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Simultaneous detection of hazardous skin whitening agents in Indian cosmetic products using a green chromatographic technique

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ABSTRACT

The present work mainly highlights the simultaneous detection of four skin whitening agents i.e. hydroquinone (HQ), resorcinol (RS), catechol (CC) and 3,3′-dichlorobenzidine (DCB) in facial creams and body lotion. Among these, the first three are positional isomers of dihydroxybenzene so simultaneous separation is difficult with the conventional reverse-phase high-performance liquid chromatographic technique (RP-HPLC). The selected skin whitening agents were detected in facial cream and body lotion using micellar liquid chromatography coupled to a photodiode array detector (MLC-PDA). In the present study, optimization of the method was accomplished using response surface methodology (RSM) with central composite design (CCD). The second-order polynomial model for predicting the optimal chromatographic run time was evaluated by the analysis of variance (ANOVA) and 3D response surface plots for the interactions between three variables were constructed. Three experimental parameters which were chosen as independent variables were; surfactant concentration (SDS), the volume percentage of organic modifier (OM) and pH of the mobile phase. The second-order polynomial model for predicting the optimal chromatographic run time was evaluated by the analysis of variance (ANOVA) and 3D response surface plots for the interactions between three variables were constructed. Three experimental parameters which were chosen as independent variables were; surfactant concentration (SDS), percentage of organic modifier (OM) and pH of the mobile phase. The second-order polynomial model for predicting the optimal chromatographic run time was evaluated by the analysis of variance (ANOVA) and 3D response surface plots for the interactions between three variables were constructed. Three experimental parameters which were chosen as independent variables were; surfactant concentration (SDS), percentage of organic modifier (OM) and pH of the mobile phase. The optimized mobile phase was 0.15 M SDS and 7% 1-butanol, buffered at pH 7 with 0.01 M NaH_2PO_4. The chromatographic run time for simultaneous determination of selected analytes was 7.5 min. The correlation coefficient (r^2) values were satisfactory between 0.998–0.999 over the linear concentration range. Limits of detection (LODs) and the limits of quantification (LOQs) for the four skin whitening agents were in the range of 0.05–0.07 μg/mg and 0.11–0.14 μg/mg, respectively. Trueness (98.4–102.7%) and precision (< 4.3%) were acceptable. The developed method was fast, cost-effective, and green which could easily analyze complex matrices (facial creams, body lotion) without any pretreatment other than filtration. The results indicated that the MLC-PDA method proved to be more suitable for the simultaneous separation of selected positional isomers.

1. Introduction

Skin tone is a fascinating aspect among women of all age groups. In Asia, skin complexion is still considered one of the most important beauty features for women. In the early nineties, skin whitening agents were used only by women with a dark complexion but lately fair complexion women also use them to tone their skin color. Nowadays, the trend shows that not only women but men are also using these skin tone whitening agents [1].

In the human body, skin color is mainly controlled by melanin pigment which is produced by melanocytes. Synthetic skin whitening agents like hydroquinone (HQ), resorcinol (RS), catechol (CC) generally decrease the production of melanocytes, which in turn reduces the amount of melanin [2] leading to discoloration of the skin. These chem-

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icals are also used as antiseptic, disinfectants, pharmaceuticals and in plastics manufacturing [1]. Globally, many national and international regulations [3,4,5] exist that ensure the use of permitted skin whitening agents in fairness cream and body lotion. European Union (Directive 2013/655) regularized the use of permitted (salicylic acid, kojic acid, arbutin, corticosteroid) skin whitening agents and non-permitted (hydroquinone, resorcinol, catechol) skin whitening agents into cosmetic products. In India, there are no such strict regulations to test these toxic agents in cosmetic products apart from heavy metals. Skin whitening products (branded and non-branded) available in the Indian market either as cosmetics or pharmaceutical preparation contain permitted and non-permitted compounds as per EU guidelines [6]. All these products can be a potential source of health hazards because they enter the body by means of cutaneous and sub-cutaneous dermal effects (if used regularly) [7]. Fairness-enhancing agents also show renal and neurological complications, cataracts, glaucoma ochronosis, dermatitis, leucoderma etc. and can also harm foetal development in pregnant women [8]. Apart from these harmful effects, if exposed to continuous UV-irradiation, these skin whitening agents may lead to cancerous growth, DNA damage, gene mutation, and immune system impairment [9]. Apart from these primary compounds, 3,3′- dichlorobenzidine, which may form during the production of cosmetic products or used to impart specific color, is an aromatic amine known to cause toxicity via dermal exposure and is reported as a carcinogen by the Scientific Committee for Consumer Safety (SCCS) [10].

