การวิเคราะห์ปริมาณเมทิลพาราเบน โพรพิลพาราเบน พีน็อกซี่เอทานอล และคลอร์เฟเนซิน ด้วยวิธีการโครมาโทกราฟีเหลวสมรรถนะสูง
Simultaneous Determination of Methylparaben, Propylparaben, Phenoxyethanol and Chlorphenesin by High Performance Liquid Chromatography

นิพนธ์ต้นฉบับ **Original Article**

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วารสารไทยเภสัชศาสตร์และวิทยาการสุขภาพ2564;16(3):263-269.

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บทคัดย่อ

วัตถุประสงค์: พัฒนาและตรวจสอบความถูกต้องของวิธีวิเคราะห์ปริมาณสารกันบูด 4 ชนิด คือ เมทิลพาราเบน (methylparaben; MP) โพรพิลพาราเบน (propylparaben; PP) พีน็อกซี่เอทานอล (phenoxyethanol; PE) และคลอร์เฟเนซิน (chlorphenesin; CH) ด้วยวิธีโครมาโทกราฟีเหลวสมรรถนะสูง (high performance liquid chromatography; HPLC) **วิธีการศึกษา:** ศึกษาและปรับเปลี่ยนสภาวะของ HPLC ให้สามารถวิเคราะห์หาปริมาณสารกันบูด 4 ชนิด ในเครื่องสำอาง และตรวจสอบความถูกต้องของวิธีวิเคราะห์ **ผลการศึกษา:** สภาวะการวิเคราะห์ด้วย HPLC คือ คอลัมน์ C18 (250 mm×4.6 mm, 5 µm) ใช้อาซีโตไนไตรล์ และกรดฟอสฟอริกความเข้มข้น 0.1 % v/v เป็นเฟสเคลื่อนที่ พร้อมกับมีการปรับอัตราส่วน อัตราไหลของเฟสเคลื่อนที่ คือ 1.5 mL/min. ปริมาณการฉีดเป็น 10 µL ใช้ความยาวคลื่น 270 nm พบ retention time ของ PE คือ 6.8 นาที MP คือ 8.1 นาที CH คือ 10.3 นาที และ PP คือ 14.5 นาที ใช้เวลาวิเคราะห์ 30 นาที พบความสัมพันธ์เชิงเส้นตรงระหว่างพื้นที่ใต้กราฟและความเข้มข้นของสารละลายมาตรฐาน (R^2 > 0.997) ซึ่ง MP, PP, PE และ CH อยู่ในช่วงความเข้มข้น 60 - 300, 12 - 60, 200 - 1000 และ 96 - 480 µg/mL ตามลำดับ ความเข้มข้นต่ำสุดของสารที่ทดสอบซึ่งเป็นที่ยอมรับได้ (LOQ) ของ MP, PP, PE และ CH 19.29, 1.50, 66.30 µg/mL และ 22.88 µg/mL ตามลำดับ ความถูกต้องของวิธีมีร้อยละการคืนกลับอยู่ในช่วง 96.10 – 102.35% ผลความแม่นยำมีค่าร้อยละส่วนเบี่ยงเบนมาตรฐานสัมพัทธ์ต่ำกว่า 2.0% และปริมาณสาร MP, PP, PE และ CH ในเครื่องสำอางตัวอย่างอยู่ในช่วงที่กระทรวงสาธารณสุขกำหนด **สรุป:** วิธีโครมาโทกราฟีเหลวสมรรถนะสูงที่พัฒนาและผ่านการตรวจสอบความถูกต้องสามารถใช้ในการวิเคราะห์ปริมาณเมทิลพาราเบน โพรพิลพาราเบน พีน็อกซี่เอทานอล คลอร์เฟเนซิน ในเครื่องสำอางได้อย่างมีประสิทธิภาพ

คำสำคัญ: โครมาโทกราฟีเหลวสมรรถนะสูง, เมทิลพาราเบน, โพรพิลพาราเบน, พีน็อกซี่เอทานอล, คลอร์เฟเนซิน, เครื่องสำอาง

