Journal of Pharmaceutical and Biomedical Analysis

A stability indicating RP-HPLC-UV assay method for the simultaneous determination of hydroquinone, tretinoin, hydrocortisone, butylated hydroxytoluene and parabens in pharmaceutical creams

--Manuscript Draft--

Manuscript Number:	JPBA-D-23-02651R1
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Keywords:	RP-HPLC; Hydroquinone; Parabens; Topical Formulations; AGREEprep and BAGI; Stability study
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Abstract:	Multicomponent drugs are medications that combine two or more active pharmaceutical ingredients in a single dosage form. These dosage forms improve the patient compliance, reduce the risk of drug interactions, and simplify dosing regimens. However, quality control of these multicomponent dosage forms can be challenging, especially if the final product contains four or more ingredients that are active (comprise stabilizers, preservatives, excipients, and other components). This problem can be more pronounced if the excipients can interfere with the analysis. In this work, a stability indicating assay method was developed and validated (according to the ICH International Guidelines) for the simultaneous determination of hydroquinone (HQ), tretinoin (TRT), hydrocortisone (HCA), butylated hydroxytoluene (BHT), methyl paraben (MP) and propyl paraben (PP) in commercially available pharmaceutical creams. The proposed method is based on gradient elution using X-Bridge C18 (150 ´ 4.6 mm, 5µm) column with a flow rate of 1 mL/min. The linear ranges (µg/mL) were 240-560 for HQ, 24-56 for MP, 132-308 for HCA, 6-14 for PP, 12-28 for BHT, 6.6-15 for TRT. During the validation process, the intra- and interday precision and trueness (evaluated as recovery) were found to be below 2.0% and between 100–102%, respectively. System suitability tests (SST) allow validating the herein proposed procedure specifically for pharmaceutical and industrial applications. SST test shows that the reported procedure fulfill with the Guidelines, allowing excellent separation of the analytes with very sensitive, accurate (precise and true) and reproducible quantitation of each analytes. The method was successfully applied in forced degradation studies of the six analytes. Specifically, acid degradation slightly affected HCA and BHT (91% recovery), while alkaline degradation drastically reduced HCA recovery (5.5%) and moderately affected BHT (85%). Photodegradation primarily influenced TRT quantity, and oxidative degradation intensified the BHT peak (130%).
Suggested Reviewers:	Victoria Samanidou Aristotle University of Thessaloniki samanidu@chem.auth.gr
	Abuzar Kabir Florida International University akabir@fiu.edu
	Halil I. Ulusoy Sivas Cumhuriyet University hiulusoy@yahoo.com
	Sibel A. Ozkan Ankara University ozkan@pharmacy.ankara.edu.tr

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Response to Reviewers:	Dear Prof. Jiang; (Editor, Journal of Pharmaceutical and Biomedical Analysis) Thank you for the review of our manuscript (JPBA-D-23-02651) entitled “A stability indicating RP-HPLC-UV assay method for the simultaneous determination of hydroquinone, tretinoin, hydrocortisone, butylated hydroxytoluene and parabens in pharmaceutical creams”. We have considered the referees' comments and made the suggested changes to the best of our ability. These revisions are highlighted in the manuscript (using “track changes mode”) and are summarized below: Reviewer #1 CommentResponse This manuscript discusses the development of a stability indicating RP-HPLC assay method for the simultaneous determination of hydroquinone, tretinoin, hydrocortisone, butylated hydroxytoluene, and parabens in pharmaceutical creams. The method was validated and found to be accurate and precise. Specificity and complete separation of all six components were performed using gradient elution. The study is well designed, and properly presented. I recommend publication after the dressing the following points:We appreciate the efforts of the referee in thoroughly reviewing our paper. All the points raised have been carefully addressed, and the corresponding modifications have been made as outlined below. The abstract should be in one paragraph, rather than being fragmented in three paragraphsThe abstract has been combined in one paragraph. The introduction: The method novelty should be highlighted in the last paragraph of the introductionThe method novelty has been highlighted in the last paragraph of the introduction as suggested. Lines 73-76 Results: the units should be presented consistently (e.g. mL min-1) should be mL/min, similar to the concentration units in µg/mL.The units have been consistently modified and presented as mL/min, and this adjustment has been applied uniformly throughout the manuscript, Lines 20, 134, and 139. Conclusion: please, change "even if its transferability could be not easily due to the gradient elution mode." to "even if its transferability could be challenging due to the gradient elution mode."We appreciate the reviewer's suggestion and have incorporated the recommended changes in the manuscript, as indicated in Lines 316-317. Reference 2 should be corrected to "O A. Ogbechie-Godec, N Elbuluk, Melasma: an Up-to-Date Comprehensive Review, Dermatol. Ther. (Heidelb). 7 (2017) 305 318.https://doi.org/10.1007/s13555-017-0194-1[3]We appreciate the reviewer's note, and we have corrected reference 2 as recommended. Line 352 Reference 6 should be corrected to "T.S. Chen, S.Y. Liou, W.W. Kuo, H.C. Wu, G.P. Jong, H.F. Wang, C.Y. Shen, V.V. Padma, C.Y. Huang, Y.L. Chang, Rapid method for the quantification of hydroquinone concentration: Chemiluminescent analysis, Luminescence. 30 (2015) 947-949"Reference 6 has been corrected as suggested. Line 365 Reference 13 should be corrected to "P. Gimeno, A.F. Maggio, M. Bancilhon, N. Lassu, H. Gornes, C. Brenier, L. Lempereur, HPLC-UV method for the identification and screening of hydroquinone, ethers of hydroquinone and corticosteroids possibly used as skin-whitening agents in illicit cosmetic products, J. Chromatogr. Sci. 54 (2016) 343-352." We have corrected reference 13 as recommended. Line 391 Reference 25 should be corrected to ICH Guideline, Validation of analytical procedures: text and methodology, Q2. 1 (2005) 5.Reference 31 (in the revised version) has also been corrected. Line 461 Reviewer #2 CommentResponse Reviewer #2: The authors in this work separated six compounds, including four active ingredients and two excipients within one run using gradient elution. The peaks are well resolved, and quite symmetric. This work has merits, and the paper deserves to be published in the Journal of Pharmaceutical and Biomedical Analysis following minor revision. Here are my comments to further improve the manuscript:We would like to express our gratitude to the reviewer for taking the time to review our paper. All the points raised have been addressed as suggested. 1. The authors used the symbol K to refer to the capacity factor. Usually, K is used to indicate the partition coefficient. Instead, we use k’ to refer to the capacity factor.We have replaced the symbol K by k’ as recommended. 2. Retention factor is a more common terminology than capacity factor. So, please, use

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Prof. Marcello Locatelli
University "G. d'Annunzio" of Chieti-Pescara,
Dept. of Pharmacy, Build B level 2, Via dei Vestini 31, 66100
Chieti (CH), Italy
E-mail: marcello.locatelli@unich.it

Dear Editor,

Please find enclosed the revised manuscript "***A stability indicating RP-HPLC-UV assay method for the simultaneous determination of hydroquinone, tretinoin, hydrocortisone, butylated hydroxytoluene and parabens in pharmaceutical creams***" resubmitted to the **Journal of Pharmaceutical and Biomedical Analysis** as a research article.

We thank the Reviewers for their very positive evaluations and for their useful suggestions and comments. All are accepted and reported in the current revised version in "track changes mode". The point-by-point response to the Reviewers comments is enclosed in this R1 version.

We hope that in the present form the paper can be accepted for publication in your esteemed journal.

*** Corresponding authors**

Prof. **Marcello Locatelli**; Department of Pharmacy, University "G. d'Annunzio" of Chieti-Pescara, Via dei Vestini 31, 66100 Chieti, Italy E-mail: marcello.locatelli@unich.it

Prof. **Fotouh R. Mansour**; Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, Elgeish Street, the medical campus of Tanta University, Tanta, Egypt 31111. E-mail: fotouhrashed@pharm.tanta.edu.eg

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(Editor, Journal of Pharmaceutical and Biomedical Analysis)

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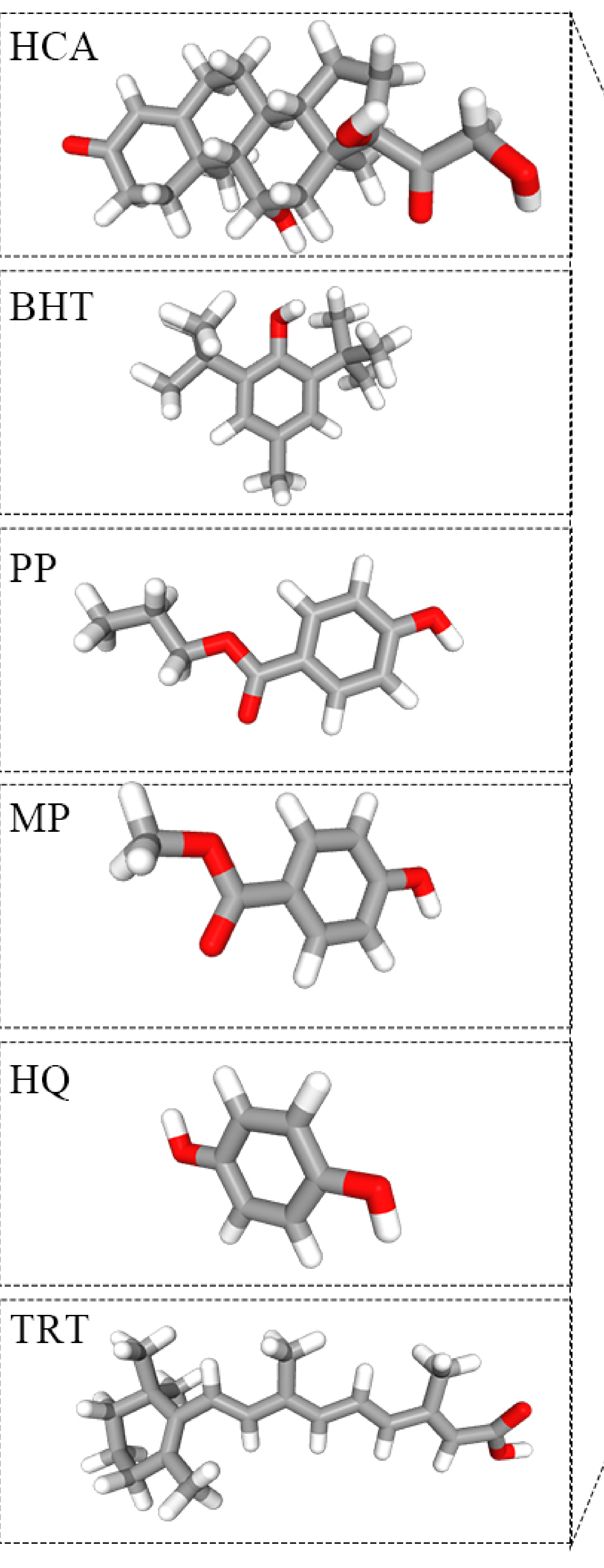
Best regards,

Highlights

- Validated HPLC method for the quantitation of hydroquinone, tretinoin, hydrocortisone, butylated hydroxytoluene and parabens
- Innovative analytical approach for multicomponent formulations
- Practical application for drug quality control in cream dosage form
- First tool to successfully quantify up to 6 components simultaneously in a topical formulation
- AGREEprep and BAGI evaluation of the proposed method