Indian cosmetic market has been growing at a rate of 15–20 percent more than that of USA or European markets and now it occupies the second position in the world [11]. A variety of beauty products are available in the market and among them, skin whitening products occupy around 50% of the market. In recent years duplicate or substandard cosmetic products find their way into the Indian market as well as there are no stringent legislation and easy technique to identify them [12]. Therefore, a rapid and sensitive method should be developed for simultaneous analysis of skin whitening agents and related substances.

In the present study hydroquinone, resorcinol, catechol and 3,3′-dichlorobenzidine are selected as the analyte of interest. Hydroquinone (benzene-1,4-diol) is a benzene derivative used in various cosmetic products to treat skin hyperpigmentation. Resorcinol which is 1,3 isomer of benzenediol works by helping to remove hard, scaly and roughened skin [13]. Catechol also known as 1,3 dihydroxybenzene, reduces the synthesis of the enzyme tyrosinase, which catalyzes the synthesis of melanin pigment and indirectly acts as a melanin inhibitor [14].

Different analytical techniques have been reported for the determination of skin whitening agents in cosmetic products. Techniques like HPLC coupled to different detectors- UV, PDA and fluorimetry have been used to detect HQ, RS, CC and DCB in cosmetic samples [14,15,16,17]. Separation and detection of these compounds have also been reported by using GC–MS [18,19], capillary electrophoresis [20], micellar electrokinetic capillary chromatography (MEKC) [21] and ultra-high performance liquid chromatography (UHPLC) [7]. The reported methods have good sensitivity but the key problem is the simultaneous determination of HQ, RS, CC and DCB. All these methods also require tedious sample extraction steps, which are sometimes cumbersome and often result in the loss of the analyte of interest. These steps can be minimized using micellar liquid chromatography (MLC), a modified form of reverse phase chromatography. The solubilizing ability of micelles is an important property that allows the analysis of complex matrices without extraction and facilitates direct injection of real samples [22].

No micellar liquid chromatographic (MLC) method has been reported to simultaneously identify and quantify non-permitted skin whitening agents (HQ, RS, CC and DCB) in cosmetic products like facial creams and body lotions. The main aim of the present work is to develop and optimize the MLC method that could simultaneously detect HQ, RS, CC and DCB in a single run. The MLC method may be simple, save time and provide the regulatory authorities with more flexible analytical techniques.

2. Experimental section

2.1. Materials

Analytical standards of hydroquinone (HQ) ≥ 98.0%, resorcinol (RS) ≥ 98.0%, catechol (CC) 98%, 3,3′-dichlorobenzidine (DCB) ≥ 98.0%, sodium dodecyl sulfate (SDS) ≥ 99.0%, and sodium dihydrogen phosphate (NaH_2PO_4) were procured from Sigma Aldrich (Mumbai, India). Ultrapure Type-I water (Indion LAB Q Ultra system) was used throughout the work. Organic solvents 1-propanol, 1-pentanol (Spectrochem Pvt. Ltd., Mumbai, India) and 1-butanol, methanol was purchased from Central Drug House (New Delhi, India). All reagents used here were of analytical grade and the solvents were of HPLC grade. Branded, as well as local cosmetic samples were purchased from local cosmetic suppliers.