Abstract

Objective: To develop and validate novel methods for a simultaneous determination of preservatives including methylparaben (MP), propylparaben (PP), phenoxyethanol (PE) and chlorphenesin (CH) by high performance liquid chromatography (HPLC). **Methods:** Cosmetics products were subject to quantification of preservatives. The system configurations and conditions of the methods were optimized. Method validations were performed for all analytical methods. The method was applied for the quantitative analyses of MP, PP, PE, and CH in cosmetic products. **Results:** The HPLC system was developed by using C18 column (250 mm×4.6 mm, 5 µm) and gradient elution with acetonitrile and 0.1% aqueous solution of phosphoric acid as a mobile phase at a flow rate of 1.5 mL/min, injection volume of 10 µl at ambient temperature and the UV detection at 270 nm. The retention time of PE, MP, CH and PP was 6.8, 8.1, 10.3, and 14.5 min, respectively with a total run time of 30 min. The linearity ranges of calibration curves were 60 – 300, 12 – 60, 200 – 1,000, and 96 - 480 µg/mL for MP, PP, PE and CH, respectively (R^2 > 0.997). Limit of quantitation (LOQ) were 19.29, 1.50, 66.30, and 22.88 µg/mL for MP, PP, PE and CH, respectively. The accuracy of this method was determined by recovery studies and mean recoveries were 96.10 – 102.35% and the %RSD for the precision study were less than 2.0%. We found acceptable ranges of MP, PP, PE, and CH in cosmetic products as mandated by the Thai FDA regulation. **Conclusion:** HPLC was developed and validated. This method was successfully applied to determine MP, PP, PE, and CH in cosmetic products.

Keywords: high performance liquid chromatography, methylparaben, propylparaben, phenoxyethanol, chlorphenesin, cosmetics

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Introduction

Preservatives are antimicrobial agents necessary for most products to prevent the products from microbial contamination by the consumer during the use. Cosmetic products contained water and other ingredients that may be contaminated with microorganisms.[1] Microbial contamination of cosmetic products is a significant risk to the health of consumers. The report in Thailand found that personal cosmetic was contaminated with bacteria (3.44%) and mold (9.19%).[2] The contaminated products could cause irritation or infection when consumer use. Preservatives are an important ingredient in

cosmetic products for preventing the contamination and growth of microorganisms. The quantity of the preservatives must be adequate to preserve the cosmetic products. However, excessive use of the preservative can be harmful to consumers.

Parabens can cause serious health hazards such as hypersensitivity, allergy, and cancer. They can be absorbed systemically from topical application and penetrate into the human circulatory system.[3] They have activities on enzymes that metabolize natural hormones. Therefore, they interfere with steroidogenesis and nuclear receptors, such as androgens, estrogens, progesterone, and glucocortico-steroids.[4] Parabens' accumulation can exert harmful effects on the human body related to their estrogenic properties which may be connected with the development of breast cancer, malignant melanoma and reduction of reproductive potential.[5] MP, ethylparaben, PE and CH were toxic to human meibomian gland epithelial cells.[6]

According to the notification of Thailand Ministry of Health, various substances have been approved as preservatives with their maximum concentrations in cosmetic products. P-hydroxybenzoate esters or parabens have been extensively used as preservatives in the food, pharmaceuticals, and cosmetic industries (see Figure 1).

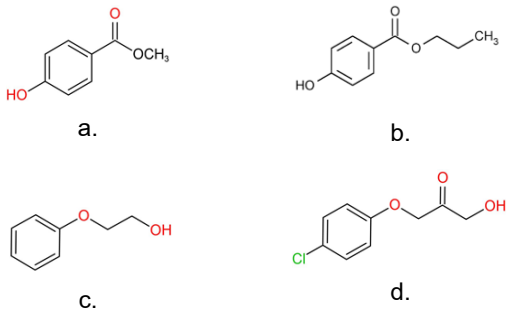

Figure 1 Preservative structures a. methylparaben, b. propylparaben, c. phenoxyethanol, and d. chlorphenesin.

Methylparaben (MP) and propylparaben (PP) are the commonly used parabens and often used together because they have synergistic effects. The maximum concentration of parabens in cosmetics is 0.4% (calculated on acid form, used only one ester) and 0.8% (calculated on acid form, combined with other esters). Phenoxyethanol or 2-phenoxyethanol (PE) is widely used as a preservative in cosmetic products to limit bacterial growth which is safe at a maximum concentration of 1.0%. Chlorphenesin (CH) is an organic compound that functions as a preservative. CH is active against bacteria, some specified fungi, and yeast at a concentration of 0.1 - 0.3%. CH is used as a preservative in cosmetics and personal care products at a maximum concentration of 0.3%.[7] Therefore, there must be a quantitative analysis to ensure not exceeding the limit. Most of the cosmetics in Thailand contain non-formaldehyde releasing preservatives such as parabens, methylisothiazolinone, and PE.[8] Moreover, several cosmetics usually combine parabens and PE as preservatives. Consumers are concerned about the safety of parabens, so paraben-free cosmetics are alternatives. The common preservatives in parabens-free products are PE and CH.

