. This characteristic enhances selectivity for aromatic compounds, making phenyl-bonded phases particularly effective for separating aromatic from non-aromatic analytes. Given that the targeted analytes—4-methylbenzylidene camphor (4-MBC), octyl methoxycinnamate (OMC) and avobenzone (AVO)—are all chemical sunscreens with aromatic structures and extended conjugation (Figure 1), the phenyl-bonded column is well-suited for their separation.

To optimize the chromatographic conditions, we evaluated the influence of mobile phase composition on the retention behavior of the targeted analytes using the Fortis Phenyl column. The parameters investigated included salt type, salt concentration, and organic modifier percentage, with the mobile phase flow rate maintained at 0.4 mL/min. Acetonitrile (ACN) was selected as the organic modifier due to its lower viscosity in aqueous mixtures compared to methanol (MeOH), minimizing back pressure during analysis. Room temperature was chosen to enhance sustainability and ensure the stability of AVO, which is more stable at ambient conditions than at elevated temperatures [39].

Physicochemical properties of the three sunscreen filters, calculated using the ADME Boxes software, provided further insights into their chromatographic behavior. Both 4-MBC and OMC are lipophilic across the entire pH range, with positive distribution coefficient (LogD) values of 4.33 and 5.71, respectively. AVO, with a stable LogD value of approximately 4.57 at pH values from 1 to 7.0, becomes significantly less lipophilic at higher pH levels, with its LogD dropping from 4.39 at pH 8.0 to 0.61 at pH 13. Moreover, AVO undergoes hydroxide-ion-catalyzed degradation in aqueous solutions at pH values between 7.4 and 10.0 [23, 39]. Therefore, a mobile phase with a pH well below 8.0 was selected to ensure effective separation of AVO from 4-MBC and OMC, while preventing degradation of AVO during analysis.

In these experiments, a mixed working standard solution containing 4-MBC, OMC, and AVO at a concentration of 0.4 μg/mL was prepared using a 90/10 (v/v) ACN/H2O mixture. The initial phase of the study aimed to assess the influence of different salts in the mobile phase, specifically ammonium formate (AMF), ammonium acetate (AMA), and ammonium bicarbonate (AMC), each tested at a concentration of 25 mM, with a fixed acetonitrile content of 55%. The elution order remained consistent across all three salts, with 4-MBC eluting first, followed by OMC and AVO. The retention times of AVO, the last eluted sunscreen, were 17.0, 18.1, and 15.9 min for AMF, AMA, and AMC, respectively. The resolution between OMC and AVO was 2.32, 1.82, and 1.26 for AMF, AMA, and AMC, respectively. Additionally, peak symmetry for all analytes was better than 1.22 in AMF, better than 1.23 in AMA, and better than 0.82 in AMC. Thus, AMF was determined to be the optimal salt, providing superior peak shapes and efficient separation of the three sunscreen filters within a short analysis time.

Building on this, further optimization was carried out by varying salt concentration, and the percentage of the organic modifier, while maintaining other conditions constant. AMF concentration was tested in the range of 5 mM to 50 mM, with the ACN content adjusted to 60% to further reduce analysis time. In all cases, the elution order remained consistent, with 4-MBC eluting first, followed by OMC and AVO, and resolution between OMC and AVO consistently ranging between 1.28 and 1.67. It was observed (Figure 3A) that increasing AMF concentration had a slight effect on retention times, while the number of theoretical plates (Figure 3B) improved up to 45 mM AMF. Peak symmetry (Figure 3C) was optimal between 15–25 mM but remained close to ideal at 45 mM. Consequently, 45 mM AMF was selected as the optimal concentration. Additional experiments explored ACN levels of 55%, 57%, 58%, and 60%, while keeping the total AMF content at 1.85 mmol/100 mL. Increasing the ACN percentage led to shorter retention times (Figure 4A), with 57% ACN offering the best compromise between analysis time and resolution. During method development, it was also found that the sample dilution solvent significantly influenced separation efficiency and peak shape (Figure 4B). The optimal mixture for sample preparation was determined to be MeOH/ACN/45 mM AMF aqueous solution (20/40/40, v/v/v), providing the highest number of theoretical plates across all analytes.