2.2. Instrumentation, chromatographic conditions and software

A Shimadzu LC-20AD Prominence liquid chromatograph equipped with a rheodyne injector valve with a 20 μL loop and an SPD-20A PDA detector was used for MLC analysis. The chromatographic separation was carried out in isocratic mode utilizing a mixture of 0.15 M SDS and 7% 1-butanol, buffered at pH 7 with 0.01 M NaH_2PO_4. The Shimadzu C_{18} column, 250 mm × 4.6, 5 μm particle size was equilibrated with the mobile phase for 30 min. at a flow rate of 1.0 mL/min.

An analytical balance Mettler-Toledo ME204 (Pocklington, United Kingdom) was used to weigh the solid standards and cosmetic samples. Homogenization of standards and cosmetic samples was carried out using a magnetic stirrer and ultrasonic bath from PCI Analytics (Mumbai, India). Contech LAB pH meter, Model pH-103 (Mumbai, India) equipped with a combined Ag/AgCl/glass electrode was used for pH measurements. Mobile phases and cosmetic samples were filtered by using a 0.45 μm membrane filter. The MLC system was equipped with LC-solution software version 1.22 SP1 used for data acquisition. The chromatographic experiment designing and data modeling were carried out by Design Expert 12 (Stat-Ease Inc., Minneapolis, Minnesota, USA) and Microsoft Excel (version, 2010). UV spectra of the individual analytes were used to find out the optimized wavelength of maximum absorbance (λ_{max}) which was 293 nm, 272 nm, 274 nm and 282 nm for HQ, RS, CC and DCB, respectively.

2.3. Standard and sample preparation

2.3.1. Standard solutions

A stock solution containing 100 μg/mL of HQ, RS, CC and DCB were prepared in a mixture of Type-I ultrapure water/methanol (1:1 v/v) and stored at 4 °C. Working solutions were freshly prepared by diluting them in the mobile phase before injection. The standard solutions were stored in the refrigerator and were found to be stable for one month.

2.3.2. Sampling and sample preparation

Cosmetic samples including skin whitening cream and body lotion were obtained from the local market of Sagar (Madhya Pradesh, India). 100 mg precisely weighed amount of cosmetic sample was taken into a centrifuge tube (15 mL), 5 mL of solvent ultra-pure water and methanol (1:1 v/v) was added in the same. The mixture was sonicated for 10 min. and the supernatant was filtered with 0.45µm nylon membrane filter to inject directly into the chromatographic column. A similar procedure was followed to prepare sample blank.

3. Result and discussion

3.1. Selection of mobile phase variables

The separation of analytes in liquid chromatography basically depends on the physicochemical properties of analyte like pKa and logP values (Figure S1). The pKa values indicate that HQ, RS and CC were

Table 1
Central composite design (CCD) matrix and results with five levels/three factors to optimize MLC-PDA method parameters.

Dependent and independent variable for response surface methodology (RSM)- central composite design (CCD) for three-factor five levels								
Exp. No	X_1 SDS (M)		X_2 OM (%)		X_3 pH		Response value (Rt)	
							Experimental values	Predicted value
1.	−1	0.05	−1	1	−1	3	19.4	16.3
2.	1	0.15	−1	1	−1	3	10.2	12.2
3.	−1	0.05	1	7	−1	3	13.6	13.3
4.	1	0.15	1	7	−1	3	7.0	9.2
5.	−1	0.05	−1	1	1	7	12.8	11.8
6.	1	0.15	−1	1	1	7	4.1	7.8
7.	−1	0.05	1	7	1	7	6.1	8.8
8.	1	0.15	1	7	1	7	7.3	7.1
9.	−1.68	0.01	0	4	0	5	12.8	13.9
10.	1.68	0.18	0	4	0	5	9.5	7.1
11.	0	0.1	−1.68	−1.04	0	5	13	13.1
12.	0	0.1	1.68	9.04	0	5	7.5	8.0
13.	0	0.1	0	4	−1.68	1.63	12.5	14.3
14.	0	0.1	0	4	1.68	8.36	5.4	6.7
15.	0	0.1	0	4	0	5	11.5	10.5
16.	0	0.1	0	4	0	5	11.5	10.5
17.	0	0.1	0	4	0	5	11.5	10.5
18.	0	0.1	0	4	0	5	11.5	10.5
19.	0	0.1	0	4	0	5	11.5	10.5
20.	0	0.1	0	4	0	5	11.5	10.5