Graphical Abstract



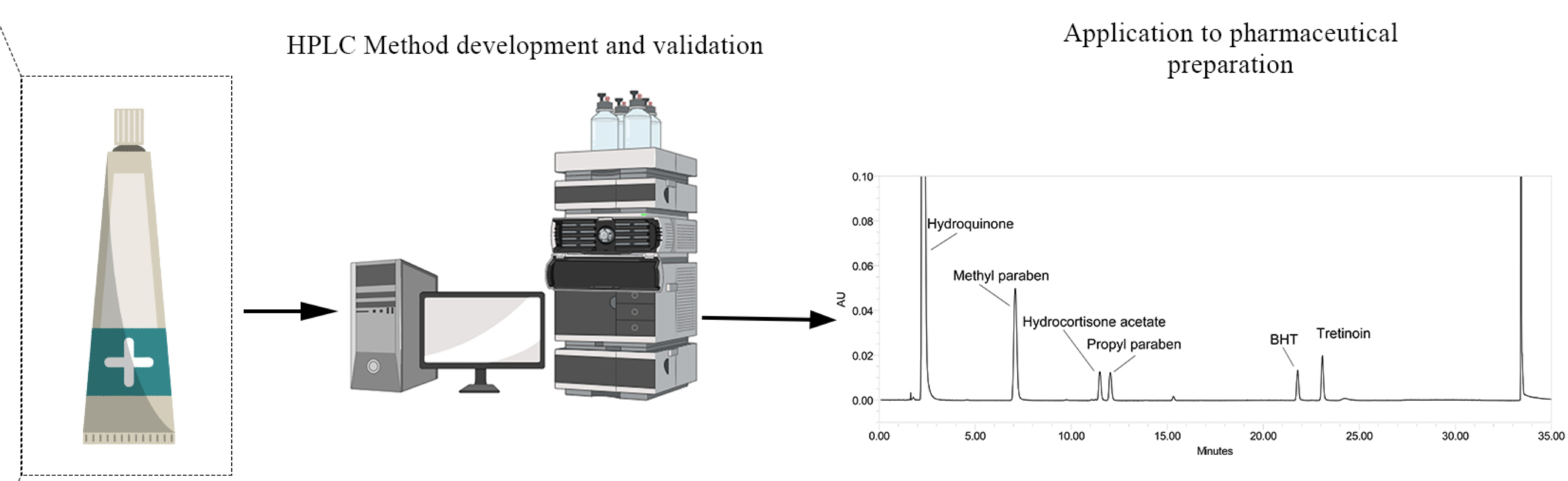


Figure 1

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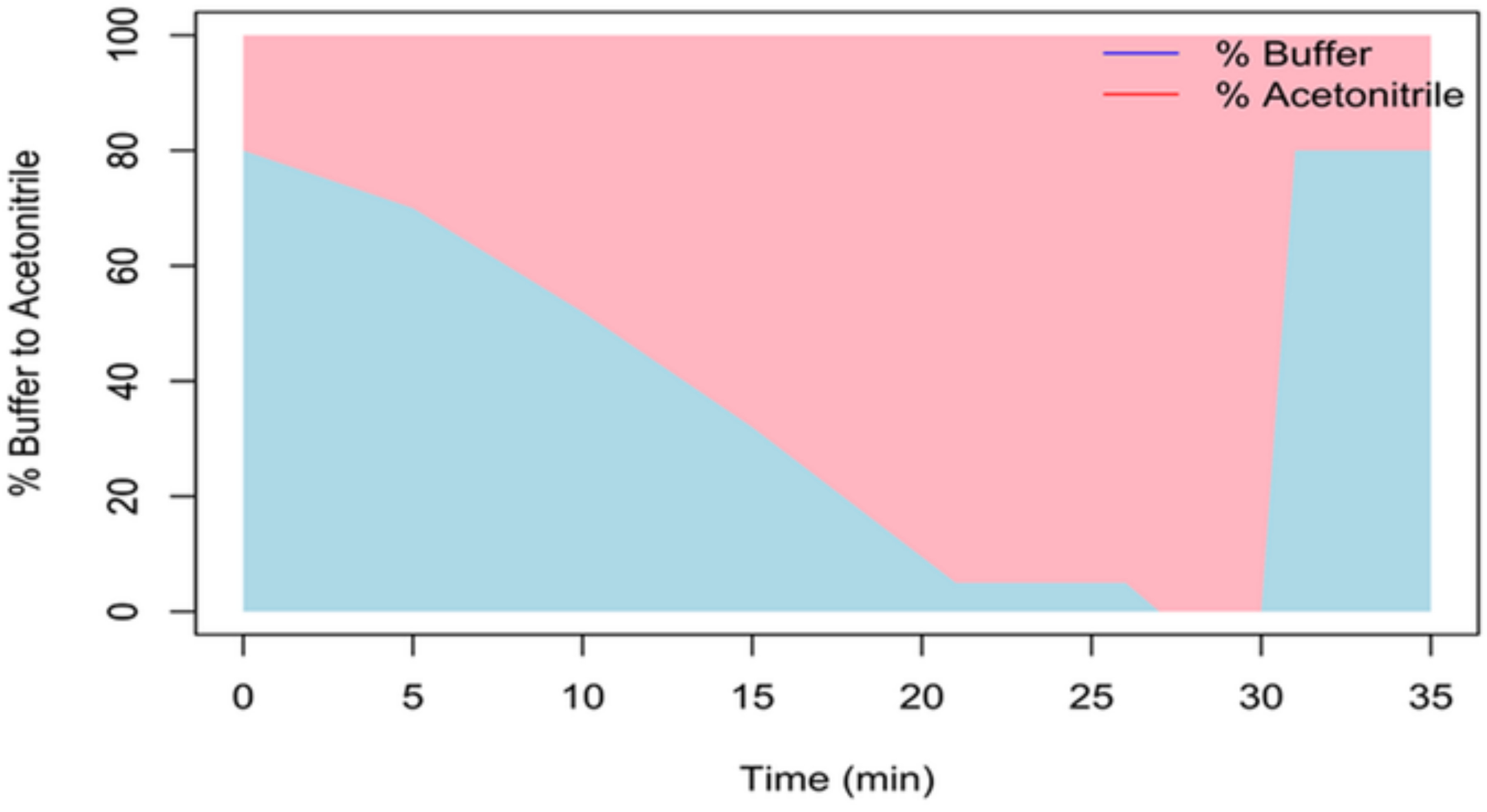

Figure 2

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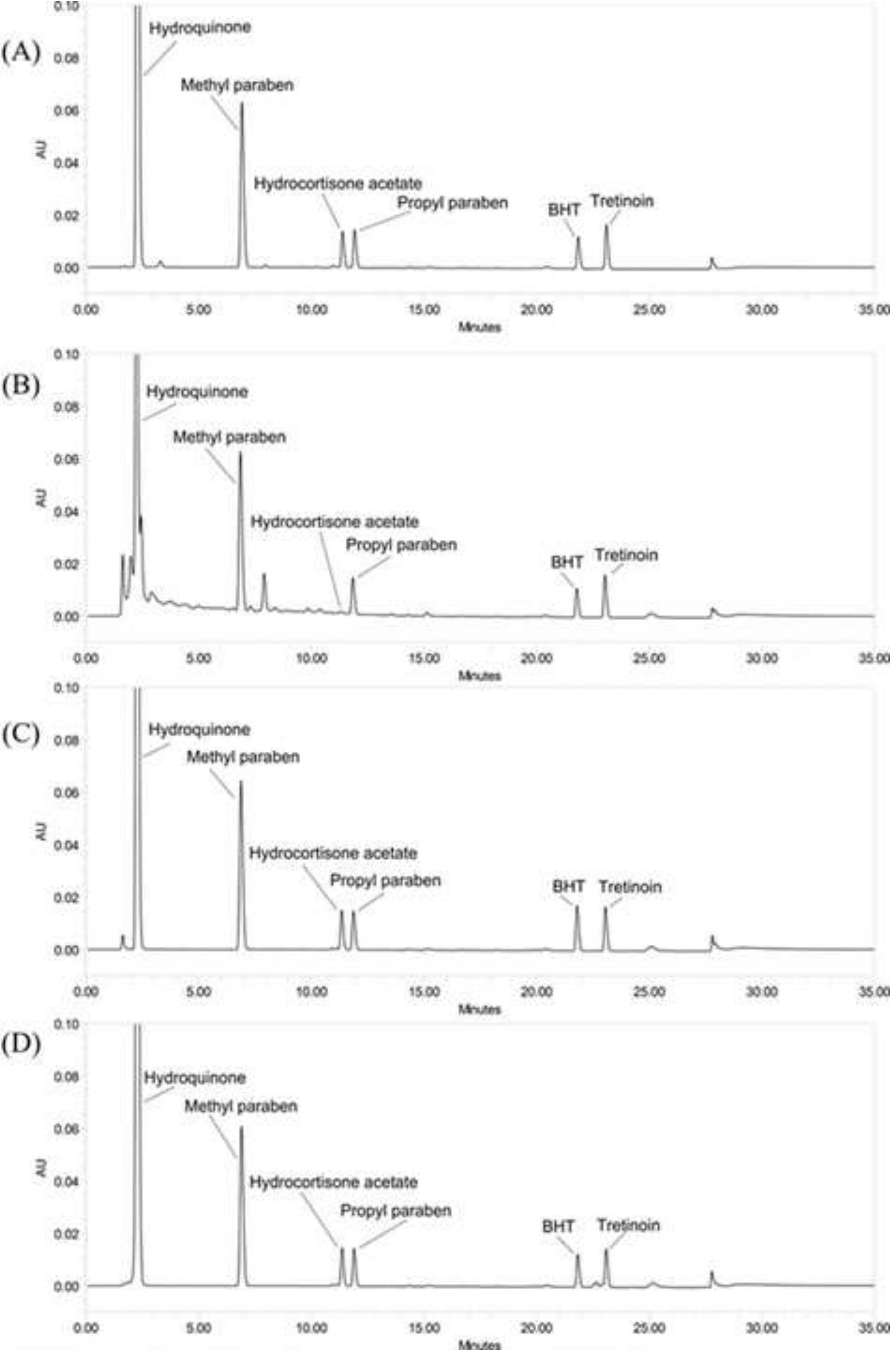

Figure 3

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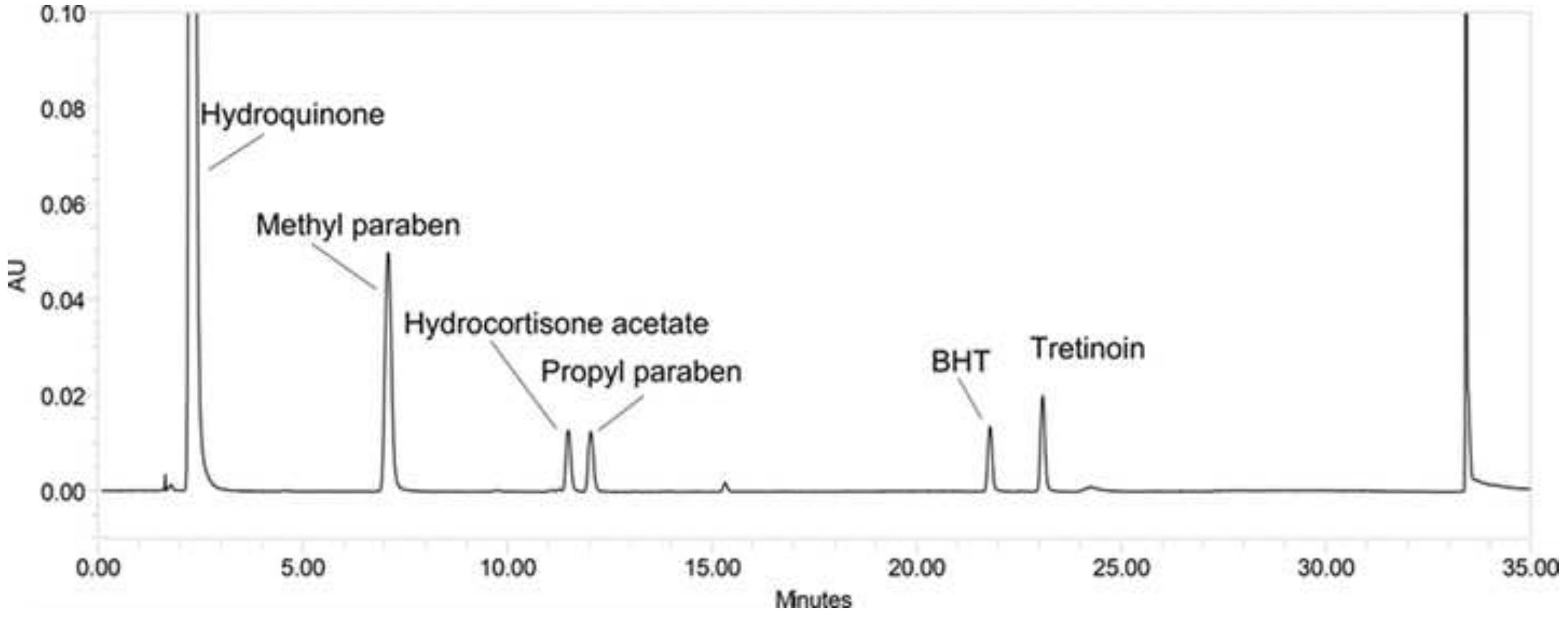