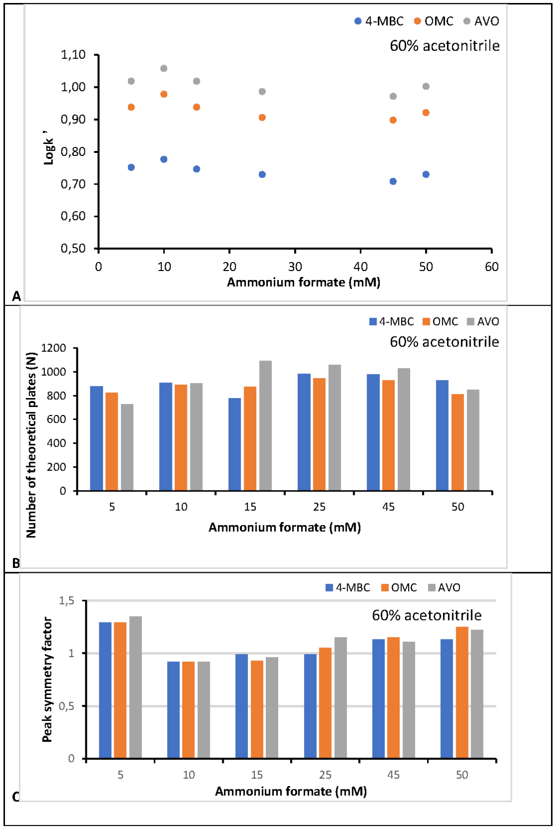

Figure 3. Impact of the concentration of ammonium formate (mM) in the aqueous eluent of mobile phase consisting of ACN/AMF, 60/40 (v/v), on the Logk’ values (**A**); number of theoretical plates (**B**);

and peak symmetry factor (**C**), of a solution of 4-MBC, OMC and AVO at 0.4 μg/mL each, dissolved in ACN/H2O, 90/10 (v/v).