neutral, whereas DCB was cationic due to the protonation of the amino group (NH_2) in the selected pH range (3–7). According to logP values HQ, RS and CC are polar while DCB is less polar. Since the selected analytes have different properties, a systematic approach should be used to optimize and find out the possible interactions of the analyte at different pH. Therefore an experimental design approach was used to optimize the MLC method [23].

In MLC, the mobile phase is an aqueous micellar mobile phase with a small amount of organic solvent acting as a modifier. Surfactants generally used in MLC are SDS, Brij-35 and CTAB. Among these surfactants, SDS was selected based on reported research work [24]. In general, short-chain alcohols (1-propanol, 1-butanol, 1-pentanol) are frequently used to increase the micellar mobile phase efficiency and elution power. In the present work, HQ, RS and CC are hydrophilic in nature, wherein using 1-propanol helps to improve the peak parameters. DCB is a slightly hydrophobic compound which if eluted in pure SDS will result in long retention times and poor peak parameters. Increasing the carbon chain length of organic modifiers will result in proper retention time and peak parameters for DCB. Together with this, the retention time for HQ, RS and CC will also reduce resulting in the loss of resolution. Therefore, in this work it was decided to use a medium carbon length alcohol i.e., 1-butanol. After the selection of surfactant (SDS) and organic modifier (1-butanol) statistical model namely response surface methodology (RSM) [25] was used for optimizing the SDS concentration, volume percentage of organic modifier (1-butanol) and find out the suitable pH for best separation. Total 20 mobile phases were prepared by varying the SDS concentration in the range of 0.05–0.15 M and organic modifier in the range of 1–7% while pH was varied in the range of 3–7.

3.2. Experimental design and data analysis

3.2.1. Response surface methodology (RSM)

In general, chromatographic method optimization is usually performed by keeping the entire separation variables fixed and changing one variable at a time to understand its influence on chromatographic response value (one-factor-at-a-time strategy). As the change occurs only in one separation variable. This optimization method does not describe the interactive effects of all separation variables. This optimization approach is cumbersome, time-consuming and requires more solvents because the number of experiments performed for optimization increased. This can be minimized using a statistical experimental design, where several factors can be simultaneously studied in few assays.

The effect of independent separation variables i.e., concentration of surfactant (SDS), volume percentage of organic modifier (OM) and pH of the mobile phase on chromatographic run time (dependent variable) was studied using RSM. The method was used to select the minimum possible chromatographic run time for the selected skin whitening agents. The concentration range for SDS and volume percentage of 1-butanol was selected based on chromatographic standpoint and physical limitations. The independent separation variable under study and their limiting values in coded and decoded form (CCD design) are given in Table S1. As shown in Table S1 the experiment was started by creating a central composite design (CCD) which comprised of a total of 20 runs (Table 1) made of augmentation of a 2^3 factorial design consisting of 8 factorial runs as cubic points with 6 replication runs as center points (for calculation of pure error) and six axial runs as star points. In order to create a rotatable design α value was fixed at 1.68. The CCD response was the chromatographic run time for four selected analytes.