Figure 4

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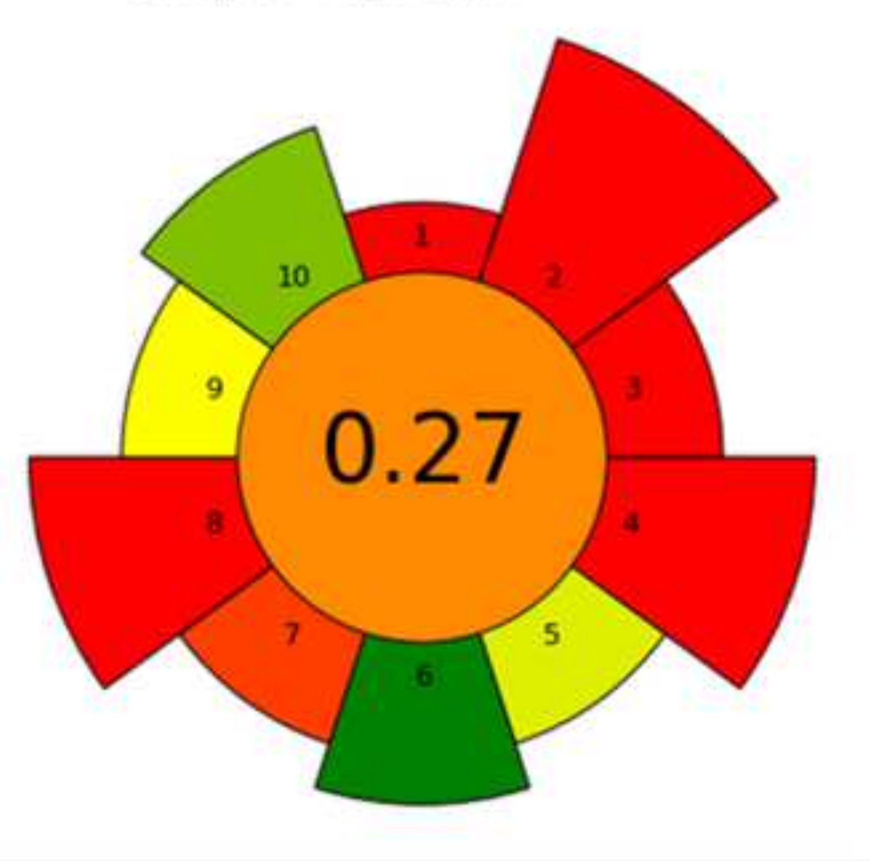

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Table 1: Chemical structures and properties of the investigated analytes

Compound	Chemical structure	Molecular Weight (g/mol)	Protein Binding	Half life	LogP	pKa (Strongest Acidic)
Methyl paraben (MP)		152.15	n.a.	n.a.	2.17	8.5
Propyl paraben (PP)		180.20	n.a.	n.a.	3.24	8.5
Hydrocortisone acetate (HCA)		404.50	95%	6-8h	2.31	12.61
Butylated hydroxytoluene (BHT)		220.35	n.a.	n.a.	5.25	11.6
Tretinoin (TRT)		300.40	≥95%	0.5-2h	5.66	4.76
Hydroquinone (HQ)		110.11	n.a.	n.a.	0.71	9.68

n.a. not available

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Table 2: Characteristic parameters of the calibration equations for the proposed HPLC method.

	Range µg/mL	**R** *(n = 7)*	**a**	**b**	S_a	S_b	**LOD µg/mL**
Hydroquinone	240 - 560	0.99944	-210619.47	11570026.78	94008.30	222043.66	26.813
Methyl paraben	24 - 56	0.99946	-30184.79	16280665.29	12711.50	307500.82	2.577
Propyl paraben	6 - 14	0.99948	-7735.3414	13510701.08	2612.97	251433.37	0.638
Hydrocortisone acetate	132 - 308	0.99942	-3030.1379	583315.978	2544.45	11409.15	14.395
Butylated hydroxytoluene	12 - 28	0.99960	-6972.8770	8515586.90	2844.79	138252.70	1.10
Tretinoin	6.6 - 15.4	0.99920	-5617.7836	14094409.25	3701.82	323825.34	0.867

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Table 3: System suitability parameters for the proposed HPLC method for simultaneous determination of Tritospot components.

	t_r	k'	α	Rs	A*s*
HQ	2.29	21.92			1.45
MP	7.16	70.74	3.23	19.02	1.13
PP	12.12	70.74	1.05	2.61	1.12
HCA	11.54	114.35	1.62	18.09	1.16
BHT	21.88	21.78	1.8	45.15	0.98
TRT	23.19	23.09	1.06	6.54	1.01

Table 3 track changes
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	t_r	k'	α	Rs	As	~~N~~
HQ	2.29	21.92			1.45	~~1607~~
MP	7.16	70.74	3.23	19.02	1.13	~~10429~~
PP	12.12	70.74	1.05	2.61	1.12	~~46997~~
HCA	11.54	114.35	1.62	18.09	1.16	~~49561~~
BHT	21.88	21.78	1.8	45.15	0.98	~~194010~~
TRT	23.19	23.09	1.06	6.54	1.01	~~199012~~

Formatted Table

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Table 4: Intraday and interday precision (RSD%) and trueness (Mean % found).

Analyte	Intraday			Interday		
	Added (μg/mL)	Found (μg/mL)	% found	Added (μg/mL)	Found (μg/mL)	% found
HQ	400.0	409.8	102.4	400.0	405.0	101.3
	400.0	415.0	103.7	400.0	406.6	101.7
	400.0	402.6	100.7	400.0	392.4	98.1
		Mean	**102.3**			**100.3**
		RSD%	**1.5**			**1.9**
MP	40.0	41.1	102.6	40.0	40.3	100.8
	40.0	40.4	101.0	40.0	40.6	101.5
	40.0	40.4	100.9	40.0	40.0	99.9
		Mean	**101.5**			**100.8**
		RSD%	**0.9**			**0.8**
PP	10.0	10.4	104.1	10.0	10.2	101.5
	10.0	10.5	105.4	10.0	10.1	101.4
	10.0	10.3	102.6	10.0	9.8	98.4
		Mean	**104.0**			**100.4**
		RSD%	**1.4**			**1.7**
HCA	220.0	223.4	101.5	220.0	223.7	101.7
	220.0	220.7	100.3	220.0	220.7	100.3
	220.0	220.5	100.2	220.0	218.8	99.5
		Mean	**100.7**			**100.5**
		RSD%	**0.7**			**1.1**
BHT	20.0	20.6	102.8	20.0	20.2	100.9
	20.0	20.5	102.7	20.0	20.6	103.0
	20.0	20.1	100.4	20.0	20.3	101.5
		Mean	**101.9**			**101.8**
		RSD%	**1.3**			**1.1**
TRT	11.0	11.2	101.6	11.0	11.0	100.2
	11.0	11.2	101.9	11.0	10.8	98.1
	11.0	11.0	100.2	11.0	11.2	101.7
		Mean	**101.2**			**100.0**
		RSD%	**0.9**			**1.8**

Table 4 track changes

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Table 4: Intraday and interday precision (RSD%) and trueness (Mean % found).

Analyte	Intraday			Interday		
	Added (µg/mL)	Found (µg/mL)	% found	Added (µg/mL)	Found (µg/mL)	% found
HQ	400.0	409.~~76~~8	102.~~44~~4	400.0	405.~~04~~0	101.~~26~~3
	400.0	~~414.96~~415.0	103.~~74~~7	400.0	406.6	101.~~65~~7
	400.0	402.6	100.~~65~~7	400.0	392.~~44~~4	98.~~11~~1
		Mean	**102.3**			**100.3**
		RSD%	**1.5**			**1.9**
MP	40.0	41.~~06~~1	102.~~64~~6	40.0	40.~~32~~3	100.~~81~~8
	40.0	40.~~41~~4	101.~~03~~0	40.0	40.~~61~~6	101.~~53~~5
	40.0	40.~~37~~4	100.~~93~~9	40.0	~~39.97~~40.0	99.~~93~~9
		Mean	**101.5**			**100.8**
		RSD%	**0.9**			**0.8**
PP	10.0	10.~~41~~4	104.~~11~~1	10.0	10.~~15~~2	101.~~54~~5
	10.0	10.~~54~~5	105.~~41~~4	10.0	10.~~13~~1	101.~~35~~4
	10.0	10.~~26~~3	102.~~56~~6	10.0	9.~~84~~8	98.~~42~~4
		Mean	**104.0**			**100.4**
		RSD%	**1.4**			**1.7**
HCA	220.0	223.~~37~~4	101.~~53~~5	220.0	223.~~74~~7	101.7
	220.0	220.~~71~~7	100.~~32~~3	220.0	220.~~68~~7	100.~~31~~3
	220.0	220.~~46~~5	100.~~21~~2	220.0	218.~~79~~8	99.~~45~~5
		Mean	**100.7**			**100.5**
		RSD%	**0.7**			**1.1**
BHT	20.0	20.~~56~~6	102.~~78~~8	20.0	20.~~18~~2	100.~~89~~9
	20.0	20.~~54~~5	102.~~68~~7	20.0	20.~~59~~6	~~102.96~~103.0
	20.0	20.~~07~~1	100.~~37~~4	20.0	20.~~29~~3	101.~~48~~5
		Mean	**101.9**			**101.8**
		RSD%	**1.3**			**1.1**
TRT	11.0	11.~~17~~2	101.~~57~~6	11.0	11.~~02~~0	100.~~18~~2
	11.0	11.~~21~~2	101.9	11.0	10.~~79~~8	98.~~11~~1
	11.0	11.~~02~~0	100.~~22~~2	11.0	11.~~19~~2	101.~~72~~7
		Mean	**101.2**			**100.0**
		RSD%	**0.9**			**1.8**

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Table 5: A comparison between our proposed method and previous published works

Analyte(s)	Instrument configuration	Elution mode	Range (µg/mL)	LOD/LOQ	Ref.
HQ TRT	HPLC-UV	Isocratic	50-250 1-5	0.4208/ 1.2750 0.2866/ 0.8686	[7]
HQ HCA	HPLC-DAD	Isocratic	25-150 25-150	2.123/ 7.077 2.003/ 6.676	[8]
HQ HCA TRT	HPLC-DAD	Isocratic	10–200 5–100 1–40	2.01/ 6.11 1.13/ 3.41 0.28×10^{-3}/ 0.87×10^{-3}	[9]
HQ TRT	HPLC-UV	Isocratic	50-300 0.5-5	6.86/ 22.89 0.18/ 0.61	[26]
HQ HCA TRT	UHPLC	Gradient	N.R	0.2993/0.8982 0.0557/0.1115 0.1116/0.3348	[27]
HQ TRT	HPLC-UV	Isocratic	100–300 0.625 to 1.875	3.75/ 11.37 0.02/ 0.07	[32]
HQ TRT	HPLC-UV	Isocratic	100-300 0.625-1.875	1.80/ 5.44 0.02/ 0.07	[33]
HQ TRT HCA BHT MP PP	HPLC-UV	Gradient	240 – 560 6.6 - 15.4 132 – 308 12 – 28 24 – 56 6 - 14	26.813/240 0.867/6.6 14.395/132 1.10/12 2.577/24 0.638/6	Current method

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~~Table 5: Comparison with literature~~

Table 5: A comparison between our proposed method and previous published works

Analyte(s)	Instrument configuration	Elution mode	Range (µg/mL)	LOD/LOQ	Ref.
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HQ HCA TRT	HPLC-DAD	Isocratic	10–200 5–100 1–40	2.01/ 6.11 1.13/ 3.41 0.28×10^{-3}/ 0.87×10^{-3}	[9]
HQ TRT	HPLC-UV	Isocratic	50-300 0.5-5	6.86/ 22.89 0.18/ 0.61	[26]
HQ HCA TRT	UHPLC	Gradient	N.R	0.2993/0.8982 0.0557/0.1115 0.1116/0.3348	[27]
HQ TRT	HPLC-UV	Isocratic	100–300 0.625 to 1.875	3.75/ 11.37 0.02/ 0.07	[~~30~~32]
HQ TRT	HPLC-UV	Isocratic	100-300 0.625-1.875	1.80/ 5.44 0.02/ 0.07	[~~31~~33]
HQ TRT HCA BHT MP PP	HPLC-UV	Gradient	240 – 560 6.6 - 15.4 132 – 308 12 – 28 24 – 56 6 - 14	26.813/240 0.867/6.6 14.395/132 1.10/12 2.577/24 0.638/6	Current method

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Declaration of interests

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

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A stability indicating RP-HPLC-UV assay method for the simultaneous determination of hydroquinone, tretinoin, hydrocortisone, butylated hydroxytoluene and parabens in pharmaceutical creams

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Abbreviations

As: asymmetry factor;
BHT: Butylated hydroxytoluene;
HCA: hydrocortisone acetate;
HQ: Hydroquinone;
SST: System suitability tests;
ICH: International Conference of Harmonization
k': retention factor;
LOD: limits of detection;
LOQ: limits of quantitation;
MP: Methyl paraben;
N: number of theoretical plates;
PP: propyl paraben;
RP-HPLC: Reversed-phase high-performance liquid chromatography;
Rs: resolution;
S/N: signal-to-noise ratio;
t_r: retention time;
TRT**:** Tretinoin;
α: selectivity;

Abstract

Multicomponent drugs are medications that combine two or more active pharmaceutical ingredients in a single dosage form. These dosage forms improve the patient compliance, reduce the risk of drug interactions, and simplify dosing regimens. However, quality control of these multicomponent dosage forms can be challenging, especially if the final product contains four or more ingredients that are active (comprise stabilizers, preservatives, excipients, and other components). This problem can be more pronounced if the excipients can interfere with the analysis. In this work, a stability indicating assay method was developed and validated (according to the ICH International Guidelines) for the simultaneous determination of hydroquinone (HQ), tretinoin (TRT), hydrocortisone (HCA), butylated hydroxytoluene (BHT), methyl paraben (MP) and propyl paraben (PP) in commercially available pharmaceutical creams. The proposed method is based on gradient elution using X-Bridge C18 (150 × 4.6 mm, 5μm) column with a flow rate of 1 mL/min. The linear ranges (μg/mL) were 240-560 for HQ, 24-56 for MP, 132-308 for HCA, 6-14 for PP, 12-28 for BHT, 6.6-15 for TRT. During the validation process, the intra- and interday precision and trueness (evaluated as recovery) were found to be below 2.0% and between 100–102%, respectively. System suitability tests (SST) allow validating the herein proposed procedure specifically for pharmaceutical and industrial applications. SST test shows that the reported procedure fulfill with the Guidelines, allowing excellent separation of the analytes with very sensitive, accurate (precise and true) and reproducible quantitation of each analytes. The method was successfully applied in forced degradation studies of the six analytes. Specifically, acid degradation slightly affected HCA and BHT (91% recovery), while alkaline degradation drastically reduced HCA recovery (5.5%) and moderately affected BHT (85%). Photodegradation primarily influenced TRT quantity, and oxidative degradation intensified the BHT peak (130%).

