

HPLC COUPLED WITH BOX–BEHNKEN DESIGN FOR THE SIMULTANEOUS QUANTIFICATION OF METHYLPARABEN AND PROPYLPARABEN IN CREAM FORMULATIONS

Hai Ngo Dang Truong*, Hien Nguyen Thi Ngoc, Ho Dang Ngoc

Dong A University, Vietnam

*Corresponding author: haindt@donga.edu.vn

(Received: May 07, 2026; Revised: August 05, 2026; Accepted: August 28, 2026)

DOI: 10.31130/ud-jst.2026.24(9).317E

Abstract - The study employed high-performance liquid chromatography (HPLC) combined with Box–Behnken design (BBD), response surface methodology (RSM), and the Derringer desirability function to simultaneously quantify methylparaben (MP) and propylparaben (PP) in cream formulations. The factors investigated were mobile phase composition, column temperature, and flow rate. The responses included PP retention time, MP–PP resolution, and MP asymmetry factor. Optimal conditions were obtained at an ACN:H₂O ratio of 51.7:48.3, column temperature of 40°C, and flow rate of 1.16 mL/min. The deviation between predicted and experimental values was below 6%, indicating good model reliability. The method was validated according to ICH guidelines and met requirements for linearity, accuracy, and precision. Limits of detection and quantification for both compounds were 0.01 and 0.03 µg/mL, respectively. The method was successfully applied to commercial cream samples. The results confirm the effectiveness of combining HPLC with BBD in reducing experimental runs and improving analytical performance.

Key words - HPLC; methylparaben; propylparaben; Box–Behnken design; response surface methodology

1. Introduction

Methylparaben (MP) and propylparaben (PP) are paraben preservatives found in pharmaceuticals and cosmetics. They offer significant benefits, including the inhibition of microbial growth at low concentrations, minimal alteration of product characteristics, and cost-effectiveness [1]. Although recent articles suggest that these compounds may be associated with endocrine disruption [2], [3], they remain prevalent, particularly in creams. Thus, strict control of their levels is required to ensure consumer safety.

Several studies have used HPLC to determine MP and PP in such formulations [4], [5], [6]. Most employ the one-factor-at-a-time (OFAT) approach, which overlooks the combined effects of chromatographic conditions. Factors such as mobile phase composition, pH, flow rate, and column temperature can interact and greatly affect separation efficiency and analytical reliability.

This research aims to establish an HPLC method for simultaneous quantification of MP and PP in cream formulations, optimized through a Box–Behnken design (BBD), response surface methodology (RSM), and the Derringer desirability function [7], [8], [9]. This approach is intended to provide robustness while enabling prediction of analytical responses under varying chromatographic conditions.

2. Materials and methods

2.1. Instruments and reagents

Analysis was performed on a Hitachi CM5000 DAD HPLC system (Japan). Additional equipment comprised an Ohaus EX324/AD analytical balance (0.1 mg readability, USA), an ultrasonic bath, and glassware.

Reference standards for MP (lot C0419108.04, assay 100.03%) and PP (lot C0319138.03, assay 100.03%) were supplied by the National Institute of Drug Quality Control. Solvents consisted of HPLC-grade methanol (MeOH) and acetonitrile (ACN), analytical-grade ethanol (EtOH), and double-distilled water.

Samples were laboratory-prepared using a conventional cream base containing gentamicin.

2.2. HPLC conditions

Chromatographic separation was achieved using a Hitachi HPLC system (Model 6530, Hitachi, Japan) equipped with a photodiode array (PDA) detector and an InertSustain C18 column (250 × 4.6 mm, 5 µm). An acetonitrile–water mixture was used as the mobile phase under isocratic elution. The injection volume was 10 µL, and chromatograms were monitored at a wavelength of 255 nm [10].

2.3. Preparation of standard and sample solutions

MP stock standard solution (S1): An accurately weighed 20 mg portion of MP reference standard was dissolved in solvent and diluted to 100 mL in a volumetric flask.

PP stock standard solution (S2): Similarly, 25 mg of PP reference standard was dissolved in solvent and diluted to 100 mL, and a 5.0 mL aliquot was further diluted to 25 mL with solvent.