In order to get the regression coefficient (R^2) the experimental data was introduced to the quadratic polynomial model. The general quadratic polynomial model used in the response surface analysis was:

$$y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_{12} + \beta_{22} X_{22} + \beta_{33} X_{32} + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{123} X_1 X_2 \quad (1)$$

Where 'y' is the response value, β_0 is a constant for line intersects the y-axis, β_1, β_2 and β_3 are regression coefficients, β_{12}, β_{13}, β_{23} and β_{123} are the regression coefficients for the interaction of variables [26]. The RSM plots were designed using Design-Expert software version 12 (Stat-Ease Inc., Minneapolis, Minnesota, USA). The influence of independent variables (SDS, OM, pH) on a dependent variable (Rt) can be demonstrated by expressing the quadratic polynomial equation in the form of three-dimensional (3D) surface plots.

3.2.2. Statistical analysis and validation of the proposed model

The general multivariate optimization approach begins with establishing a regression model between independents (SDS, 1-butanol; OM, and mobile phase pH) and dependent variables (chromatographic run time; Rt). The quadratic polynomial model with different statistical values such as F-value, P-Value and regression coefficient (R^2) was utilized. As the main aim of the present study was simultaneous separation and

Table 2
Retention time, detection wavelength and calibration data obtained for HQ, RS, CC and DCB in sample matrix using MLC-PDA detector.

Analyte	Rt	λ max (nm)	Range (μg/mL)	r^2	Linear Regression	In sample matrix (μg/mL)		RSD% (n = 6)
						LODs	LOQs	
HQ	3.0	293	0.15–12	0.999	y = 0.679–0.016	0.07	0.14	3.9
RS	4.1	272	0.12–10	0.998	y = 2.253+0.212	0.07	0.11	5.2
CC	5.7	274	0.12–10	0.999	y = 2.161+0.144	0.05	0.12	2.7
DCB	7.3	282	0.12–10	0.999	y = 6.208–0.092	0.06	0.13	2.7

resolution of selected analytes in minimum time, therefore, analysis of variance (ANOVA) and some related important statistical parameters (F-value, P-value and regression coefficient) shown in Table S2 was constructed for the chromatographic run time [27]. The statistical values obtained for the quadratic polynomial model included all independent variables (SDS, OM, pH) as well as their binary interactions (SDS × OM, SDS × pH, OM × pH) and self-interactions (SDS^2, OM^2, pH^2). The obtained variables can be represented in the following equation (in terms of coded factors):

$$Rt = +11.52 - 2.09 \times SDS - 2.04 \times OM - 2.07 \times pH + 0.34 \times SDS^2 - 0.54 \times OM^2 + 4.5 \times pH^2 + 1.15\ SDS \times OM + 2.10\ SDS \times pH + 0.28\ OM \times pH \quad (2)$$

The achieved F and P-values from ANOVA analysis revealed that the model was valid and adequately explained the primary response (i.e. chromatographic run time). As per Eq. (2), out of 11 terms in the fitted model two interaction variables including OM and SDS × pH can be excluded from this model during variable selection. The reason being that the P-values lower than 0.05 were significant, whereas the values greater than 0.1 were insignificant. The bar chart of these factors tends to lie above the *t*-value (2.16). The *t*-value lower than 2.16 has a minimum influence on the chromatographic run time. Therefore the parameters having *t*-value above 2.16 were the best selection for optimization studies.

ANOVA of the quadratic polynomial model for chromatographic run time shows that the F experimental value for the model is 1.58 which means that it is significant. The CCD model is also significant and adequate with experimental conditions for all response values. One of the essential statistical parameters that can be used to validate a model is the regression coefficient of the model (R^2). Here, different regression statistical values including the adjusted correlation coefficient (R^2 adjusted) and the regression coefficient of prediction (R^2 predicted) are presented in Table S2 to show the suggested model fitness and prediction ability. Obtained experimental values (performed with the instrument) and predicted values (obtained from the model) of chromatographic run time revealed that the model is good and can optimize the MLC mobile phase for simultaneous separation and detection of selected analytes. Table 1 indicates that among all 20 runs, run no. 8 has provided a minimum chromatographic run time for studied skin whitening agents with good resolution.