The quantitative determination of these preservatives is important for product quality and consumer protection. The analytical methods for determination of parabens, PE, and CH were reversed-phase HPLC (RP-HPLC)[9-12], thin layer chromatography (TLC)[12-15], and gas chromatography (GC).[16,17] The method for simultaneous determination of MP, PP, PE, and CH in cosmetic products is deficient. Therefore, this study aimed to develop and validate a simple RP-HPLC method for a simultaneous determination of MP, PP, PE, and CH in cosmetic products. The developed method was applied to the quantitative analysis of these preservatives in cosmetic samples.

Methods

Chemicals and reagents

Methyl paraben was purchased from Sigma-Aldrich, Japan and propyl paraben was purchased from Sigma-Aldrich, India (>99% purity). Phenoxyethanol was purchased from Chemipan, Thailand. Chlorphenesin was purchased from MySkinRecipes, Thailand. Acetonitrile and methanol HPLC grade were purchased from LiChrosolve, Germany. All reagents were of analytical grade or HPLC grade. Skin cream and lotion were purchased from SWU drug store and supermarket in Ongkharak, Nakhonayok. Hair serum was purchased from a convenience store.

Preparation of stock solutions and calibration standards

Stock standard solution of MP, PP, PE, and CH were prepared in methanol at 1.5, 0.3, 5 and 2.4 mg/mL, respectively. For the standard curve of HPLC analysis, the working solutions were prepared by diluting with methanol to

make final concentrations of MP, PP, PE, and CH are 60 - 30, 12 - 60, 200 – 1,000, and 96 - 480 µg/mL, respectively.

Method developments and validations

High-performance liquid chromatography (HPLC)

Analytical separation was carried out on Agilent model 1260 Infinity II composed of 1260 DAD WR detector. (Agilent, Germany). The chromatographic condition was optimized. The separation was carried out on an ACE5 C18-AR column, 250 mm x 4.6 mm, 5 µm with 5 µm guard cartridge (ACE, UK) at ambient temperature. The mobile phase was acetonitrile and 0.1% aqueous solution of phosphoric acid. The gradient elution was applied as follows: 0 - 11 min in 25% acetonitrile, 11 - 13 min linear gradient from 25 to 100% acetonitrile and 13 - 20 min in 100% acetonitrile, 20 - 21 min linear gradient from 100 to 25% acetonitrile and 21 - 30 in 25% acetonitrile. The flow rate was 1.5 mL/min with the total run time of 30 min. The injection volume was 10 µL. The detection wavelength was 270 nm. The column temperature was ambient. Agilent OpenLab CDS 2.X software (Agilent, Germany) was used for HPLC operation.

Method validation

Method validations followed the ICH Guidelines [18] and the validation parameters included specificity, linearity, range, accuracy, precision and LOQ and LOD. Specificity of the method was evaluated by injecting standard solutions of MP, PP, PE and CH, mobile phase, blank, and sample separately. The linearity test was performed using five different concentrations of MP, PP, PE, and CH in the range 60 - 300, 12 - 60, 200 – 1,000, and 96 - 480 µg/mL, respectively. Three injections from each concentration were analyzed under the same conditions. Linear regressions were determined as coefficient of variations (R^2). Precision of the method was determined by repeatability (intra-day precision) and intermediate precision (inter-day precision) at three concentration levels (high, medium, low). The accuracy of the method was determined by recovery studies at same concentration levels and the intermediate precision was studied on different days. The accuracy and precision of the method was expressed by percent recovery (%recovery) and percent relative standard deviation (%RSD), respectively.