Standard mixture solution: Aliquots (5.0 mL each) of S1 and S2 were transferred to a 50 mL volumetric flask and diluted to volume with solvent (20 µg/mL MP and 5 µg/mL PP) before filtration through a 0.45 µm membrane filter.

Sample solution: The cream sample (~0.5 g) was extracted with 30 mL of EtOH at 60 °C for 10 min, cooled, made up to 50 mL, diluted as necessary to fall within the calibration range, and filtered through a 0.45 µm membrane.

2.4. Box–Behnken design for optimization

Three factors were selected: mobile phase composition (A), column temperature (B), and flow rate (C). A Box–Behnken design (BBD) was generated using Statgraphics

Centurion XIX software, which was also used for data processing and statistical analyses. The design consisted of 15 experimental runs (12 edge points and 3 center-point replicates) and was repeated in three independent blocks, resulting in a total of 45 experimental runs for statistical analysis. Table 1 outlines the levels of each factor.

Table 1. Experimental matrix of the BBD

Factor	Code	Unit	Level		
			Low (-1)	Center (0)	High (+1)
Mobile phase composition	A	% ACN	40	50	60
Column temperature	B	°C	25	30	35
Flow rate	C	mL/min	0.8	1.0	1.2

The monitored responses included the retention time of PP (t_R (PP)), the resolution between MP and PP (R_s (MP–PP)), and the asymmetry factors of MP and PP (A_s (MP) and A_s (PP)). Any responses with poor model fit were excluded from optimization. The optimal conditions were then determined by the Derringer desirability function.

2.5. Method validation

Method validation followed the ICH guidelines, assessing specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, and accuracy. System suitability testing was conducted prior to each analysis [11].

3. Results

3.1. Factor – response relationships

Experimental runs followed the BBD model's randomized settings to minimize systematic bias. Table 2 displays the goodness-of-fit statistics of the models.

Table 2. Model goodness-of-fit

Model statistics	t_R (PP)	R_s (MP–PP)	A_s (MP)	A_s (PP)
R^2 (%)	99.6162	99.8967	85.3047	45.7060
Adjusted R^2 (%)	99.4882	99.8623	80.4063	27.6081
Predicted R^2 (%)	99.2298	99.7930	71.9203	0.000
Standard error of estimate	0.2724	0.1865	0.0257	0.0153
Mean absolute error	0.1806	0.1276	0.0182	0.0104
Durbin–Watson statistic (p)	2.3553 (0.7961)	1.8761 (0.2399)	1.7704 (0.1479)	1.7457 (0.1303)

Among the four responses evaluated, t_R (PP), R_s (MP–PP), and A_s (MP) were adequately modeled. For the A_s (PP) response, although some terms were statistically significant, the overall quadratic model showed poor predictive performance ($R^2 = 45.71\%$, adjusted $R^2 = 27.61\%$, and predicted $R^2 = 0\%$). Moreover, the observed A_s (PP) values varied only slightly from 1.21 to 1.28 (Supplementary Information) and remained well within the commonly accepted chromatographic system suitability criterion for peak asymmetry ($A_s < 1.5$). This limited variation indicates that the investigated factors had only a negligible influence on A_s (PP). Consistent with this finding, the fitted quadratic regression equation for A_s (PP) is presented below:

$$A_s \text{ (PP)} = 0.7510 + 0.0181A + 0.0050B - 0.0976C - 0.0001AB - 0.0039AC + 0.0016BC - 0.0001A^2 - 0.0001B^2 + 0.1195C^2$$

As a result, A_s (PP) was excluded from the multi-response optimization, and only responses with acceptable predictive performance were included [12], [13]. Table 3 and Figure 1 present the relationships between factors A, B, and C and the remaining responses: t_R (PP), R_s (MP–PP), and A_s (MP).