3.2.3. Graphical evaluation

The effect of independent separation variables (SDS, OM, pH) on the dependent separation variable (chromatographic run time) is graphically (RSM 3D graph) presented in Fig. 1A-C. Among these three independent separation variables, one variable was kept constant and the effect of the other two separation variables on the chromatographic run time (dependent variable) was studied. Fig. 1A-C shows that minimum chromatographic run time was obtained at high SDS concentration (0.15 M), high pH (7) and high OM concentration (7%) because the slope of all curves was steeper toward their highest values. Therefore 0.15 M SDS and 7% 1-butanol, buffered at pH 7 with 0.01 M NaH_2PO_4 was selected as a mobile phase for simultaneous determination of selected skin whitening agents with a minimum chromatographic run time.

Regression analysis with R^2 values and response surface 3D graph indicated a satisfactory correlation between the independent and dependent variables. The results obtained from the ANOVA test also confirm that the model can be successfully used to predict the optimum chromatographic run time for selected analytes. Therefore, the proposed model provides an efficient, automated and robust analytical method for detecting skin whitening agents in the cream formulation and body lotion. It may also be suitable for a number of other applications and analytical method developments.

3.3. Performance of the method

The method validation has been achieved according to ICH guideline Q2 (R1) [18]. The validation parameters were: calibration and sensitivity, the limit of detection and limit of quantification, trueness, precision and robustness.

3.3.1. Selectivity

The cream base (rose water; 75 mL, glycerine; 5 mL, distilled water; 20 mL) kindly donated by the cosmetic division of Sagar Institute of Pharmaceutical Sciences, India, was used to examine the selectivity of the developed method. The cream base showed some peaks up to 2.5 min., which corresponded to other excipients of cream formulation (Fig. 2A). However, no peaks were observed in the separation window (3.0 min. to 7.5 min.) of selected analytes, ensuring the absence of interference.

In order to further confirm the matrix effect, the cream base was spiked with HQ, RS, CC and DCB (5 µg/100 mg) and chromatographed (Fig. 2B). The chromatogram obtained for the four analytes did not show any interference from the matrix.

3.3.2. Calibration and sensitivity

Calibration measurements were performed in triplicate using eight standard solutions of analytes in the range of 0.12–10 μg/mL (RS, CC and DCB) and 0.15–12 μg/mL (HQ). Slope, y-intercept and determination coefficients (r^2) were obtained by plotting the peak area versus concentration using the least-square linear regression method. A fresh standard solution was prepared for every calibration analysis and spread over a period of two months. The obtained results for calibration curves are shown in Table 2. Good linearity ($r^2 > 0.998$) was obtained for the selected analytes.

3.3.3. Limits of quantification (LOQs) and limit of detection (LODs)

Limits of detection and quantification were obtained using signal to noise (S/N) ratio and calculated using the equations: 3.3 σ/S for LODs and 10 σ/S for LOQs where sigma (σ) is the standard deviation of the y-intercept of the regression lines (standard deviation of the response) and 'S' is the slope of the calibration curve [28]. Limits of detection (LODs) and the limits of quantification (LOQs) for the four skin whitening agents were in the range of 0.05–0.07 μg/mg and 0.11–0.14 μg/mg, respectively (Table 2).

3.3.4. Precision and trueness

The intra- and inter-day trueness and precision of selected analytes were determined by analyzing them at three different concentrations using spiked blank samples. For HQ three concentrations were 0.15, 5 and 12 μg/mL and for RS, CC, DCB the concentrations were 0.12, 5 and