Keywords: RP-HPLC; Hydroquinone; Parabens; Topical Formulations; AGREEprep and BAGI; Stability study.

1. INTRODUCTION

Topical pharmaceutical formulations that contain a combination of hydroquinone (HQ), corticosteroids, and tretinoin (TRT) are used to treat melasma, a specific skin condition that affects the face, precisely the cheeks, forehead, and upper lip, and is generally characterized by abnormal pigmentation. This disease is more prevalent in women, accounting for 90% of reported cases [1,2]. Hydroquinone (benzene-1,4-diol; HQ) is the main depigmenter used and it acts through the inhibition of the tyrosinase enzyme which prevents the conversion of DOPA to melanin. Tretinoin (all-trans retinoic acid; TRT) is used to enhance and improve the cell regeneration while hydrocortisone acetate (HCA) reduces associated UV-induced inflammation. The use of the three agents together is known as "triple combination therapy" which was shown to be more effective in treating melasma [3]. Methyl and propyl parabens (MP and PP) are often used in pharmaceutical formulations as preservatives, especially for their low toxicity and wide antibacterial and antifungal activities. Butylated hydroxytoluene (BHT) was added to the formulation to benefit from its antioxidant effects. The chemical structures for all these components are reported in **Table 1**.

Table 1: Chemical structures and properties of the investigated analytes

The assay used for HQ, as reported in the official USP, is widely reported alone [4–6], in combinations [7–14], as well as in the presence of its main degradation products [15–17]. In all these procedures the mainly used instrument configuration was high performance liquid chromatography (HPLC), although spectrophotometric [4,5,12,14,17], and chemiluminescence [6] were also applied. The second component, HCA, was similarly widely quantified using various techniques alone or in combinations of topical formulations. Several methods were also reported for the quantitation of TRT in dermatological preparations in the presence of other compounds [18,19], degradation products [16,18–23], or metabolites [22,24,25].

By a systematic literature survey, only few works report simultaneous separation and quantitation of all active principles of the triple combination therapy formulations (HQ, TRT, and a corticosteroid) [7–9,26,27] let alone preservatives too [15]. In addition, the study of the stability is crucial for the final commercial product before releasing in the market, so forced degradation studies must be performed. The process of forced degradation entails subjecting drug substances and products to harsher conditions than those used in accelerated conditions

69 [28]. This leads to the formation of degradation products, which can be analyzed to assess the
70 stability of the molecule. Also help in developing stability indicating methods which must
71 demonstrate high level of specificity, while also offering a better understanding of the
72 degradation pathways and products of the drug substance [29].
73 To the best of our knowledge, the herein proposed method is the first to successfully
74 quantify all six components simultaneously in a commercially available topical formulation.
75 The inclusion of preservatives in the analysis is a novel aspect, as previous methods have
76 mostly focused on the quantification of the active ingredients alone. The challenge faced during
77 method development was to successfully separate all six components which varied so much in
78 polarity from the polar HQ (Octanol/Water Partition Coefficient; LogP= 0.71) to highly non-
79 polar TRT (Octanol/Water Partition Coefficient; LogP= 5.66). What was even more
80 challenging was that some of the constituents' polarities were very close (HCA/MP and
81 BHT/TRT). Another important challenge was the staggering difference in components'
82 concentrations, where HQ was almost 40 times that of TRT, highlighting that this multianalytes
83 method allows the quantification of the compounds in a wide large concentration range.
84

85 2. MATERIALS AND METHODS

86 2.1. Materials and solvents

87 All chemicals used throughout this study were of analytical grade. The solvents were
88 HPLC grade (Fisher Scientific, NJ, USA). Global NAPI Pharmaceuticals (Cairo, Egypt) kindly
89 gifted high purity reference material of HQ, TRT, HCA, MP, PP, BHT as well as Tritospot®
90 cream (labeled to contain 3% HQ, 0.02% TRT, 1% HCA and 4% eusolex).
91

92 2.2. Instrument and software

93 The employed HPLC system was a Waters 2695 HPLC system (Waters Corp., Milford,
94 USA) connected to VWD-3400RS UV detector set at 280 nm and WPS-3000TPLRS
95 autosampler, using Empower v.2 software for data acquisition and processing. The HPLC
96 column was an X-Bridge C18 (4.6 x 150 mm, 5um, Waters Corp., Milford, USA). The buffer's
97 pH was adjusted using Hanna HI 8314 pH-meter (Hanna, Padua, Italy)
98

99 2.3. Procedures

100 *2.3.1. Standard Solutions*

101 The diluent used was a mixture of methanol: acetonitrile: tetrahydrofuran: phosphoric
102 acid (ratio 50:30:20:0.2). Stock standard solution was prepared by transferring accurately: 22.0
103 mg TRT reference material, 40.0 mg BHT reference material, 80.0 mg MP reference material
104 and 20.0 mg PP reference material into 100 mL dark colored volumetric flask. The powders
105 were dissolved, and the volume completed to the mark with the diluent.

106 The Working standard solutions were prepared by transferring accurately 40.0 mg HQ
107 reference material and 22.0 mg HCA reference material into 100 mL volumetric flask to be
108 dissolved with 50 mL diluent. From the stock standard solution, 5.0 mL were added, mixed,
109 and the volume completed to the mark with diluent. Aliquots from the working standard
110 solution were diluted to produce the different concentrations of HQ (240-560 µg/mL), MP (24-
111 56 µg/mL), HCA (132-308 µg/mL), PP (6-14 µg/mL), BHT (12-28 µg/mL), and TRT (6.6-15
112 µg/mL). 10 µL of the sample was injected and chromatographed using the specified
113 chromatographic conditions. The peak responses of all six components were recorded
114 simultaneously and plotted against their corresponding concentration.

115

116 ***2.3.2. Chromatographic conditions***

117 Different mobile phases, gradient profiles, pH, and detection wavelengths were tried.
118 More stable baselines were observed when phosphate buffer was employed instead of formate
119 buffer. Better peak shapes were obtained when acetonitrile was employed as a mobile phase
120 modifier, compared with methanol. The resolution between peaks improved by decreasing the
121 buffer pH. Accordingly, the use of phosphate buffer (pH 2.1)-acetonitrile as a mobile phase
122 resulted in better separation with improved peak sharpness, larger area, quicker retention time,
123 and enhanced resolution. Using these phases, different gradient elution profiles were evaluated,
124 starting from high polarity to lower, in order to resolve the analytes, obtain good peak
125 symmetries, and to obtain an adequate total runtime (important in pharmaceutical industry and
126 related to the concept of the high throughput).

127 For the detection wavelength, 210, 254 and 280 nm were investigated. Parabens (MP
128 and PP) had high absorptivity at shorter wavelengths while TRT did not, which made both 210
129 and 254 nm not suitable for TRT quantification especially at such low concentration compared
130 to other five constituents. The wavelength of 280 nm, on the other hand, gave satisfactory
131 response for all six analytes. In addition, using different detection wavelengths for different
132 phases of gradient/separation led to unacceptable noise/drifting of the baseline.

133 Gradient elution (**Figure 1**) (from high polarity to lower) was preferred to ensure best
134 separation at reasonable time. Flow rate was chosen at 1 mL/min. Faster flow rates resulted in

135 overlap of BHT and TRT peaks, which were very sensitive to changes in organic phase percent.
136 Because of the proximity of their polarities, they required enough time to interact with the
137 stationary phase in a way that resolve them completely (higher selectivity factor). Slower flow
138 rates on the other hand were not advantageous since they led to deteriorating peak shapes and
139 prolonged analysis time for no improvement in resolution (1 mL/min showed resolution of 2.6
140 that was more than enough for a successful complete separation). The injection volume was 10
141 µL. The employed buffer was prepared by mixing 10 mL glacial acetic acid and 10 mL
142 phosphoric acid 85 % in 2 L of water. Then, 2 mL triethylamine were added, and the pH was
143 adjusted to 2.1 by adding ammonia. Before using the mobile phase, it was filtered and degassed.
144
145 **Figure 1:** Mobile phase gradient composition
146
147 ***2.3.3. Validation***
148 2.3.3.1. Specificity
149 The ability to assess unequivocally the analyte in the presence of components which
150 might be expected to be present by determining and calculating system suitability parameters
151 including retention time (t_r), retention factor (k'), selectivity (α), resolution (Rs), and
152 asymmetry factor (As).
153
154 2.3.3.2. Linearity and range
155 Several concentrations were prepared and used to create a calibration curve for each
156 component. Each concentration was injected three times and the area under the curve is plotted
157 against the concentration. After that, the regression equation was calculated for each ingredient.
158
159 2.3.3.3. Trueness
160 The mean percentage recovery of three replicates for three different concentration
161 levels within the linear range (nine measurements) were calculated for all six analytes.
162
163 2.3.3.4. Precision repeatability
164 The relative standard deviation was calculated for three different concentrations of all
165 ingredients by injecting each concentration three times. On the same day under the same
166 experimental conditions.
167
168 2.3.3.5. Intermediate precision

The RSD was calculated using the same procedure of mentioned in Precision repeatability except for that injections are done on three different days.

2.3.3.6. Limit of quantitation and limit of detection

The limit of detection (LOD) and limit of quantification (LOQ) were validated by means of signal-to-noise ratio (S/N) equal to 3 and 10, respectively [30,31]. Additionally, the LOQs were validated considering the back-calculated concentrations and evaluating their BIAS% with respect to the theoretical concentration.

2.3.4. Forced degradation

Forced degradation are carried out to achieve to generate a degradation profile that is comparable to what would be seen under normal stability study.

2.3.4.1. Acid degradation

Samples of the working standard solution were treated with 1 M HCl at 60°C for 1h. During that time, every 20 min a 10.0 mL aliquot was pipetted out of the mix, neutralized and assayed using the proposed HPLC method.

2.3.4.2. Base degradation

The stock standard and materials mixture were dissolved in 5 M NaOH and heating the solution to 60°C for 1 h. Every 20 min, 10 mL of the mixture was taken, neutralized with 5M HCl, and analyzed by the proposed HPLC method.

2.3.4.3. Oxidative degradation

The oxidative degradation was investigated by treating the standard solution of raw material with 3% H_2O_2 and heating the solution for 1 h at 60°C. The area under curve of the peaks was monitored using the same chromatographic conditions.

2.3.4.4. Photodegradation

Was studied by applying the radiation of a UV lamp to the working standard solution in a quartz cell for 24 h and similarly taking samples every 20 min to be assayed.