Table 3. ANOVA result and fitted quadratic regression equations for the BBD responses

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-value*
tr (PP)					
A	491.234	1	491.234	6617.860	< 0.001
B	11.648	1	11.648	156.920	< 0.001
C	77.760	1	77.760	1047.580	< 0.001
AA	38.683	1	38.683	521.14	< 0.001
AB	4.095	1	4.095	55.17	< 0.001
AC	10.887	1	10.887	146.67	< 0.001
BB	0.047	1	0.047	0.64	0.4299
BC	0.476	1	0.476	6.41	0.0163
CC	1.881	1	1.881	25.34	< 0.001
Blocks	0.00849	2	0.00425	0.06	0.9445
Total error	2.450	33	0.074	-	-
Corrected total	638.150	44	-	-	-
Regression equation	tr (PP) = 134.696 – 2.9727A – 0.5009B – 56.3552C + 0.0058AB + 0.4759AC + 0.0999BC + 0.0187A ² + 0.0007B ² + 10.2812C ²				
Rs (MP–PP)					
A	1100.260	1	1100.260	31617.990	< 0.001
B	4.352	1	4.352	125.060	< 0.001
C	2.030	1	2.030	58.340	< 0.001
AA	1.093	1	1.093	31.420	< 0.001
AB	0.617	1	0.617	17.720	< 0.001
AC	2.376	1	2.376	68.290	< 0.001
BB	0.022	1	0.022	0.620	0.4363
BC	0.124	1	0.124	3.560	0.0679
CC	0.053	1	0.053	1.520	0.2259
Blocks	0.01030	2	0.00515	0.150	0.8630
Total error	1.148	33	0.035	-	-
Corrected total	1112.120	44	-	-	-
Regression equation	Rs (MP–PP) = 71.6534 – 1.2824A – 0.2331B – 10.7071C + 0.0023AB + 0.2227AC + 0.0509BC + 0.0031A ² + 0.0004B ² – 1.7059C ²				
As (MP)					
A	0.091	1	0.091	138.700	< 0.001
B	0.001	1	0.001	2.050	0.1614
C	0.001	1	0.001	1.620	0.2118
AA	0.014	1	0.014	20.950	< 0.001
AB	0.000	1	0.000	0.000	1.0000
AC	0.009	1	0.009	12.970	< 0.001
BB	0.007	1	0.007	10.760	0.0025
BC	0.0001	1	0.0001	0.200	0.6555
CC	0.0019	1	0.0019	2.870	0.0997
Blocks	0.00086	2	0.00043	0.650	0.5277
Total error	0.021	33	0.001	-	-
Corrected total	0.148	44	-	-	-
Regression equation	As (MP) = 0.0431 – 0.0425A – 0.0172B – 0.0800C + 0.00002AB – 0.0133AC – 0.0022BC – 0.0004A ² – 0.0003B ² + 0.3079C ²				
* Significant factor at α = 0.05.					

* Significant factor at $\alpha = 0.05$.

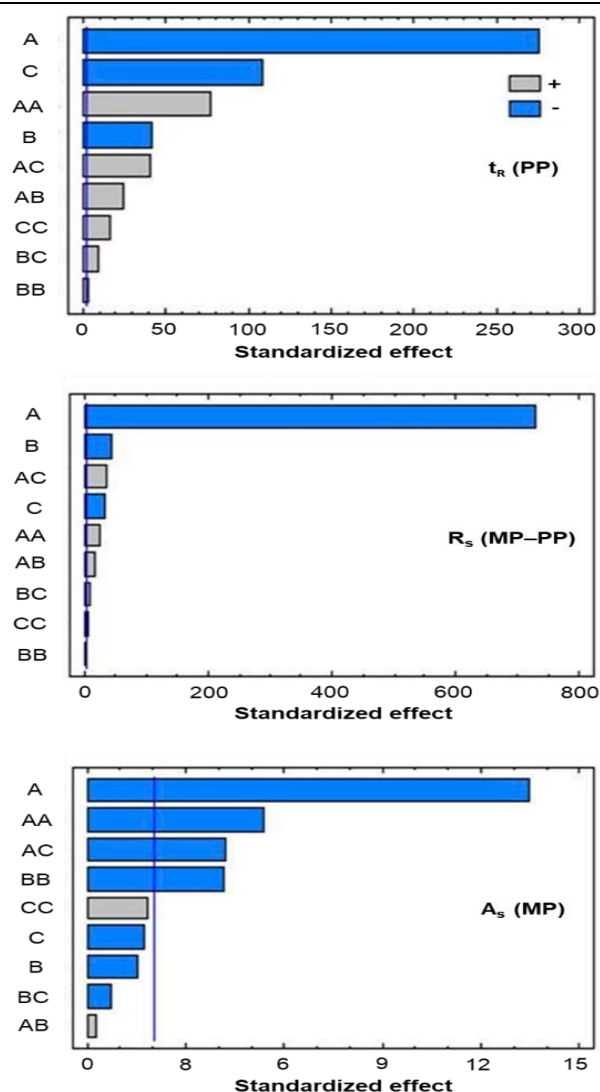


Figure 1. Pareto charts of the standardized effects of factors A (mobile phase composition, ACN:H₂O), B (column temperature), and C (flow rate) on the responses: t_R (PP), R_s (MP-PP), and A_s (MP)