Sample preparations

Approximately 1 g of skin cream, lotion or hair serum was weighed into centrifuge tube before adding 10 mL of methanol. These samples were mixed by vortex for 5 minutes and sonicated in an ultrasonic bath for 45 minutes, centrifuged at 6,000 rpm for 15 minutes. After filtering through a 0.45 µm nylons membrane filter (CNW Technologies, China), the filtrates were analyzed by HPLC. This sample preparation was adopted from previous research.[19,20]

Data and statistical analysis

The study results are described by mean, standard deviation (SD) and relative standard deviation (RSD) of concentration. Standard regression curve analysis was performed by using Microsoft Office 365 Excel.

Results

High performance liquid chromatography system

Selection of appropriate wavelength

In this research, selection for appropriate wavelength by using UV-Visible spectrophotometer. Solutions of MP, PP, PE, and CH were prepared separately in methanol and their absorbance was measured over the wavelength range of 200 - 315 nm using a UV-Vis spectrophotometer. The UV absorption spectra of the preservatives are shown in Figure 2. Each preservative has a different maximum absorption wavelength of 257 nm for MP and PP, 220 and 271 nm for PE, and 230 and 280 nm for CH. In this research, a wavelength of 270 nm was selected as the optimum wavelength for all analyses.

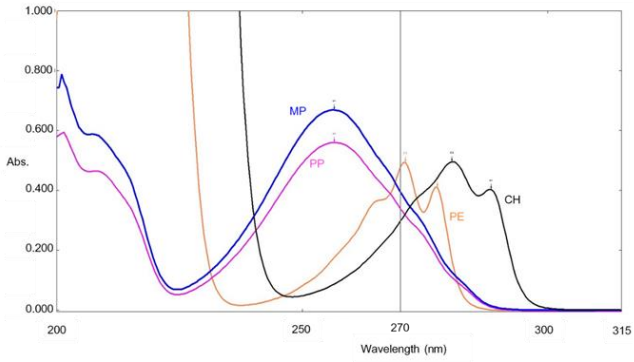

Figure 2 UV absorption spectra of PE, MP, CH and PP (42.0, 6.0, 60.0 and 6.0 µg/mL, respectively).

System suitability

The chromatographic separation of MP, PP, PE, and CH was carried out in the gradient mode using a mixture of acetonitrile and 0.1% aqueous solution of phosphoric acid as mobile phase. The retention time for PE, MP, CH and PP were 6.8, 8.1, 10.3 and 14.5 minutes, respectively (Figure 3).

System suitability test was developed for the routine application of the assay method. System suitability test was performed from six replicate injections of a solution containing 180, 36, 600 and 288 µg/ml of MP, PP, PE, and CH, respectively. All peaks were well resolved and the precision of injections for all preservative peaks were acceptable. The percent relative standard deviation (RSD) of the retention time and peak area responses were measured, giving an average between 0.04 - 0.79% and 0.29 - 0.74%, respectively. The tailing factor (T), capacity factor (K) and theoretical plate number (N) were also calculated. The results of system suitability in comparison with the required limits are shown in Table 1. The proposed method met these requirements within the accepted limits.

Table 1 The system suitability parameters for MP, PP, PE, and CH.

Parameters	Recommended limits[21]	Results			
		MP	PP	PE	CH
Retention times (min)	-	8.00	14.50	6.71	10.17
%RSD of peak area*	%RSD < 1%	0.29	0.74	0.30	0.48
Resolution (Rs)	Rs > 1.5	4.99	14.52	20.26	6.77
Capacity factor (K')	K' > 1.5	12.34	23.17	10.18	15.95
Tailing factor (T)	T < 2	0.82	1.05	0.85	0.87
Theoretical plate number (N)	N > 2000	12441.93	448025.66	13270.58	13181.01

Method validation

Specificity of the method was tested using methanol and hair serum without preservative sample. Both substances did not interfere with the analyses of the MP, PP, PE, and CH (Figure 3). Calibration curves were linear over the concentration range of 60 - 300 µg/mL for MP, 12 - 60 µg/mL for PP, 200 – 1,000 µg/mL for PE, and 96 - 480 µg/mL for CH. The results were presented in Table 3 and showed a good correlation between the peak area of analyses and concentration with R^2 > 0.997.[22] % recovery ranged from of 96.10 to 102.35% with %RSD of less than 2.0% indicating that the method was reliable and reproducible[23] (Table 2). The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated based on 3.3σ/slope and 10σ/slope, respectively, where σ was the standard deviation of the response of the curve and slop was that of the calibration curve. The results of LOD and LOQ are present in Table 3.