2.3.5. Application to pharmaceutical preparation

202 Accurately weighed 2.0 gm of test sample (Tritospot cream) were transferred to an
203 amber conical flask. 70 mL diluent were then added, shaken by mechanical means for 15
204 minutes, and Sonicated for 30 minutes at 40° C. The contents were left to cool down to room
205 temperature before they were filtered into 100 mL volumetric flask where volume was
206 completed to the mark with diluent.
207

208 # 3. RESULTS AND DISCUSSION

209 RP-HPLC was an obvious answer to the challenges imposed by this pharmaceutical
210 preparation because of its high separation power, versatility, sensitivity and reproducibility.
211 The wide range of polarities and concentrations encompassed together with the proximity of
212 the polarities of (HCA & PP) and (BHT & TRT) meant that separation conditions had to be
213 carefully tuned to achieve best separation at reasonable time with acceptable sensitivity and
214 reproducibility.
215

216 ## 3.1. Method Validation

217 The developed method was validated in accordance with the ICH guidelines [31]. The
218 results of the validation study showed that the method is accurate and precise, as summarized
219 in **Table 2**.
220

221 **Table 2**: Characteristic parameters of the calibration equations for the proposed HPLC
222 method.
223

224 ### *3.1.1. Specificity*

225 Various system suitability parameters were calculated to ensure its specificity and
226 complete separation of all six components. They were summed up in **Table 3**. Resolution of all
227 peaks was greater than 1.5, which indicated complete separation. The peaks symmetry was
228 within acceptable range. Understandably, HQ peak symmetry factor was the largest because of
229 its very high concentration compared to other components.
230

231 **Table 3**: System suitability parameters for the proposed HPLC method for simultaneous
232 determination of Tritospot components.
233

234 ### *3.1.2. Linearity and range*

235 To test the linearity of the method, six different concentrations were utilized. The
236 correlation coefficients obtained for all six components were >0.9992 as shown in **Table 2.**
237
238 **3.1.3. Precision and trueness**
239 The mean percentage recovery used to evaluate the trueness of the herein proposed
240 method were calculated for all six analytes and summed in **Table 4**. All these values were
241 between 100 – 102% for all analytes except PP (104%) which was still acceptable for routine
242 quality control work (admitted values must be within ±15%). The relative standard deviation
243 percentages (RSD%) for replicate injections (intra and inter-day) was always below 2.0%.
244 These results in terms of precision and trueness (both intra- and interday) were very useful for
245 quality control requirements, which require such precision and trueness to release products to
246 the market with confidence in their test results.
247
248 **Table 4**: Intraday and interday precision (RSD%) and trueness (Mean % found).
249
250 ***3.1.4. Limit of detection and limit of quantitation***
251 The signal-to-noise ratio (S/N) was employed to establish the limits of detection (LOD)
252 and limits of quantitation (LOQ) in the chromatographic techniques, coupled also with the
253 evaluation of the BIAS% at these concentration levels. The LOD values were 26.813 µg/mL,
254 2.577 µg/mL, 0.638 µg/mL, 14.395 µg/mL, 1.10 µg/mL, and 0.867 µg/mL for HQ, MP, PP,
255 HCA, BHT, and TRT, respectively. LODs were very suited to the method's use for
256 pharmaceutical preparation analysis.
257
258 **3.2. Forced degradation**
259 The focus was on all components using the same proposed chromatographic conditions
260 and following the changes in peaks response and shape. Acid degradation **(Figure 2A)** slightly
261 affected HCA and BHT (mean percent recovery 91%), while alkaline degradation **(Figure 2B)**
262 showed massive decrease in percent recovery of HCA (5.5%) and too much lesser extent BHT
263 (85%). Oxidative degradation **(Figure 2C)** intensified the BHT peak (130%), while
264 photodegradation **(Figure 2D)**, as expected, mainly affected TRT.
265
266 **Figure 2:** Chromatogram of photodegradation of all six components of Tritospot® cream (A)
267 Acid degradation (B) Alkaline degradation (C) Oxidative degradation (D) Photodegradation

268
269 ### 3.3. Application to pharmaceutical preparation

270 The proposed method was used to analyze the commercial product Tritospot®. Mean
271 percent recoveries for all studied six components were reasonable and RSD of six replicates
272 was less than 2% for all studied analytes indicating the applicability of the proposed method to
273 pharmaceutical dosage form analysis with satisfactory trueness and precision **Figure 3**.
274
275 **Figure 3:** Chromatogram of all six components of Tritospot® cream in a real sample analysis
276
277 ### 3.4. Comparison with other reported methods

278 The simultaneous separation of six analytes in one HPLC run is quite challenging. A
279 few reports have been found for the concurrent analysis of HQ, HCA, and TRT [9,27]. Other
280 methods could determine two of the active ingredients [7,8,26,32,33], as shown in **Table 5**. To
281 the best of our knowledge, no analytical method has been reported yet for the simultaneous
282 analysis of these six analytes. Determination of multiple analytes using a single method in a
283 single run is congruent with the current trend of developing analytical methods that save time
284 and reduce organic solvents consumption, without sacrificing the method reliability. The
285 developed method is more suitable for the routine chemical analysis of these compounds in
286 dosage forms.
287
288 **Table 5**: A comparison between our proposed method and previous published works
289
290 ### 3.5. AGREEprep and BAGI evaluation

291 The method proposed and validated here was evaluated using the AGREEprep tool
292 [34,35] relating to the evaluation of its green profile and environmental compatibility.
293 Furthermore, it was also evaluated using the BAGI tool [36] in order to evaluate
294 practicality of an analytical method, and it can be combined with the most common about Green
295 Chemistry.
296 The pictograms relating to the AGREEprep and BAGI tools are shown in **Figure 4**,
297 highlighting how this procedure responds to the main applicability requirements.
298
299 **Figure 4:** AGREEprep and BAGI pictogram for the herein reported method
300

301 Certainly, based on the AGREEprep pictogram, the method lends itself to many
302 possible improvements in terms of environmental impact. In fact, for its evaluation, only 1
303 sample per hour was considered (criterion 6) prepared ex situ (criterion 1) by manual procedure
304 (criterion 7) with high sample quantities (criteria 2, 4, and 5).
305 A possible improvement consists in modifying everything through an online approach
306 on (proportionately) smaller quantities and with the aid of automatic preparation systems
307 (automatic preparers).
308 The evaluation using BAGI certainly went better. In fact, with this tool its applicability
309 appears in terms of practicality to further improve and to compare the performance of different
310 methods.
311
312 ## 4. Conclusion
313 The proposed method successfully separated and simultaneously quantified all six components
314 of a topical formulation used for treatment of hyperpigmentation for the first time. The analysis
315 procedure was relatively simple and fast, even if its transferability could be challenging due to
316 the gradient elution mode. Various validation parameters were calculated and found to meet
317 the specified ICH standards allowing the application of the herein proposed method to clinical
318 and pharmaceutical fields. The proposed method, assessed through AGREEprep and BAGI
319 tools, exhibits potential for environmental enhancements and practicality. It proves excellent
320 for quality control and serves as a promising foundation for further improvements, particularly
321 in terms of eco-compatibility and high throughput.
322
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327
328 **Author contributions**
329 All Authors contributed equally to Conceptualization; Investigation; Project
330 administration; Resources; Supervision; Roles/Writing - original draft; and Writing - review &
331 editing.
332
333 **Conflict of interest statement**

The authors declare no conflict of interest.

Declaration of interests

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

Data availability

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References

[1] A.C. Handel, L.D.B. Miot, H.A. Miot, Melasma: a clinical and epidemiological review, An. Bras. Dermatol. 89 (2014) 771. https://doi.org/10.1590/ABD1806-4841.20143063.

[2] O.A. Ogbechie-Godec, N. Elbuluk, Melasma: an Up-to-Date Comprehensive Review, Dermatol. Ther. (Heidelb). 7 (2017) 305–318. https://doi.org/10.1007/s13555-017-0194-1.

[3] H.M. Torok, A comprehensive review of the long-term and short-term treatment of melasma with a triple combination cream, Am. J. Clin. Dermatol. 7 (2006) 223–230. https://doi.org/10.2165/00128071-200607040-00003.

[4] P.O. Odumosu, T.O. Ekwe, Identification and spectrophometric determination of hydroquinone levels in some cosmetic creams, African J. Pharm. Pharmacol. 4 (2010) 231–234.

[5] S. Kaur, T. Kaur, G. Kaur, S. Verma, Development and validation of UV-spectrophotometric method for estimation of hydroquinone in bulk, marketed cream and preapared NLC formulation, Int. J. Appl. Pharm. 9 (2017) 102–108. https://doi.org/10.22159/ijap.2017v9i5.20467.

[6] T. Chen, S. Liou, W. Kuo, H. Wu, G. Jong, H. Wang, C. Shen, V.V. Padma, C. Huang, Y. Chang, Rapid method for the quantification of hydroquinone concentration: chemiluminescent analysis, Luminescence. 30 (2015) 947–949.

367 https://doi.org/10.1002/bio.2842.
368 [7] K. Sheliya, K. Shah, P. Kapupara, Development and validation of analytical method
369 for simultaneous estimation of mometasone furoate, hydroquinone and tretinoin in
370 topical formulation by RP-HPLC, J. Chem. Pharm. Res. 6 (2014) 934–940.
371 [8] E. Rahmayuni, H. Harmita, H. Suryadi, Development and validation method for
372 simultaneous analysis of retinoic acid, hydroquinone and corticosteroid in cream
373 formula by high-performance liquid chromatography, J. Appl. Pharm. Sci. 8 (2018)
374 87–92. https://doi.org/10.7324/JAPS.2018.8913.
375 [9] F. Ibrahim, M.K. Sharaf El-Din, A.K. El-Deen, K. Shimizu, A New HPLC-DAD
376 Method for the Concurrent Determination of Hydroquinone, Hydrocortisone Acetate
377 and Tretinoin in Different Pharmaceuticals for Melasma Treatment, J. Chromatogr.
378 Sci. 57 (2019) 495–501. https://doi.org/10.1093/chromsci/bmz020.
379 [10] H. Barange, Development of analytical method for simultaneous estimation of
380 hydroquinone and monobenzone in topical formulation by RP-HPLC, World J. Pharm.
381 Res. 6 (2017) 742–753. https://doi.org/10.20959/wjpr20178-9115.
382 [11] B.P. Maggadani, Y. Harahap, H.L.N. Hutabalian, Simultaneous identification and
383 quantification of hydroquinone, tretinoin and betamethasone in cosmetic products bu
384 isocratic RP-HPLC, Int. J. Appl. Pharm. 11 (2019) 181–185.
385 [12] Z. Moldovan, D.E. Popa, I.G. David, M. Buleandra, I.A. Badea, A Derivative
386 Spectrometric Method for Hydroquinone Determination in the Presence of Kojic Acid,
387 Glycolic Acid, and Ascorbic Acid, J. Spectrosc. 2017 (2017).
388 https://doi.org/10.1155/2017/6929520.
389 [13] P. Gimeno, A.-F. Maggio, M. Bancilhon, N. Lassu, H. Gornes, C. Brenier, L.
390 Lempereur, HPLC–UV Method for the Identification and Screening of Hydroquinone,
391 Ethers of Hydroquinone and Corticosteroids Possibly Used as Skin-Whitening Agents
392 in Illicit Cosmetic Products, J. Chromatogr. Sci. 54 (2016) 343–352.
393 https://doi.org/10.1093/chromsci/bmv147.
394 [14] M. Esteki, S. Nouroozi, Z. Shahsavari, A fast and direct spectrophotometric method
395 for the simultaneous determination of methyl paraben and hydroquinone in cosmetic
396 products using successive projections algorithm, Int. J. Cosmet. Sci. 38 (2016).
397 https://doi.org/10.1111/ics.12241.
398 [15] S.S. Abbas, M.R. Elghobashy, L.I. Bebawy, R.F. Shokry, Stability-indicating
399 chromatographic determination of hydroquinone in combination with tretinoin and
400 fluocinolone acetonide in pharmaceutical formulations with a photodegradation kinetic