Mobile phase composition (factor A) had the strongest effect across all three responses ($p < 0.001$) and should therefore be prioritized during optimization. For the t_R (PP) response, flow rate (factor C) was more influential than column temperature (factor B), whereas the trend was opposite for R_s (MP-PP). By contrast, flow rate and column temperature had only minor, statistically insignificant effects on A_s (MP) ($p > 0.05$).

The direction and relative magnitude of these effects were also evident from the Pareto charts. Mobile phase composition had the greatest negative linear effect, indicating that increasing its level decreased all three responses. The interaction between mobile phase composition and flow rate (AC) was the dominant interaction, and the quadratic effect of mobile phase composition (AA) was the most pronounced.

3.2. Chromatographic optimization

Chromatographic conditions were optimized using RSM to balance chromatographic performance with analytical efficiency. The t_R (PP) and A_s (MP) were

minimized, while R_s (MP-PP) was constrained within the experimental range (5.55–20.19), which exceeded the minimum required for baseline separation. Because further increases in resolution would prolong the analysis time without substantially improving separation, the optimum conditions were selected based on the overall desirability function (Table 4).

Table 4. Constraints and optimum values used for multi-response desirability optimization

Response	Lower limit	Upper limit	Goal	Optimum
t_R (PP) (min)	4.43	17.72	Minimize	5.71
R_s (MP-PP)	5.55	20.19	In range	10.85
A_s (MP)	1.07	1.27	Minimize	1.17
Overall desirability (D) = 0.7986				

Figure 2 illustrates the desirability response surfaces used to identify the optimum chromatographic conditions.

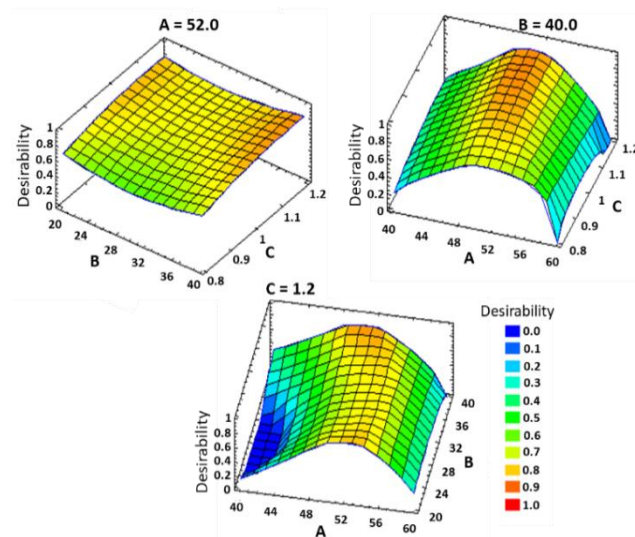


Figure 2. Response surface plots of the overall desirability as a function of factors: A (mobile phase composition, ACN:H₂O, v/v; 52:48), B (column temperature, 40 °C), and C (flow rate, 1.2 mL/min)

The optimum conditions were an ACN:H₂O mobile phase ratio of 51.7:48.3, a column temperature of 40 °C, and a flow rate of 1.16 mL/min. A notable decline in desirability (< 0.65) was observed outside this region, suggesting that these combined conditions provided the best overall balance among the chromatographic responses.

A comparison of experimental values with model predictions (Table 5) revealed minor deviations: 1.40% for t_R (PP), 1.71% for A_s (MP), and 5.81% for R_s (MP-PP). This indicates that the optimization model established via the Box-Behnken design is accurate and predictive, making it a reliable tool for efficiently guiding analytical conditions for MP and PP analysis.