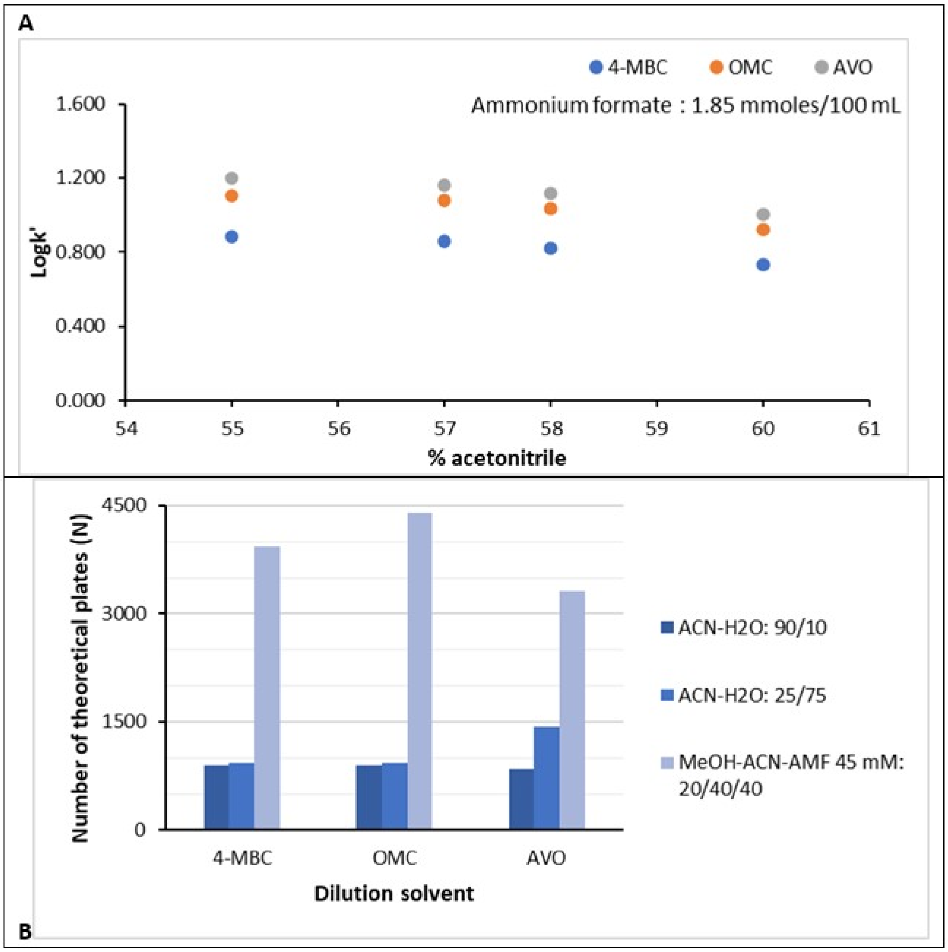

Figure 4. (**A**) Impact of the % acetonitrile in the mobile phase on the Logk' factors of the targeted analytes, (**B**) Impact of the dilution solvent of a sample analyzed under optimum chromatographic conditions on the number of theoretical plates of the targeted analytes.

Based on the optimization studies above, the RP-HPLC-PDA conditions were established for the simultaneous quantitation of the three sunscreen filters: Chromatographic separation was achieved using a Fortis Phenyl analytical column (150.0 x 2.1 mm, 5 μm), with isocratic elution at a flow rate of 0.4 mL/min, at room temperature. The optimum mobile phase is composed of 57/43 (v/v) ACN/45 mM AMF aqueous solution. Figure 5 presents a typical chromatogram obtained upon the analysis of a mixed working solution containing all three sunscreen filters (4-MBC, OMC, AVO) at concentrations of 2.0, 6.0 and 1.6 μg/mL, respectively. Based on the HPLC-PDA chromatogram presented in Figure 5, 4-MBC and OMC were detected at 300 nm and AVO was detected at 359 nm, for quantitation purposes.

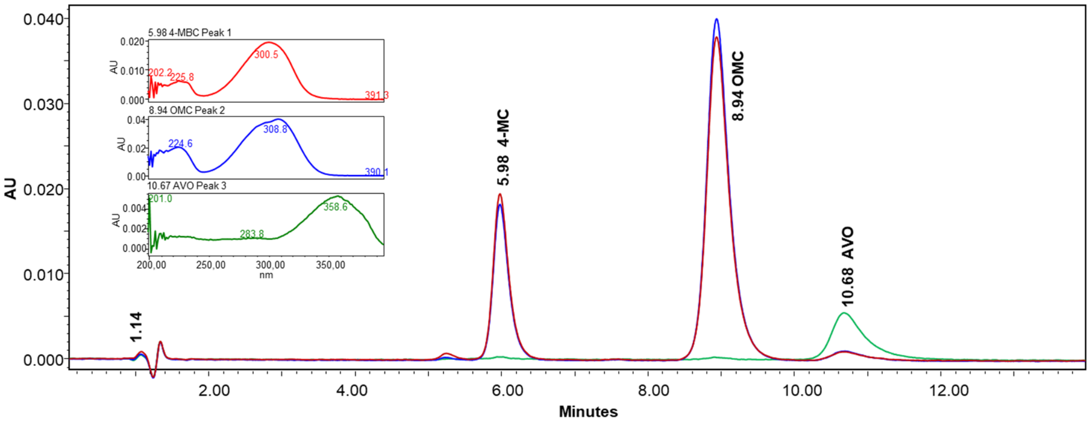

Figure 5. Typical HPLC-PDA chromatogram obtained upon the analysis of a mixed working solution containing all three sunscreen filters (4-MBC, OMC, AVO) at concentrations of 2.0, 6.0 and 1.6 μg/mL, extracted at 300 nm (red line), 309 nm (blue line), and 359 nm (green line), alongside with UV spectra of 4-MBC (λmax 300 nm; red line), OMC (λmax 309 nm; blue line) and AVO (λmax 359 nm; red line) over the wavelength range 200 to 390 nm.