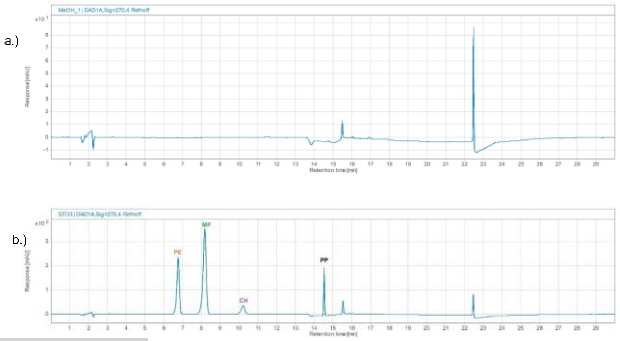

Figure 3 HPLC chromatograms a.) methanol (extraction solvent) and b.)ค่า conc พวกนี้เป็นค่าอะไร PE 600 µg/mL, MP 180 µg/mL, CH 280 µg/mL, and PP 35 µg/mL. The retention time of PE, MP, CH, and PP was 6.8, 8.1, 10.3 and 14.5 min, respectively.

Table 2 The accuracy (%recovery) and precision results for MP, PP, PE and CH.

Preservatives	Concentration (µg/mL)	Mean recovery (%)	Intra-day precision (%RSD)	Inter-day precision (%RSD)
MP	60	101.61	1.69	1.81
	180	100.34	1.22	1.70
	300	101.39	1.67	1.33
PP	12	96.10	0.45	1.88
	35	99.46	0.54	1.17
	60	100.33	0.25	0.47
PE	200	102.35	0.76	0.59
	600	100.47	0.14	0.63
	1000	101.56	0.92	0.60
CH	96	101.54	0.97	1.70
	280	101.99	1.21	1.19
	480	102.16	1.95	1.70

Table 3 Summary of validation parameters of MP, PP, PE and CH.

Parameter	MP	PP	PE	CH
Concentration (µg/mL)	60 - 300	12 - 60	200 – 1,000	96 - 480
Linearity (R^2)	0.9999	0.9999	0.9998	0.9996
Equation	y = 23.537x+12.308	y = 18.370x+18.912	y = 3.657x+18.987	y = 1.760x+8.162
LOD (µg/mL)	6.37	0.50	21.88	7.55
LOQ (µg/mL)	19.29	1.50	66.30	22.88

Determination of MP, PP, PE and CH in cosmetic samples

This method was applied to determine the quantity of preservatives in cosmetic products with various matrices. The seven samples including cream, lotion, gel, and hair serum

were tested by this method. Peak identification of preservatives in various cosmetics was based on the comparison between the retention time of standard compounds to the sample. Quantification was calculated by using calibration curves fitted by linear regression analysis. The preservatives detected in samples were MP, PP, PE, and CH and their amounts were observed to be in the ranges 34.29 - 210.46, 6.42 - 222.53, 23.07 - 529.37, and 72.89 µg/mL, respectively. The chromatograms of cosmetic samples are shown in Figure 4. The result of quantity of preservatives in cosmetic are presented in Table 4.

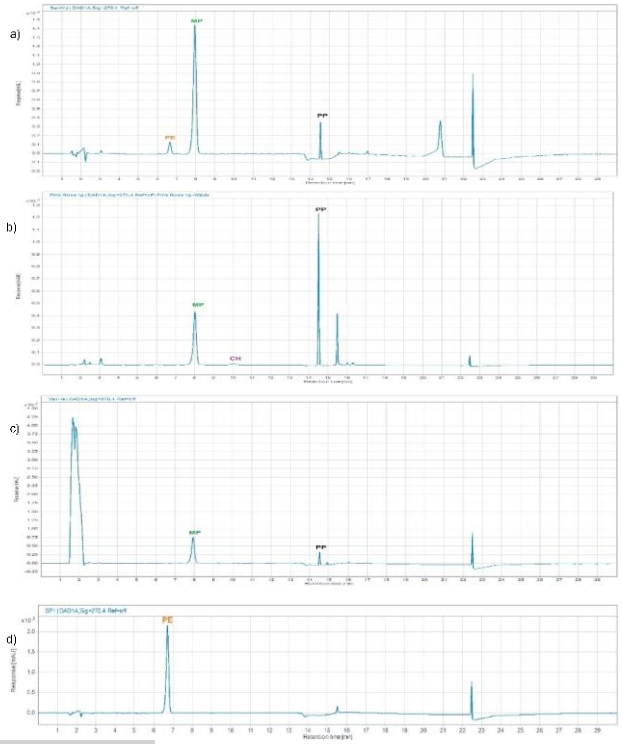

Figure 4 The chromatograms of cosmetic samples, a) cream S, b) lotion P, c) gel Y, and d) hair serum 1

Table 4 Results from determination of MP, PP, PE and CH in cosmetic products.