401 study, RSC Adv. 5 (2015) 43178–43194. https://doi.org/10.1039/c5ra07083j.
402 [16] A. Akhavan, J. Levitt, Assessing retinol stability in a hydroquinone 4%/retinol 0.3%
403 cream in the presence of antioxidants and sunscreen under simulated-use conditions: a
404 pilot study, Clin. Ther. 30 (2008) 543–547.
405 https://doi.org/10.1016/j.clinthera.2008.03.010.
406 [17] M.R. Elghobashy, L.I. Bebawy, R.F. Shokry, S.S. Abbas, Successive ratio subtraction
407 coupled with constant multiplication spectrophotometric method for determination of
408 hydroquinone in complex mixture with its degradation products, tretinoin and methyl
409 paraben, Spectrochim. Acta - Part A Mol. Biomol. Spectrosc. 157 (2016) 116–123.
410 https://doi.org/10.1016/j.saa.2015.12.019.
411 [18] Y.R. Ye, E. Bektic, R. Buchta, R. Houlden, B. Hunt, Simultaneous determination of
412 tretinoin and clindamycin phosphate and their degradation products in topical
413 formulations by reverse phase HPLC, J. Sep. Sci. 27 (2004) 71–77.
414 https://doi.org/10.1002/jssc.200301652.
415 [19] A. Zarghi, M. Jenabi, A.J. Ebrahimian, HPLC determination of the stability of
416 tretinoin in tretinoin-minoxidil solution, Pharm. Acta Helv. 73 (1998) 163–165.
417 https://doi.org/10.1016/S0031-6865(98)00014-4.
418 [20] B.M. Tashtoush, E.L. Jacobson, M.K. Jacobson, A rapid HPLC method for
419 simultaneous determination of tretinoin and isotretinoin in dermatological
420 formulations, J. Pharm. Biomed. Anal. 43 (2007) 859–864.
421 https://doi.org/10.1016/j.jpba.2006.08.027.
422 [21] R. Gatti, M.G. Gioia, V. Cavrini, Analysis and stability study of retinoids in
423 pharmaceuticals by LC with fluorescence detection, J. Pharm. Biomed. Anal. 23
424 (2000) 147–159. https://doi.org/10.1016/S0731-7085(00)00285-5.
425 [22] C.M. Teglia, M.D. Gil García, M.M. Galera, H.C. Goicoechea, Enhanced high-
426 performance liquid chromatography method for the determination of retinoic acid in
427 plasma. Development, optimization and validation, J. Chromatogr. A. 1353 (2014) 40–
428 48. https://doi.org/10.1016/j.chroma.2014.01.013.
429 [23] D.P. Sinica, C. Roy, J. Chakrabarty, Pelagia Research Library Stability indicating RP-
430 HPLC method development and validation for determination of potential degradation
431 impurities of tretinoin in tretinoin topical pharmaceutical formulation, Der Pharm. Sin.
432 4 (2013) 6–14.
433 [24] B. Disdier, H. Bun, J. Catalin, A. Durand, Simultaneous determination of all-trans-,
434 13-cis-, 9-cis-retinoic acid and their 4-oxo-metabolites in plasma by high-performance

435 liquid chromatography, J. Chromatogr. B Biomed. Appl. 683 (1996) 143–154.
436 https://doi.org/10.1016/0378-4347(96)00112-0.
437 [25] C. Lanvers, G. Hempel, G. Blaschke, J. Boos, Simultaneous determination of all-trans-
438 , 13-cis- and 9-cis-retinoic acid, their 4-oxo metabolites and all-trans-retinol in human
439 plasma by high-performance liquid chromatography, J. Chromatogr. B Biomed. Appl.
440 685 (1996) 233–240. https://doi.org/10.1016/S0378-4347(96)00192-2.
441 [26] B. Maggadani, H. Harmita, Y. Harahap, H. Hutabalian, Simultaneous identification
442 and quantification of hydroquinone, tretinoin and betamethasone in cosmetic products
443 by isocratic reversed phase high performance liquid chromatography, Int. J. Appl.
444 Pharm. 11 (2019) 181–185. https://doi.org/10.22159/ijap.2019v11i3.32297.
445 [27] B. Desmedt, V. Rogiers, P. Courselle, J.O. De Beer, K. De Paepe, E. Deconinck, J.O.
446 De Beer, K. De Paepe, E. Deconinck, Development and validation of a fast
447 chromatographic method for screening and quantification of legal and illegal skin
448 whitening agents, J. Pharm. Biomed. Anal. 83 (2013) 82–88.
449 https://doi.org/10.1016/j.jpba.2013.04.020.
450 [28] D. Pokar, A.K. Sahu, P. Sengupta, LC-Q-TOF-MS driven identification of potential
451 degradation impurities of venetoclax, mechanistic explanation on degradation pathway
452 and establishment of a quantitative analytical assay method, J. Anal. Sci. Technol. 11
453 (2020) 54. https://doi.org/10.1186/s40543-020-00252-4.
454 [29] M. Blessy, R.D. Patel, P.N. Prajapati, Y.K. Agrawal, Development of forced
455 degradation and stability indicating studies of drugs—A review, J. Pharm. Anal. 4
456 (2014) 159. https://doi.org/10.1016/J.JPHA.2013.09.003.
457 [30] L. Calò, L. Anzillotti, C. Maccari, R. Cecchi, R. Andreoli, Validation of a
458 Bioanalytical Method for the Determination of Synthetic and Natural Cannabinoids
459 (New Psychoactive Substances) in Oral Fluid Samples by Means of HPLC-MS/MS,
460 Front. Chem. 8 (2020). https://doi.org/10.3389/fchem.2020.00439.
461 [31] ICH Guideline, Validation of analytical procedures: text and methodology, Q2. 1
462 (2005) 5.
463 [32] R. SHILU, K. PANKAJ, A stability indicationg reverse phase high performance liquid
464 chromatography method for simultaneous estimation of allantoin, hydroquinone and
465 tretinoin in cream formulation, Int. J. Pharm. Sci. Drug Res. 14 (2020) 195–201.
466 https://doi.org/10.25004/IJPSDR.2022.140206.
467 [33] R. Shilu, P. Kapupara, Advanced HPLC method development, validation and force
468 degradation study for simultaneous analysis of allantoin, hydroquinone and tretenoin

469 in melasma topical cream formation, Indian Drugs. 59 (2022) 40–47.
470 https://doi.org/10.53879/id.59.05.13065.
471 [34] W. Wojnowski, M. Tobiszewski, F. Pena-Pereira, E. Psillakis, AGREEprep –
472 Analytical greenness metric for sample preparation, TrAC Trends Anal. Chem. 149
473 (2022) 116553. https://doi.org/10.1016/j.trac.2022.116553.
474 [35] M. Locatelli, A. Kabir, M. Perrucci, S. Ulusoy, H.I. Ulusoy, I. Ali, Green profile tools:
475 Current status and future perspectives, Adv. Sample Prep. 6 (2023) 100068.
476 https://doi.org/10.1016/j.sampre.2023.100068.
477 [36] N. Manousi, W. Wojnowski, J. Płotka-Wasylka, V. Samanidou, Blue applicability
478 grade index (BAGI) and software: a new tool for the evaluation of method practicality,
479 Green Chem. 25 (2023) 7598–7604. https://doi.org/10.1039/D3GC02347H.
480

A stability indicating RP-HPLC-UV assay method for the simultaneous determination of hydroquinone, tretinoin, hydrocortisone, butylated hydroxytoluene and parabens in pharmaceutical creams

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Abbreviations

As: asymmetry factor;
BHT: Butylated hydroxytoluene;
HCA: hydrocortisone acetate;
HQ: Hydroquinone;
SST: System suitability tests;
ICH: International Conference of Harmonization
~~K: capacity~~k': retention factor;
LOD: limits of detection;
LOQ: limits of quantitation;
MP: Methyl paraben;
N: number of theoretical plates;
PP: propyl paraben;
RP-HPLC: Reversed-phase high-performance liquid chromatography;
Rs: resolution;
S/N: signal-to-noise ratio;
t_r: retention time;
TRT**:** Tretinoin;
α: selectivity;

Abstract

Multicomponent drugs are medications that combine two or more active pharmaceutical ingredients in a single dosage form. These dosage forms improve the patient compliance, reduce the risk of drug interactions, and simplify dosing regimens. However, quality control of these multicomponent dosage forms can be challenging, especially if the final product contains four or more ingredients that are active (comprise stabilizers, preservatives, excipients, and other components). This problem can be more pronounced if the excipients can interfere with the analysis.

In this work, a stability indicating assay method was developed and validated (according to the ICH International Guidelines) for the simultaneous determination of hydroquinone (HQ), tretinoin (TRT), hydrocortisone (HCA), butylated hydroxytoluene (BHT), methyl paraben (MP) and propyl paraben (PP) in commercially available pharmaceutical creams. The proposed method is based on gradient elution using X-Bridge C18 (150 × 4.6 mm, 5μm) column with a flow rate of 1 mL/min. The linear ranges (μg/mL) were 240-560 for HQ, 24-56 for MP, 132-308 for HCA, 6-14 for PP, 12-28 for BHT, 6.6-15 for TRT. During the validation process, the intra- and interday precision and trueness (evaluated as recovery) were found to be below 2.0% and between 100–102%, respectively. System suitability tests (SST) allow validating the herein proposed procedure specifically for pharmaceutical and industrial applications. SST test shows that the reported procedure fulfill with the Guidelines, allowing excellent separation of the analytes with very sensitive, accurate (precise and true) and reproducible quantitation of each analytes.

The method was successfully applied in forced degradation studies of the six analytes. Specifically, acid degradation slightly affected HCA and BHT (91% recovery), while alkaline degradation drastically reduced HCA recovery (5.5%) and moderately affected BHT (85%). Photodegradation primarily influenced TRT quantity, and oxidative degradation intensified the BHT peak (130%).

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Keywords: RP-HPLC; Hydroquinone; Parabens; Topical Formulations; AGREEprep and BAGI; Stability study.

~~**Keywords:** RP-HPLC, Method validation, Parabens, Topical Formulations, AGREEprep, BAGI, stability study~~

1. INTRODUCTION

Topical pharmaceutical formulations that contain a combination of hydroquinone (HQ), corticosteroids, and tretinoin (TRT) are used to treat melasma, a specific skin condition that affects the face, precisely the cheeks, forehead, and upper lip, and is generally characterized by abnormal pigmentation. This disease is more prevalent in women, accounting for 90% of reported cases [1,2].[1,2]. Hydroquinone (benzene-1,4-diol; HQ) is the main depigmenter used and it acts through the inhibition of the tyrosinase enzyme which prevents the conversion of DOPA to melanin. Tretinoin (all-trans retinoic acid; TRT) is used to enhance and improve the cell regeneration while hydrocortisone acetate (HCA) reduces associated UV-induced inflammation. The use of the three agents together is known as “triple combination therapy” which was shown to be more effective in treating melasma [3]. Methyl and propyl parabens (MP and PP) are often used in pharmaceutical formulations as preservatives, especially for their low toxicity and wide antibacterial and antifungal activities. Butylated hydroxytoluene (BHT) was added to the formulation to benefit from its antioxidant effects. The chemical structures for all these components are reported in **Table 1**.

Table 1: Chemical structures and properties of the investigated analytes

The assay used for HQ, as reported in the official USP, is widely reported alone [4–6][4–6], in combinations [7–14][7–14], as well as in the presence of its main degradation products [15–17]. In all these procedures the mainly used instrument configuration was high performance liquid chromatography (HPLC), although spectrophotometric [4,5,12,14,17], and chemiluminescence [6][6] were also applied. The second component, HCA, was similarly widely quantified using various techniques alone or in combinations of topical formulations. Several methods were also reported for the quantitation of TRT in dermatological preparations in the presence of other compounds [18,19], degradation products [16,18–23], or metabolites [22,24,25].

By a systematic literature survey, only few works report simultaneous separation and quantitation of all active principles of the triple combination therapy formulations (HQ, TRT, and a corticosteroid) [7–9,26,27] let alone preservatives too [15]. In addition, the study of the stability is crucial for the final commercial product before releasing in the market, so forced degradation studies must be performed. The process of forced degradation entails subjecting drug substances and products to harsher conditions than those used in accelerated conditions.

[28]. This leads to the formation of degradation products, which can be analyzed to assess the stability of the molecule. Also help in developing stability indicating methods which must demonstrate high level of specificity, while also offering a better understanding of the degradation pathways and products of the drug substance [~~28~~29].

Field Code Changed

To the best of our knowledge, the herein proposed method is the first to successfully quantify all six components simultaneously in a commercially available topical formulation. The inclusion of preservatives in the analysis is a novel aspect, as previous methods have mostly focused on the quantification of the active ingredients alone. The challenge faced during method development was to successfully separate all six components which varied so much in polarity from the polar HQ (Octanol/Water Partition Coefficient; LogP= 0.71) to highly non-polar TRT (Octanol/Water Partition Coefficient; LogP= 5.66). What was even more challenging was that some of the constituents' polarities were very close (HCA/MP and BHT/TRT). Another important challenge was the staggering difference in components' concentrations, where HQ was almost 40 times that of TRT, highlighting that this multianalytes method allows the quantification of the compounds in a wide large concentration range.

2. MATERIALS AND METHODS

2.1. Materials and solvents

All chemicals used throughout this study were of analytical grade. The solvents were HPLC grade (Fisher Scientific, NJ, USA). Global NAPI Pharmaceuticals (Cairo, Egypt) kindly gifted high purity reference material of HQ, TRT, HCA, MP, PP, BHT as well as Tritospot® cream (labeled to contain 3% HQ, 0.02% TRT, 1% HCA and 4% eusolex).