Table 5. Optimized responses: predicted versus experimental values

Response	Predicted	Experimental	Relative deviation (%)
t_R (PP) (min)	5.71	5.63	1.40
R_s (MP-PP)	10.85	10.22	5.81
A_s (MP)	1.17	1.15	1.71

3.3. Method validation

The analytical method was validated for specificity, linearity, range, accuracy, precision, LOD, LOQ, and system suitability in accordance with ICH guidelines. Validation samples consisted of a self-prepared 0.3% gentamicin cream using a conventional cream base. Accuracy was assessed using placebo samples spiked with the reference standard at 80%, 100%, and 120% of the nominal assay concentration. Since the spiked placebo samples underwent the entire procedure, including ethanol extraction before HPLC analysis, the recovery values reflect the overall performance of the analytical procedure.

Representative chromatograms of the placebo and mixed standard solution are shown in Figure 3. The mixed standard displayed two well-resolved peaks at approximately 3.6 min for MP and 5.7 min for PP, while the placebo showed no interfering peaks at these retention times, confirming the method’s specificity for quantifying MP and PP in the cream matrix.

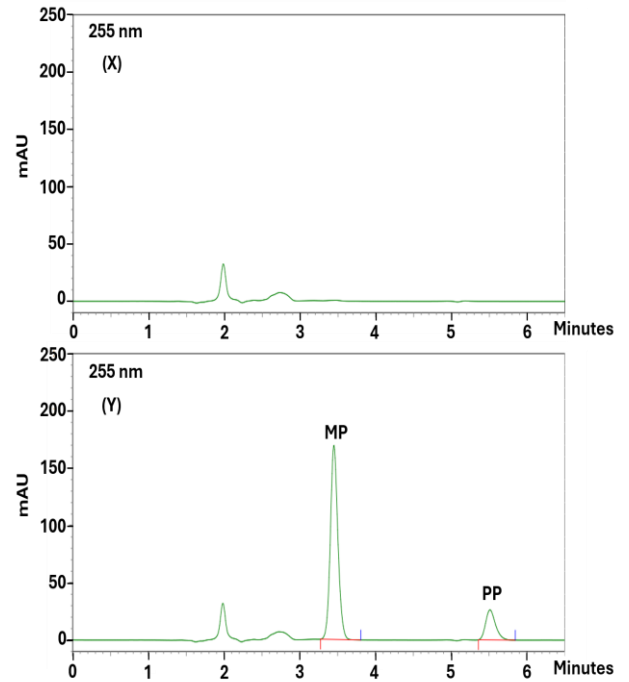


Figure 3. Chromatograms of the placebo (X) and mixed standard solution (Y; MP 20 µg/mL and PP 5 µg/mL)

Table 6 summarizes the validation results.

Table 5. Validation results for MP and PP quantification in the developed cream formulation		
Validation parameter	MP	PP
System suitability (n = 6, %RSD)	0.26	0.38
Assay linear range (µg/mL)	4.0–30.0	1.0–7.5
Regression equation	y = 233964x – 18287	y = 190695x – 21711.2
Correlation coefficient	0.99998	0.999998
Trace linear range (µg/mL)	0.25–4.00	0.06–1.00
Regression equation	y = 230810x + 10829	y = 186138x + 445.1
Correlation coefficient	0.9999	0.9998
LOD (µg/mL)	0.0102	0.0101

LOQ (µg/mL)	0.0335	0.0333
Repeatability (%) (n = 6, %RSD)	99.46 (RSD 0.75%)	100.90 (RSD 0.31%)
Intermediate precision (%) (n = 6, %RSD)	99.92 (RSD 0.39%)	99.38 (RSD 0.38%)
Accuracy (% recovery range, %RSD)	99.93–101.75 (RSD 0.71%)	98.45–101.44 (RSD 1.10%)
Working range (%)	80.0–120.0	80.0–120.0

System suitability met the requirements of the Association of Official Analytical Chemists (AOAC) [14]. Excellent linearity was obtained over both the assay and trace concentration ranges. The LOD and LOQ for both compounds were about 0.01 µg/mL and 0.03 µg/mL, respectively. Precision (%RSD < 2.0%) as well as accuracy (recoveries of 98.0–102.0%) satisfied AOAC criteria.

The analytical range was established at 80.0–120.0% of the nominal assay concentration.