Cosmetic products	Preservative concentration (µg/mL) (%w/w)			
	MP	PP	PE	CH
Cream S	67.67 (0.068)	7.17 (0.007)	23.07 (0.023)	-
Cream J	62.48 (0.062)	50.53 (0.051)	-	-
Lotion B	134.62 (0.135)	23.44 (0.023)	310.98 (0.311)	-
Lotion P	210.46 (0.210)	222.53 (0.223)	-	72.89 (0.073)
Gel Y	34.29 (0.034)	6.42 (0.006)	-	-
Hair serum 1	-	-	437.06 (0.437)	-
Hair serum 2	-	-	529.37 (0.529)	-

Discussions and Conclusion

In this study, the novel HPLC method was developed and validated for simultaneous analysis of the 4 preservatives in cosmetic products. The detection wavelength was selected with a single wavelength that all preservatives could be analyzed. Each preservative possessed different maximum absorption wavelengths, specifically parabens (MP and PP) at 257 nm, PE at 271 nm, and CH at 280 nm. In consideration of these maximum absorption wavelengths, the detection wavelength was fixed at 270 nm. For this method, acidic mobile phase was used to protect ester hydrolysis in parabens. Moreover, their ionization occurs over the pH range defined by their pKa, specifically 8.4, 8.5, 15.1, and 13.6, for MP, PP, PE, and CH, respectively. The mobile phase was 0.1% phosphoric acid: acetonitrile gradient elution. The isocratic elution, 0.1% phosphoric acid:acetonitrile (75:25 v/v), was effective for separation of PE, MP, and CH. For PP, it took much longer to elute (i.e., more than 36 min). Also, isocratic elution could not separate the other cosmetic ingredients from preservatives. The percentage of acetonitrile was increased to shorten the elution time of PP. Therefore, the mobile phase was changed to 100% acetonitrile after PE, MP, and CH was eluted. This gradient elution chromatographic analysis was simple and convenient to operate. The mobile phase composition in this method obtained a peak resolution more than 1.5. This method was a successful, complete separation of 4 preservatives. The methanol and preservatives chromato-grams showed no interfering peak. Thus, this method was specific. The range of quantitative analysis for MP, PP, PE, and CH in this study covered the quantity of preservatives in cosmetic samples. The standard curves of all preservatives were with good linearity (R^2 > 0.997 for all).[22] Accuracy was in the range of 96.10 - 102.35%, which was within the 97-103% as recommended by AOAC.[23] %RSD was acceptable with values of less than 1.95 and 1.88 for intra-day and inter-day precision, respectively. It was found that the developed HPLC was specific to MP, PP, PE, and CH. It provided better sensitivity with LOQ of 19.29, 1.50, 66.30, and 22.88 µg/mL, respectively.

Methanol was used in extraction because these preservatives were very soluble in methanol. Despite being dissolved in methanol, some ingredients did not interfere with absorption of these preservatives because their wavelengths were different from those of the preservatives. In addition, they were separated by HPLC condition. Aoyama study also suggests that the same sample preparation method was able to analyze the substance without interference.[9] The results of

our study found that concentrations tested preservatives of all cosmetic samples were higher than LOQ, except for phenoxyethanol concentration in cream S that was lower than LOQ but still higher than LOD.

In conclusion, the HPLC method was successfully developed for the simultaneous determination of 4 preservatives, specifically methylparaben, propylparaben, phenoxyethanol and chlorphenesin in cosmetic products. The peaks were separated with acceptable linearity, recovery and precision. The developed method could be effectively applied for preservatives determination in cosmetic products such as cream, lotion, gel and serum. Concentrations of the 4 preservatives did not exceed the allowed maximum concentrations. This HPLC method is simple and useful for the determination of preservatives including methylparaben, propylparaben, phenoxyethanol and chlorphenesin in cosmetic products for quality control.

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