2.2. Instrument and software

The employed HPLC system was a Waters 2695 HPLC system (Waters Corp., Milford, USA) connected to VWD-3400RS UV detector set at 280 nm and WPS-3000TPLRS autosampler, using Empower v.2 software for data acquisition and processing. The HPLC column was an X-Bridge C18 (4.6 x 150 mm, 5um, Waters Corp., Milford, USA). The buffer's pH was adjusted using Hanna HI 8314 pH-meter (Hanna, Padua, Italy)

2.3. Procedures

2.3.1. Standard Solutions

The diluent used was a mixture of methanol: acetonitrile: tetrahydrofuran: phosphoric acid (ratio 50:30:20:0.2). Stock standard solution was prepared by transferring accurately: 22.0 mg TRT reference material, 40.0 mg BHT reference material, 80.0 mg MP reference material and 20.0 mg PP reference material into 100 mL dark colored volumetric flask. The powders were dissolved, and the volume completed to the mark with the diluent.

The Working standard solutions were prepared by transferring accurately 40.0 mg HQ reference material and 22.0 mg HCA reference material into 100 mL volumetric flask to be dissolved with 50 mL diluent. From the stock standard solution, 5.0 mL were added, mixed, and the volume completed to the mark with diluent. Aliquots from the working standard solution were diluted to produce the different concentrations of HQ (240-560 µg/mL), MP (24-56 µg/mL), HCA (132-308 µg/mL), PP (6-14 µg/mL), BHT (12-28 µg/mL), and TRT (6.6-15 µg/mL). 10 µL of the sample was injected and chromatographed using the specified chromatographic conditions. The peak responses of all six components were recorded simultaneously and plotted against their corresponding concentration.

2.3.2. Chromatographic conditions

Different mobile phases, gradient profiles, pH, and detection wavelengths were tried. More stable baselines were observed when phosphate buffer was employed instead of formate buffer. Better peak shapes were obtained when acetonitrile was employed as a mobile phase modifier, compared with methanol. The resolution between peaks improved by decreasing the buffer pH. Accordingly, the use of phosphate buffer (pH 2.1)-acetonitrile as a mobile phase resulted in better separation with improved peak sharpness, larger area, quicker retention time, and enhanced resolution. Using these phases, different gradient elution profiles were evaluated, starting from high polarity to lower, in order to resolve the analytes, obtain good peak symmetries, and to obtain an adequate total runtime (important in pharmaceutical industry and related to the concept of the high throughput).

For the detection wavelength, 210, 254 and 280 nm were investigated. Parabens (MP and PP) had high absorptivity at shorter wavelengths while TRT did not, which made both 210 and 254 nm not suitable for TRT quantification especially at such low concentration compared to other five constituents. The wavelength of 280 nm, on the other hand, gave satisfactory response for all six analytes. In addition, using different detection wavelengths for different phases of gradient/separation led to unacceptable noise/drifting of the baseline.

Gradient elution (**Figure 1**) (from high polarity to lower) was preferred to ensure best separation at reasonable time. Flow rate was chosen at 1 mL/min. Faster flow rates resulted

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in overlap of BHT and TRT peaks, which were very sensitive to changes in organic phase percent. Because of the proximity of their polarities, they required enough time to interact with the stationary phase in a way that resolve them completely (higher selectivity factor). Slower flow rates on the other hand were not advantageous since they led to deteriorating peak shapes and prolonged analysis time for no improvement in resolution (1 mL~~-~~/min~~$^{-1}$~~ showed resolution of 2.6 that was more than enough for a successful complete separation). The injection volume was 10 µL. The employed buffer was prepared by mixing 10 mL glacial acetic acid and 10 mL phosphoric acid 85 % in 2 L of water. Then, 2 mL triethylamine were added, and the pH was adjusted to 2.1 by adding ammonia. Before using the mobile phase, it was filtered and degassed.

Figure 1: Mobile phase gradient composition

2.3.3. Validation

2.3.3.1. Specificity

The ability to assess unequivocally the analyte in the presence of components which might be expected to be present by determining and calculating system suitability parameters including retention time (t_r), ~~capacity~~retention factor (~~K~~k’), selectivity (α), resolution (Rs), and asymmetry factor (As~~), and number of theoretical plates (N~~).

2.3.3.2. Linearity and range

Several concentrations were prepared and used to create a calibration curve for each component. Each concentration was injected three times and the area under the curve is plotted against the concentration. After that, the regression equation was calculated for each ingredient.

2.3.3.3. Trueness

The mean percentage recovery of three replicates for three different concentration levels within the linear range (nine measurements) were calculated for all six analytes.

2.3.3.4. Precision repeatability

The relative standard deviation was calculated for three different concentrations of all ingredients by injecting each concentration three times. On the same day under the same experimental conditions.

2.3.3.5. Intermediate precision

173 The RSD was calculated using the same procedure of mentioned in Precision repeatability
174 except for that injections are done on three different days.
175
176 2.3.3.6. Limit of quantitation and limit of detection
177 The limit of detection (LOD) and limit of quantification (LOQ) were validated by
178 means of signal-to-noise ratio (S/N) equal to 3 and 10, respectively. [30,31]. Additionally, the
179 LOQs were validated considering also the back--calculated concentrations and by evaluating
180 their BIAS% with respect to the theoretical concentration.
181
182 ***2.3.4. Forced degradation***
183 Forced degradation are carried out to achieve to generate a degradation profile that is
184 comparable to what would be seen under normal stability study.
185
186 2.3.4.1. Acid degradation
187 Samples of the working standard solution were treated with 1 M HCl at 60°C for 1h.
188 During that time, every 20 min a 10.0 mL aliquot was pipetted out of the mix, neutralized and
189 assayed using the proposed HPLC method.
190
191 2.3.4.2. Base degradation
192 The stock standard and materials mixture were dissolved in 5 M NaOH and heating the
193 solution to 60°C for 1 h. Every 20 min, 10 mL of the mixture was taken, neutralized with 5M
194 HCl, and analyzed by the proposed HPLC method.
195
196 2.3.4.3. Oxidative degradation
197 The oxidative degradation was investigated by treating the standard solution of raw
198 material with 3% H_2O_2 and heating the solution for 1 h at 60°C. The area under curve of the
199 peaks was monitored using the same chromatographic conditions.
200
201 2.3.4.4. Photodegradation
202 Was studied by applying the radiation of a UV lamp to the working standard solution
203 in a quartz cell for 24 h and similarly taking samples every 20 min to be assayed.
204
205 ***2.3.5. Application to pharmaceutical preparation***

Accurately weighed 2.0 gm of test sample (Tritospot cream) were transferred to an amber conical flask. 70 mL diluent were then added, shaken by mechanical means for 15 minutes, and Sonicated for 30 minutes at 40° C. The contents were left to cool down to room temperature before they were filtered into 100 mL volumetric flask where volume was completed to the mark with diluent.

3. RESULTS AND DISCUSSION

RP-HPLC was an obvious answer to the challenges imposed by this pharmaceutical preparation because of its high separation power, versatility, sensitivity and reproducibility. The wide range of polarities and concentrations encompassed together with the proximity of the polarities of (HCA & PP) and (BHT & TRT) meant that separation conditions had to be carefully tuned to achieve best separation at reasonable time with acceptable sensitivity and reproducibility.

3.1. Method Validation

The developed method was validated in accordance with the ICH guidelines ~~[29].~~[31]. The results of the validation study showed that the method is accurate and precise, as summarized in **Table 2**.

Table 2: Characteristic parameters of the calibration equations for the proposed HPLC method.

3.1.1. Specificity

Various system suitability parameters were calculated to ensure its specificity and complete separation of all six components. They were summed up in **Table 3**. Resolution of all peaks was greater than 1.5, which indicated complete separation. The peaks symmetry was within acceptable range. Understandably, HQ peak symmetry factor was the largest because of its very high concentration compared to other components.

Table 3: System suitability parameters for the proposed HPLC method for simultaneous determination of Tritospot components.

3.1.2. Linearity and range

To test the linearity of the method, six different concentrations were utilized. The correlation coefficients obtained for all six components were >0.9992 as shown in **Table 2.**

3.1.3. Precision and trueness

The mean percentage recovery used to evaluate the trueness of the herein proposed method were calculated for all six analytes and summed in **Table 4**. All these values were between 100 – 102% for all analytes except PP (104%) which was still acceptable for routine quality control work (admitted values must be within ±15%). The relative standard deviation percentages (RSD%) for replicate injections (intra and inter-day) was always below 2.0%. These results in terms of precision and trueness (both intra- and interday) were very useful for quality control requirements, which require such precision and trueness to release products to the market with confidence in their test results.

Table 4: Intraday and interday precision (RSD%) and trueness (Mean % found).

3.1.4. Limit of detection and limit of quantitation

The signal-to-noise ratio (S/N) was employed to establish the limits of detection (LOD) and limits of quantitation (LOQ) in the chromatographic techniques, coupled also with the evaluation of the BIAS% at these concentration levels. The LOD values were 26.813 µg/mL, 2.577 µg/mL, 0.638 µg/mL, 14.395 µg/mL, 1.10 µg/mL, and 0.867 µg/mL for HQ, MP, PP, HCA, BHT, and TRT, respectively. LODs were very suited to the method’s use for pharmaceutical preparation analysis.

3.2. Forced degradation

The focus was on all components using the same proposed chromatographic conditions and following the changes in peaks response and shape. Acid degradation **(Figure 2A)** slightly affected HCA and BHT (mean percent recovery 91%), while alkaline degradation **(Figure 2B)** showed massive decrease in percent recovery of HCA (5.5%) and too much lesser extent BHT (85%). Oxidative degradation **(Figure 2C)** intensified the BHT peak (130%), while photodegradation **(Figure 2D)**, as expected, mainly affected TRT.

Figure 2: Chromatogram of photodegradation of all six components of Tritospot® cream (A) Acid degradation (B) Alkaline degradation (C) Oxidative degradation (D) Photodegradation

3.3. Application to pharmaceutical preparation

The proposed method was used to analyze the commercial product Tritospot®. Mean percent recoveries for all studied six components were reasonable and RSD of six replicates was less than 2% for all studied analytes indicating the applicability of the proposed method to pharmaceutical dosage form analysis with satisfactory trueness and precision **Figure 3**.

Figure 3: Chromatogram of all six components of Tritospot® cream in a real sample analysis

3.4. Comparison with other reported methods

The simultaneous separation of six analytes in one HPLC run is quite challenging. A few reports have been found for the concurrent analysis of HQ, HCA, and TRT [9,27]. Other methods could determine two of the active ingredients ~~[7,8,26,30,31], as shown in **Table 5**.~~[7,8,26,32,33], as shown in **Table 5**. To the best of our knowledge, no analytical method has been reported yet for the simultaneous analysis of these six analytes. Determination of multiple analytes using a single method in a single run is congruent with the current trend of developing analytical methods that save time and reduce organic solvents consumption, without sacrificing the method reliability. The developed method is more suitable for the routine chemical analysis of these compounds in dosage forms.

Table 5: A comparison between our proposed method and previous published works~~**Table 5**: Comparison with literature~~

3.5. AGREEprep and BAGI evaluation

The method proposed and validated here was evaluated using the AGREEprep tool [~~32,33~~34,35] relating to the evaluation of its green profile and environmental compatibility.

~~Furthermore, it was also evaluated using the BAGI tool [34] in order to evaluate practicality of an analytical method, and it can be combined with the most common about Green Chemistry.~~

Furthermore, it was also evaluated using the BAGI tool [36] in order to evaluate practicality of an analytical method, and it can be combined with the most common about Green Chemistry.

The pictograms relating to the AGREEprep and BAGI tools are shown in **Figure 4**, highlighting how this procedure responds to the main applicability requirements.

Figure 4: AGREEprep and BAGI pictogram for the herein reported method

Certainly, based on the AGREEprep pictogram, the method lends itself to many possible improvements in terms of environmental impact. In fact, for its evaluation, only 1 sample per hour was considered (criterion 6) prepared ex situ (criterion 1) by manual procedure (criterion 7) with high sample quantities (criteria 2, 4, and 5).

A possible improvement consists in modifying everything through an online approach on (proportionately) smaller quantities and with the aid of automatic preparation systems (automatic preparers).

The evaluation using BAGI certainly went better. In fact, with this tool its applicability appears in terms of practicality to further improve and to compare the performance of different methods.

4. Conclusion

The proposed method successfully separated and simultaneously quantified all six components of a topical formulation used for treatment of hyperpigmentation for the first time. The analysis procedure was relatively simple and fast, even if its transferability could be ~~not easily~~challenging due to the gradient elution mode. Various validation parameters were calculated and found to meet the specified ICH standards allowing the application of the herein proposed method to clinical and pharmaceutical fields. The proposed method~~ is~~, assessed through AGREEprep and BAGI tools, exhibits potential for environmental enhancements and practicality. It proves excellent for quality control ~~purposes~~ and ~~could be~~serves as a ~~valid starting point to~~promising foundation for further improvements, ~~especially~~particularly in terms of eco~~-~~compatibility and high throughput.