Parameters that describe column performance upon analysis of the targeted analytes are summarized in Table 1. Resolution was in all cases greater than 2.85 indicating satisfactory resolution between adjacent peaks. The Symmetry Factor was for all peaks between 1.23 and 1.26, which fulfills the acceptance criteria of 0.8 to 1.8, according to Ph. Eur. guidelines [41]. The values of these two parameters demonstrate the suitability of the proposed system for the analysis of solutions containing simultaneously 4-MBC, OMC and AVO.

Table 1. System suitability parameters of the HPLC-PDA method upon analysis of a mixed working solution containing 4-MBC, OMC and AVO at 2.0, 6.0 and 1.6 μg/mL, respectively.

Compound	System suitability parameter (±SD)[1]			
	Rt	Resolution[2]	Symmetry Factor	N[3]
t0	1.052 (±0.032)			
4-MBC	6.01 (±0.17)	13.76 (±0.43)	1.23 (±0.03)	3800 (±99)
OMC	9.00 (±0.33)	6.34 (±0.16)	1.23 (±0.03)	4243 (±156)
AVO	10.72(±0.41)	2.85 (±0.14)	1.26 (±0.11)	4454 (±173)

[1] SD values were based on ten replicate determinations. [2] Resolution is calculated between consecutive peak pairs. [3] EP count number.

3.2. Linearity Evaluation

To assess the linearity of the proposed HPLC-PDA method for quantifying the targeted analytes, data from three calibration curves constructed on three different days over a four-week period were used. The evaluation involved the following steps: Mixed working solutions containing the three sunscreen filters (4-MBC, OMC, AVO) were prepared at five concentration levels. Each working solution was injected twice into the HPLC-PDA system. The peak area responses from the two injections were averaged and used for the regression analysis. The average statistical parameters for the calibration curves, including the correlation coefficient (r), standard error of the estimation (Sr), and standard deviations of the intercepts and slopes, are summarized in Table 2. The correlation coefficients for all calibration curves are greater than 0.9992 indicating strong linear relationships between the response and concentration. Additionally, the low values of the standard error of estimation (Sr) and the standard deviations of the intercept and slope suggest high precision and reliability in the calibration curves. A statistical test was performed on the calibration curves to assess whether the y-intercept was significantly different from zero, using Student's t-test. The t-test results were below the critical value of 3.182 (for df=n-2=3 degrees of freedom, P = 0.05), indicating that the y-intercepts are not statistically different from zero at the 5% significance level. This supports the validity of the regression models and allows for quantitation during routine analysis using only a single calibration standard.

Table 2. Linearity data for the quantitation of 4-MBC, OMC and AVO by the proposed HPLC-PDA method.

Analyte [1]	Linearity Range (μg/mL)	Linear Equation [2]	R [3]	Standard Deviation		Sr [4]	t_{exp} [5]
				Slope	y-intercept		
4-MBC	0.8–4.0	$S_{MBC}=161660 \times C_{MBC}-931$	0.9997	2291	5407	5709	0.172
OMC	2.4–12.0	$S_{OMC}=132599 \times C_{OMC}-4556$	0.9997	1880	13314	14057	0.342
AVO	0.64–3.20	$S_{AVO}=127784 \times C_{AVO}-9396$	0.9992	2958	868	5585	1.68

[1] 4-MBC and OMC were detected at 300 nm; AVO was detected at 359 nm. [2] Peak area signal of each analyte versus the corresponding concentration. [3] Correlation coefficient. [4] Standard error of the estimation. [5] Experimental t_{value}; theoretical value of t at P= 0.05 and df = n-2 =3 is 3.182.

Limit of Detection (LOD) and Limit of Quantification (LOQ) values for the targeted analytes were calculated experimentally by serial dilutions of the sample in the mobile phase as the concentrations for which the signal-to-noise ratios were 3:1 and 10:1, respectively. LODs for all the targeted analytes were 0.1 μg/mL, and LOQs were 0.3 μg/mL. At the Lower Limit of Quantification (LLOQ), which was 0.8, 2.4 and 0.64 μg/mL, for 4-MBC, OMC and AVO, respectively, the % relative analytical error (Er%) was less than ± 5.0 %, ± 3.5 %, and ± 8.0 %, respectively, all well within the European Medicines Agency (EMA) specification of ± 20%. For the remaining concentrations, the Er% also met the EMA's criteria of ± 15 % for all filters [40]. These results confirm the accuracy and reliability of the method across the tested concentration range. The consistently low Er% values demonstrate the method's precision, while the Coefficient of Variation (CV%), was below 3.4 % for all but one concentration level, where it reached 12.43 %, indicating acceptable reproducibility.