3.4. Method application

The method was utilized to measure MP and PP in 10 commercial cream products - 6 pharmaceuticals, 2 dietary supplements, and 2 cosmetics. Table 7 provides information on the commercial cream products investigated.

Table 6. Characteristics of the commercial cream samples

No.	Product category*	Active ingredient(s)	Expiry date
1	PH01	Fluocinolone acetonide, neomycin sulfate	150426
2	PH02	Ketoconazole	031227
3	PH03	D-Panthenol	030128
4	PH04	D-Panthenol, vitamin E	020527
5	PH05	Cetrimide, curcumin extract	040227
6	PH06	Sulfadiazine	220226
7	DS01	Commiphora myrrha extract, Krameria triandra root extract, troxerutin, vitamin E	112027
8	DS02	Centella asiatica extract, vitamin E, D-Panthenol	150128
9	COS01	Aloe barbadensis extract, vitamin E	260427
10	COS02	Coix lacryma-jobi extract	190127

* PH, pharmaceuticals; DS, dietary supplements; COS, cosmetics.

The quantitative results are presented in Table 8.

Table 7. Analysis of preservatives in selected commercial cream formulations

No.	Samples	MP content (mg/g, mean ± SD)	PP content (mg/g, mean ± SD)
1	PH01	1.2935 ± 0.0024	0.2646 ± 0.0002
2	PH02	1.3410 ± 0.0347	0.4374 ± 0.0057
3	PH03	2.5204 ± 0.0007	1.3651 ± 0.0009
4	PH04	1.7820 ± 0.0128	0.2761 ± 0.0000
5	PH05	0.8053 ± 0.0003	0.8671 ± 0.0008
6	PH06	1.6892 ± 0.0004	0.9410 ± 0.0023
7	DS01	0.2536 ± 0.0023	< LOD
8	DS02	0.7902 ± 0.0348	< LOD
9	COS01	0.3626 ± 0.0017	< LOD
10	COS02	1.8661 ± 0.0076	< LOD
LOD, limit of detection.			

Among the surveyed pharmaceuticals, MP and PP were detected at 0.3–2.5 mg/g and 0.3–1.4 mg/g, respectively. The presence of active ingredients, such as fluocinolone, ketoconazole, panthenol, and sulfadiazine, did not interfere with the measurement of MP and PP. In cosmetics and dietary supplements, MP was at low levels per label claims, while PP was not detected.

4. Discussion

Many investigations have described HPLC techniques for quantifying MP and PP. However, only a few have employed RSM and BBD for experimental modeling and chromatographic optimization. In this work, an integrated HPLC–BBD–RSM analytical strategy was developed for simultaneous quantification of MP and PP.

Analytical conditions, including ACN:H₂O ratio, flow rate, and column temperature, were systematically optimized for efficiency. The retention time of PP (5.6 minutes) was shorter than that reported in a previous study (6.1 minutes) [15]. The approach yielded good resolution and improved peak shape. The asymmetry factor (1.15) was lower than the earlier reported value of 1.34 [10].

The research clarified how various chromatographic conditions interact and influence analytical performance. Of these factors, the mobile phase ratio proved to be the primary determinant of accurate quantification. In comparison, column temperature and flow rate had relatively minor impacts, particularly on the MP asymmetry factor. Consequently, when adjusting chromatographic conditions for the simultaneous determination of MP, PP, or similar compounds, the mobile phase ratio should take precedence.

The study ultimately confirmed the reliability of the predictive model throughout data processing. The deviation between model predictions and experimental outcomes was minimal, not exceeding 6.0%. These results are highly relevant for data analysis and support the broad applicability of the method to a variety of sample matrices.

5. Conclusion

The study developed an optimized protocol for the simultaneous quantification of MP and PP by HPLC, coupled with BBD and RSM. The method achieved rapid analysis, strong resolution, and well-defined peaks under the following conditions: mobile phase ratio of ACN:H₂O (51.7:48.3), flow rate of 1.16 mL/min, and column temperature of 40°C. Validated in accordance with ICH guidelines, it was applied to quantify MP and PP in multiple commercial cream samples.