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Author contributions

All Authors contributed equally to Conceptualization; Investigation; Project administration; Resources; Supervision; Roles/Writing - original draft; and Writing - review & editing.

Conflict of interest statement

The authors declare no conflict of interest.

Declaration of interests

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

Data availability

Data will be made available on request.

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References

[1] A.C. Handel, L.D.B. Miot, H.A. Miot, Melasma: a clinical and epidemiological review, An. Bras. Dermatol. 89 (2014) 771. https://doi.org/10.1590/ABD1806-4841.20143063.

[2] O.A.~~O.~~ Ogbechie-Godec, N. Elbuluk, Melasma~~:~~: an Up-to-Date Comprehensive Review, Dermatol. Ther. (Heidelb). 7 (2017) 305–318. https://doi.org/10.1007/s13555-017-0194-1.

[3] H.M. Torok, A comprehensive review of the long-term and short-term treatment of melasma with a triple combination cream, Am. J. Clin. Dermatol. 7 (2006) 223–230. https://doi.org/10.2165/00128071-200607040-00003.

367 [4] P.O. Odumosu, T.O. Ekwe, Identification and spectrophotometric determination of
368 hydroquinone levels in some cosmetic creams, African J. Pharm. Pharmacol. 4 (2010)
369 231–234.
370 [5] S. Kaur, T. Kaur, G. Kaur, S. Verma, Development and validation of UV-
371 spectrophotometric method for estimation of hydroquinone in bulk, marketed cream
372 and preapared NLC formulation, Int. J. Appl. Pharm. 9 (2017) 102–108.
373 https://doi.org/10.22159/ijap.2017v9i5.20467.
374 [6] T.S. Chen, S.Y. Liou, W.W. Kuo, H.C. Wu, G.P. Jong, H.F. Wang, C.Y. Shen, V.V.
375 Padma, C.Y. Huang, Y.L. Chang, Rapid method for the quantification of hydroquinone
376 concentration: Chemiluminescentchemiluminescent analysis, Luminescence. 30
377 (2015).) 947–949. https://doi.org/10.1002/bio.2842.
378 [7] K. Sheliya, K. Shah, P. Kapupara, Development and validation of analytical method
379 for simultaneous estimation of mometasone furoate, hydroquinone and tretinoin in
380 topical formulation by RP-HPLC, J. Chem. Pharm. Res. 6 (2014) 934–940.
381 [8] E. Rahmayuni, H. Harmita, H. Suryadi, Development and validation method for
382 simultaneous analysis of retinoic acid, hydroquinone and corticosteroid in cream
383 formula by high-performance liquid chromatography, J. Appl. Pharm. Sci. 8 (2018)
384 87–92. https://doi.org/10.7324/JAPS.2018.8913.
385 [9] F. Ibrahim, M.K. Sharaf El-Din, A.K. El-Deen, K. Shimizu, A New HPLC-DAD
386 Method for the Concurrent Determination of Hydroquinone, Hydrocortisone Acetate
387 and Tretinoin in Different Pharmaceuticals for Melasma Treatment, J. Chromatogr.
388 Sci. 57 (2019) 495–501. https://doi.org/10.1093/chromsci/bmz020.
389 [10] H. Barange, Development of analytical method for simultaneous estimation of
390 hydroquinone and monobenzone in topical formulation by RP-HPLC, World J. Pharm.
391 Res. 6 (2017) 742–753. https://doi.org/10.20959/wjpr20178-9115.
392 [11] B.P. Maggadani, Y. Harahap, H.L.N. Hutabalian, Simultaneous identification and
393 quantification of hydroquinone, tretinoin and betamethasone in cosmetic products bu
394 isocratic RP-HPLC, Int. J. Appl. Pharm. 11 (2019) 181–185.
395 [12] Z. Moldovan, D.E. Popa, I.G. David, M. Buleandra, I.A. Badea, A Derivative
396 Spectrometric Method for Hydroquinone Determination in the Presence of Kojic Acid,
397 Glycolic Acid, and Ascorbic Acid, J. Spectrosc. 2017 (2017).
398 https://doi.org/10.1155/2017/6929520.
399 [13] P. Gimeno, A.-.-F. Maggio, M. Bancilhon, N. Lassu, H. Gornes, C. Brenier, L.
400 Lempereur, HPLC–-UV methodMethod for the identificationIdentification and

~~screening~~Screening of ~~hydroquinone, ethers~~Hydroquinone, Ethers of ~~hydroquinone~~Hydroquinone and ~~corticosteroids possibly used~~Corticosteroids Possibly Used as ~~skin-whitening agents~~Skin-Whitening Agents in ~~illicit cosmetic products~~Illicit Cosmetic Products, J. Chromatogr. Sci. 54 (2016~~).~~) 343–352. https://doi.org/10.1093/chromsci/bmv147.

[14] M. Esteki, S. Nouroozi, Z. Shahsavari, A fast and direct spectrophotometric method for the simultaneous determination of methyl paraben and hydroquinone in cosmetic products using successive projections algorithm, Int. J. Cosmet. Sci. 38 (2016). https://doi.org/10.1111/ics.12241.

[15] S.S. Abbas, M.R. Elghobashy, L.I. Bebawy, R.F. Shokry, Stability-indicating chromatographic determination of hydroquinone in combination with tretinoin and fluocinolone acetonide in pharmaceutical formulations with a photodegradation kinetic study, RSC Adv. 5 (2015) 43178–43194. https://doi.org/10.1039/c5ra07083j.

[16] A. Akhavan, J. Levitt, Assessing retinol stability in a hydroquinone 4%/retinol 0.3% cream in the presence of antioxidants and sunscreen under simulated-use conditions: a pilot study, Clin. Ther. 30 (2008) 543–547. https://doi.org/10.1016/j.clinthera.2008.03.010.

[17] M.R. Elghobashy, L.I. Bebawy, R.F. Shokry, S.S. Abbas, Successive ratio subtraction coupled with constant multiplication spectrophotometric method for determination of hydroquinone in complex mixture with its degradation products, tretinoin and methyl paraben, Spectrochim. Acta - Part A Mol. Biomol. Spectrosc. 157 (2016) 116–123. https://doi.org/10.1016/j.saa.2015.12.019.

[18] Y.R. Ye, E. Bektic, R. Buchta, R. Houlden, B. Hunt, Simultaneous determination of tretinoin and clindamycin phosphate and their degradation products in topical formulations by reverse phase HPLC, J. Sep. Sci. 27 (2004) 71–77. https://doi.org/10.1002/jssc.200301652.

[19] A. Zarghi, M. Jenabi, A.J. Ebrahimian, HPLC determination of the stability of tretinoin in tretinoin-minoxidil solution, Pharm. Acta Helv. 73 (1998) 163–165. https://doi.org/10.1016/S0031-6865(98)00014-4.

[20] B.M. Tashtoush, E.L. Jacobson, M.K. Jacobson, A rapid HPLC method for simultaneous determination of tretinoin and isotretinoin in dermatological formulations, J. Pharm. Biomed. Anal. 43 (2007) 859–864. https://doi.org/10.1016/j.jpba.2006.08.027.

[21] R. Gatti, M.G. Gioia, V. Cavrini, Analysis and stability study of retinoids in

pharmaceuticals by LC with fluorescence detection, J. Pharm. Biomed. Anal. 23 (2000) 147–159. https://doi.org/10.1016/S0731-7085(00)00285-5.

[22] C.M. Teglia, M.D. Gil García, M.M. Galera, H.C. Goicoechea, Enhanced high-performance liquid chromatography method for the determination of retinoic acid in plasma. Development, optimization and validation, J. Chromatogr. A. 1353 (2014) 40–48. https://doi.org/10.1016/j.chroma.2014.01.013.

[23] D.P. Sinica, C. Roy, J. Chakrabarty, Pelagia Research Library Stability indicating RP-HPLC method development and validation for determination of potential degradation impurities of tretinoin in tretinoin topical pharmaceutical formulation, Der Pharm. Sin. 4 (2013) 6–14.

[24] B. Disdier, H. Bun, J. Catalin, A. Durand, Simultaneous determination of all-trans-, 13-cis-, 9-cis-retinoic acid and their 4-oxo-metabolites in plasma by high-performance liquid chromatography, J. Chromatogr. B Biomed. Appl. 683 (1996) 143–154. https://doi.org/10.1016/0378-4347(96)00112-0.

[25] C. Lanvers, G. Hempel, G. Blaschke, J. Boos, Simultaneous determination of all-trans-, 13-cis- and 9-cis-retinoic acid, their 4-oxo metabolites and all-trans-retinol in human plasma by high-performance liquid chromatography, J. Chromatogr. B Biomed. Appl. 685 (1996) 233–240. https://doi.org/10.1016/S0378-4347(96)00192-2.

[26] B. Maggadani, H. Harmita, Y. Harahap, H. Hutabalian, Simultaneous identification and quantification of hydroquinone, tretinoin and betamethasone in cosmetic products by isocratic reversed phase high performance liquid chromatography, Int. J. Appl. Pharm. 11 (2019) 181–185. https://doi.org/10.22159/ijap.2019v11i3.32297.

[27] B. Desmedt, V. Rogiers, P. Courselle, J.O. De Beer, K. De Paepe, E. Deconinck, J.O. De Beer, K. De Paepe, E. Deconinck, Development and validation of a fast chromatographic method for screening and quantification of legal and illegal skin whitening agents, J. Pharm. Biomed. Anal. 83 (2013) 82–88. https://doi.org/10.1016/j.jpba.2013.04.020.

[28] ~~M. Blessy, R.~~D. ~~Patel~~Pokar, A.K. Sahu, P.~~N. Prajapati, Y.K. Agrawal, Development~~ Sengupta, LC-Q-TOF-MS driven identification of ~~forced~~potential degradation impurities of venetoclax, mechanistic explanation on degradation pathway and ~~stability indicating studies of drugs—A review~~establishment of a quantitative analytical assay method, J. ~~Pharm.~~ Anal. ~~4 (2014) 159.~~Sci. Technol. 11 (2020) 54. https://doi.org/10.~~1016/J.JPHA.2013.09.003~~1186/s40543-020-00252-4.

[29] ~~1.~~M. Blessy, R.D. Patel, P.N. Prajapati, Y.K. Agrawal, Development of forced

degradation and stability indicating studies of drugs—A review, J. Pharm. Anal. 4 (2014) 159. https://doi.org/10.1016/J.JPHA.2013.09.003.

[30] L. Calò, L. Anzillotti, C.H.H.T. Maccari, R. Cecchi, R. Andreoli, Validation of a Bioanalytical Method for the Determination of Synthetic and Natural Cannabinoids (New Psychoactive Substances) in Oral Fluid Samples by Means of HPLC-MS/MS, Front. Chem. 8 (2020). https://doi.org/10.3389/fchem.2020.00439.

[31] ICH Guideline, Validation of analytical procedures: text and methodology, Q2. 1 (2005) 5.

[3032]R. ShiluSHILU, K. Pankaj, aPANKAJ, A stability indicatingindicationg reverse phase high performance liquid chromatography method for simultaneous estimation of allantoin, hydroquinone and tretenointretinoin in cream formulation.,, Int. J. Pharm. Sci. Drug Res. 14 (2020) 195–201. https://doi.org/10.25004/IJPSDR.2022.140206.

[3133]R. Shilu, P. Kapupara, Advanced HPLC method development, validation and force degradation study for simultaneous analysis of allantoin, hydroquinone and tretenoin in melasma topical cream formation, Indian Drugs. 59 (2022) 40–47. https://doi.org/10.53879/id.59.05.13065.

[3234]W. Wojnowski, M. Tobiszewski, F. Pena-Pereira, E. Psillakis, AGREEprep – Analytical greenness metric for sample preparation, TrAC Trends Anal. Chem. 149 (2022) 116553. https://doi.org/10.1016/j.trac.2022.116553.

[3335]M. Locatelli, A. Kabir, M. Perrucci, S. Ulusoy, H.I. Ulusoy, I. Ali, Green profile tools: Current status and future perspectives, Adv. Sample Prep. 6 (2023) 100068. https://doi.org/10.1016/j.sampre.2023.100068.

[3436]N. Manousi, W. Wojnowski, J. Płotka-Wasylka, V. Samanidou, Blue applicability grade index (BAGI) and software: a new tool for the evaluation of method practicality, Green Chem. 25 (2023) 7598–7604. https://doi.org/10.1039/D3GC02347H.

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