3.3. *Accuracy and Precision*

Accuracy and precision study was conducted to evaluate the proposed method. Quality control samples, prepared as outlined in Section 2.3., were analyzed at three concentration levels: low, medium, and high. Five replicates were analyzed on each of three separate days, resulting in a total of 15 measurements per concentration level. Table 3 summarizes the data obtained during the accuracy and precision evaluation. It presents the following information for each concentration level (low, medium, and high): Calculated Concentration values obtained from the analysis of the quality control samples (QCs), CV%, as a measure of the intra-run and total precision of the method at each concentration level and % Recovery, which assesses the accuracy of the method. Based on the obtained results, % CV referring to total precision for 4-MBC ranged from 3.2 to 4.1, for OMC from 3.5 to 4.3, and for AVO from 3.5 to 4.6. The % recovery for 4-MBC ranged from 98.8 to 99.4, for OMC from 98.6 to 100.4, and for AVO from 99.4 to 100.4. These values indicate the acceptable precision of the method in terms of day-to-day variability and suggest that the method is reasonably accurate for all three analytes, according to EMA specifications [40].

Table 3. Accuracy and precision data of the HPLC-PDA method obtained from the analysis of QC samples (n=3 runs in 5 replicates).

Compound	Concentration Level (μg/mL)	Intra-run Precision (CV%) [1]	Total Precision (CV%) [1]	Mean Concentration (μg/mL)	Total Accuracy (Recovery %)
4-MBC	0.8	3.8	4.1	0.790	98.8
	2.0	2.8	3.6	1.988	99.4
	4.0	2.6	3.2	3.973	99.3
OMC	2.4	3.3	4.2	2.366	98.6
	6.0	2.4	3.5	5.99	99.9
	12.0	2.6	4.3	12.04	100.4
AVO	0.64	2.9	4.6	0.636	99.4
	1.6	2.4	3.6	1.606	100.4
	3.2	2.7	3.5	3.198	99.9

[1] Coefficient of variation; intra- and inter-run CVs (calculated by ANOVA).

3.4. Robustness Evaluation

To evaluate the robustness of the proposed method, minor changes were introduced to AMF salt concentration and ACN percentage in the mobile phase, as well as to the monitoring wavelength value. A mixed working solution containing 2.4 μg/mL 4-MBC, 7.2 μg/mL OMC and 1.94 μg/mL AVO was analyzed following the proposed HPLC-PDA method, where AMF salt concentration varied by ± 1 mM, ACN percentage varied by ± 1 %, while the monitoring wavelength for each analyte varied by ± 1nm. The impact of these changes on % recovery was evaluated. As shown in Table 4, small variations in these parameters did not significantly affect the % recovery of the analytes, with CV % remaining below 2 % in all cases. Therefore, it can be concluded that the method is robust.

Table 4. Robustness evaluation: % recovery of 4-MBC, OMC and AVO upon slight variation of three factors at three levels (1, 0, -1); each time a factor was changed from level (0) the other factors remained at level (0).

Factors	**[AMF][1]** 45 ± 1 mM			**λ[2]** λ ± 1 nm			**[ACN][3]** 57 ± 1 %		
Analyte	Average	$SD_{n=3}$	%CV	Average	$SD_{n=3}$	%CV	Average	$SD_{n=3}$	%CV
4-MBC	99.2	1.8	1.78	99.2	1.8	1.78	99.1	1.8	1.77
OMC	102.5	0.6	0.54	102.4	1.2	1.15	100.8	2.0	1.96
AVO	99.5	0.2	0.20	99.7	0.4	0.40	100.1	0.8	0.82

[1] Ammonium formate concentration in the mobile phase ([AMF], varied by ± 1mM), [2] Detection wavelength (λ, which varied by ± 1nm), [3] Acetonitrile percentage in the mobile phase ([ACN]%, which varied by ± 1%).