Acknowledgements: This research was funded by Dong A University

REFERENCES

- [1] J. Lincho *et al.*, “Paraben compounds—Part I: An overview of their characteristics, detection, and impacts,” *Appl. Sci.*, vol. 11, no. 5, Art. no. 2307, 2021.
- [2] A. Karwacka *et al.*, “Exposure to modern, widespread environmental endocrine disrupting chemicals and their effect on the reproductive potential of women: An overview of current epidemiological evidence,” *Hum. Fertil. (Camb.)*, vol. 22, no. 1, pp. 2–25, 2019.
- [3] Z. Petric, J. Ružić, and I. Žuntar, “The controversies of parabens—An overview nowadays,” *Acta Pharm.*, vol. 71, no. 1, pp. 17–32, 2021.
- [4] S. Zinoski, Z. Arsova-Sarafinovska, R. Petkovska, L. Petrushevska Tozi, and L. Anastasova, “Determination of methylparaben and propylparaben in cosmetic products using HPLC,” *Maced. Pharm. Bull.*, vol. 71, no. 1, pp. 71–78, 2025.
- [5] M. A. Korany, R. M. Youssef, M. A. Ragab, M. T. Amine, and M. A. Afify, “Development and validation of stability indicating HPLC–DAD assay of adapalene, methylparaben and propylparaben in a cosmeceutical gel matrix with blueness, greenness and whiteness assessment via relative ranking: an ab initio-sub finem juxtaposed non-traditional approaches,” *Microchem. J.*, vol. 205, Art. no. 111342, 2024.
- [6] M. A. Khairy, A. Hamad, M. Hamed, M. Locatelli, and F. R. Mansour, “A stability indicating RP–HPLC–UV assay method for the simultaneous determination of hydroquinone, tretinoin, hydrocortisone, butylated hydroxytoluene and parabens in pharmaceutical creams,” *J. Pharm. Biomed. Anal.*, vol. 242, Art. no. 116021, 2024.
- [7] Ş. Dinç-Zor and Ö. A. Dönmez, “Box–Behnken design–desirability function approach in optimization of HPLC method for simultaneous determination of ibuprofen along with additives in syrup formulation,” *J. AOAC Int.*, vol. 104, no. 1, pp. 78–83, 2021.
- [8] S. Lamidi, N. Olaleye, Y. Bankole, A. Obalola, E. Aribike, and I. Adigun, “Applications of response surface methodology (RSM) in product design, development, and process optimization,” in *Response Surface Methodology: Research Advances and Applications*. London, U.K.: IntechOpen, 2022.
- [9] H. T. Hao, D. M. Nhat, and M. T. H. Thanh, “Optimization of heat-moisture treatment conditions to obtain the highest resistant starch concentration from jackfruit seed starch using the surface method,” *The University of Danang - Journal of Science and Technology*, vol. 22, no. 5A, pp. 75–80, 2024.
- [10] N. H. L. Thuy, “Simultaneous determination of triamcinolone, methylparaben and propylparaben in gel formulation by high-performance liquid chromatography,” *Y Hoc TP. Ho Chi Minh*, vol. 20, 2016 (in Vietnamese).
- [11] ICH, “*Validation of analytical procedures*,” ICH Harmonised Guideline, 2023, pp. 1–14.
- [12] Y. Dong *et al.*, “Perspectives in modeling and model validation during analytical quality by design chromatographic method evaluation: A case study,” *AAPS Open*, vol. 7, no. 1, Art. no. 3, 2021.
- [13] Y. Dong, A. S. Marzali, M. A. Sifir, and K. Zhang, “Analytical Quality by Design (AQbD) approach for the development of a robust liquid chromatography method: A case study,” *Organic Process Research & Development*, vol. 23, no. 11, pp. 2445–2452, 2019.
- [14] AOAC INTERNATIONAL, “AK-1: Guidelines for dietary supplements and botanicals,” in *Official Methods of Analysis of AOAC INTERNATIONAL*, G. W. Latimer Jr., Ed. Oxford, U.K.: Oxford Univ. Press, 2023.
- [15] N. Goswami, V. R. M. Gupta, and H. A. Jogia, “Development and validation of a novel stability-indicating RP–HPLC method for the simultaneous determination of halometasone, fusidic acid, methylparaben, and propylparaben in topical pharmaceutical formulation,” *Scientia Pharmaceutica*, vol. 81, no. 2, pp. 505–518, 2013.