3.5. Application to the Analysis of Real Samples

To further assess the applicability of the method, the quantitation of the targeted analytes was performed in two different lots of moisturizing sunscreen cream. As mentioned in section 2.1, the cream is labeled to contain 2.5 % w/w 4-MBC, 7.5% w/w OMC, and 2.0% w/w AVO.

Sample preparation was carried out according to the procedure outlined in section 2.5 and schematically illustrated in Figure 2. A typical chromatogram of the commercial sample, analyzed using the proposed method, is shown in Figure 6. Resolution was greater than 2.85 in all cases, indicating satisfactory separation between adjacent peaks. The symmetry factor was within the acceptance range of 0.8 to 1.8 as per the Ph. Eur. guidelines [41], demonstrating the suitability of the method for analyzing cosmetic cream samples containing 4-MBC, OMC, and AVO. The results of the sunscreen analysis, shown in Table 5, indicate satisfactory recovery for all three targeted analytes.

Specifically, mean recovery for 4-MBC ranged from 96.9 % to 98.2 %, for OMC from 98.1 % to 99.8 %, and for AVO from 94.6 % to 94.7 %. These results confirm that the analytical method is suitable for the analysis of sunscreen products, providing accurate and reliable results with a simple sample preparation process.

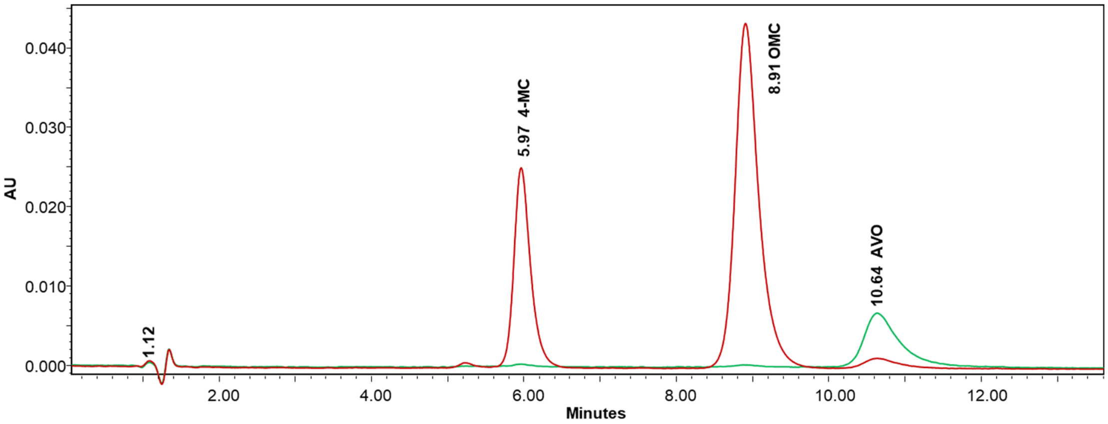

Figure 6. Typical HPLC-PDA chromatogram of a cosmetic cream (Lot 1) prepared according to the optimum sample preparation procedure and extracted at 300 nm (red line) and 359 nm (green line).

Table 5. Analysis of two different lots of the sunscreen cream containing 2.5% w/w 4-MBC, 7.5% w/w OMC and 2.0% w/w AVO by the proposed HPLC-PDA method.

Lot Number	Analyte	(% w/w) Mean ± SD $_{(n=3)}$	Recovery (%) Mean ± SD $_{(n=3)}$
1	4-MBC	2.474 ± 0.042	98.2 ±1.3
	OMC	7.36 ± 0.17	98.1 ± 2.3
	AVO	1.893 ± 0.061	94.6 ± 3.1
2	4-MBC	2.422 ± 0.097	96.9 ± 3.9
	OMC	7.483 ± 0.049	99.8 ± 0.66
	AVO	1.893 ± 0.049	94.7 ± 2.5

3.6. Comparison with Other Analytical Methods

The proposed HPLC-PDA method has been compared with other methods for the analysis of 4-MBC, AVO, and OMC in cosmetics reported in the literature. Notably, Rastogi et al. (1998) developed an ion-pair HPLC method using tetrabutylammonium hydroxide in the mobile phase, coupled with UV detection, for the identification of twenty UV filters, including the three analytes. However, their method focuses solely on the identification rather than quantitation of the analytes with a long run time (up to 45 min), and manual interpretation of overlapping peaks adds complexity to the analysis [18]. Scalia et al. (2000), employed a more complex sample preparation technique, supercritical fluid extraction (SFE) [26], compared to the simpler serial dilution approach used in the proposed method. Hauri et al. (2003) developed an HPLC-PDA method for screening and quantitation of twenty-one organic sunscreen filters, including 4-MBC, OMC, and AVO. The method employed a Kromasil C18 column and used multiple solvent systems for extraction, with gradient elution, 30 min run time and flow rates varying up to 2 mL/min. Although the method was selective and robust, the extraction process required different solvents or conditions depending on the polarity of the sunscreen filters, making the extraction process more complex [21]. Schakel et al. (2004) has published a gradient HPLC-PDA method with a more complex sample preparation involving Tween 80 and EDTA for breaking emulsions and improving separation of sixteen UV filters. While their approach targets more analytes, our method is avoiding additives like EDTA and achieving efficient separation of three specific sunscreen filters with a faster analysis time [22]. Salvador et al. (2005) has published an

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HPLC method for the determination of eighteen UV filters in cosmetics. The analytes were divided into fat-soluble and water-soluble groups, and the method involves two separate chromatographic runs and more complex mobile phase adjustments [19]. In a method proposed by De Orsi et al. (2006) both 4-MBC and OMC were analyzed together by the same method, which used a Thermo Hypsersil C18 analytical column and a gradient elution. Nevertheless, AVO was analyzed separately under a method using a Discovery RP-amide C16 analytical column with isocratic elution [23]. Escamilla et al. (2009) developed a rapid LC method for twelve UV filters, including 4-MBC, OMC, and AVO, using a Chromolith Performance RP-18e column and an ethanol/water mixture as a sustainable mobile phase. While they addressed the initial peak overlap between several filters, including 4-MBC and OMC, by adjusting the gradient and lowering the column temperature, no specific resolution data were provided. Additionally, their method utilized a high flow rate of 3 mL/min, which, while reducing analysis time to 5.5 min, may result in higher solvent consumption [24]. Kim et al. (2011) developed an HPLC method for the simultaneous quantitation of nine UV filters, including OMC, 4-MBC, and AVO, and four preservatives in commercial suncare products [25]. Wharton et al. (2015) has also developed an HPLC method for the quantitation of ten sunscreens, which involves a more complex system of multiple columns to separate different groups of organic filters [20]. While the studies by Kim et al. and Wharton et al. were applied to the analysis of cosmetic formulations, only our proposed method addresses the simultaneous analysis of all three targeted analytes (OMC, 4-MBC, and AVO) in the presence of low molecular weight (LMW) hyaluronic acid, glucans and plant extracts.

4. Conclusions

The proposed method offers a reliable, selective, and simultaneous quantitation of three key sunscreen filters, 4-methylbenzylidene camphor (4-MBC), octyl methoxycinnamate (OMC), and avobenzone (AVO), in a moisturizing sunscreen for acne-prone skin containing also [β-(1.3), (1.6)-D-glucan], low molecular weight (LMW) hyaluronic acid and plant extracts. Utilizing isocratic reversed-phase high-performance liquid chromatography (RP-HPLC) on a Fortis Phenyl column, the method achieves efficient separation of the analytes within 14 min. The sample preparation involves a simple methanol extraction followed by serial dilutions, ensuring a straightforward and time-efficient process. Additionally, the method effectively separates the sunscreen filters from glucans, plant extracts, LMW hyaluronic acid and other moisturizing ingredients, ensuring accurate analysis. This ease of preparation, combined with the short analysis time, makes the method well-suited for routine quality control in laboratory settings. The method was thoroughly evaluated using mixed working solutions of the three targeted analytes, demonstrating excellent linearity, accuracy, precision, and robustness with minimal sensitivity to parameter variations. It can, hence, be applied to quality control to check if legal restrictions concerning the 4-MBC, OMC and AVO are met.

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