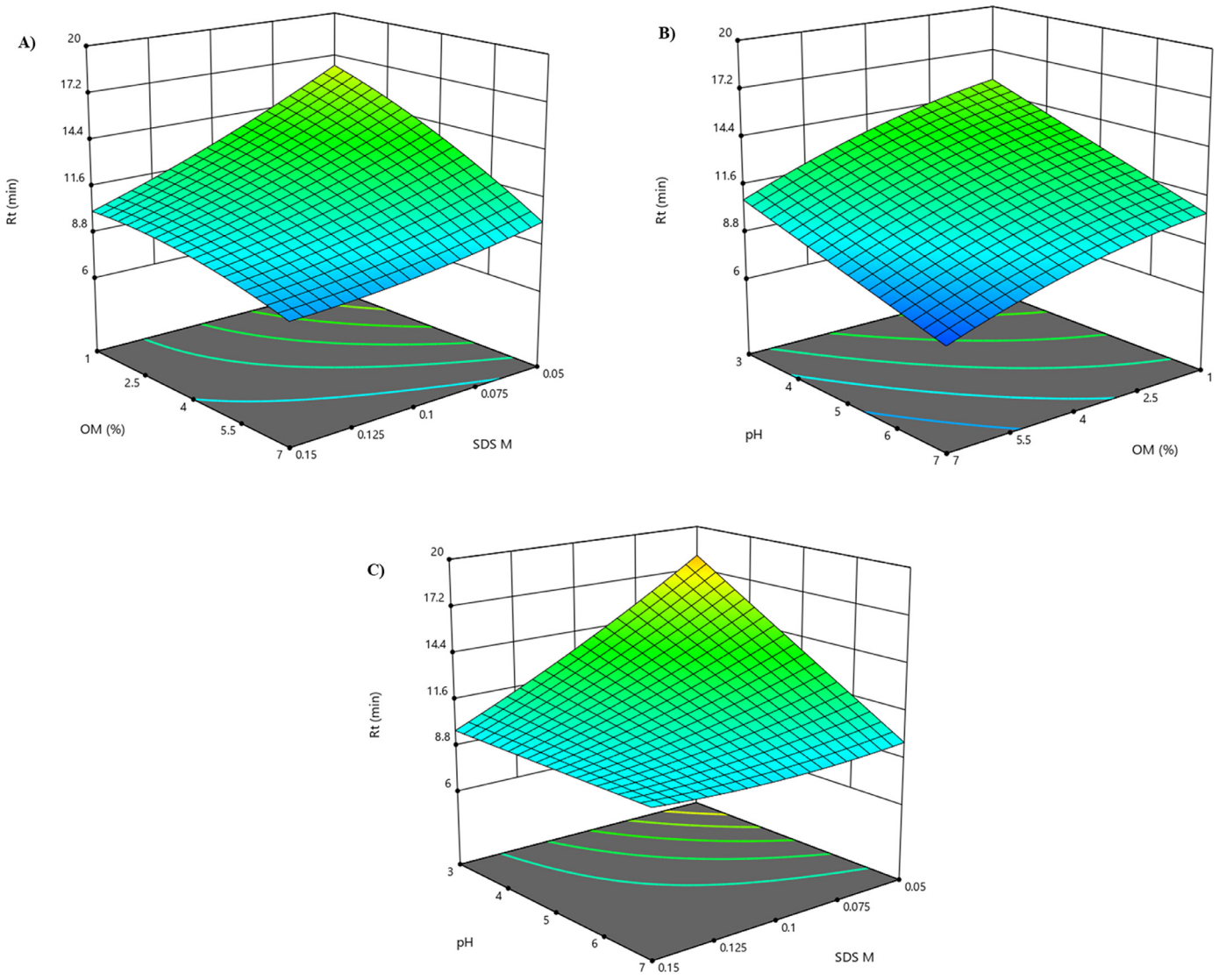

Fig. 1. Three-dimensional (3D) response surface plots showing the effects of SDS, OM, pH on chromatographic run time using MLC-PDA method.

10 μg/mL. The intra-day analysis was performed by analyzing the selected concentration 6 times on the same day consecutively, whereas inter-day analysis was calculated by taking the average of five measurements of intra-day values taken on 5 days non-consecutively over a period of three months. Trueness was the closeness between the average value of selected analyte concentration provided by the method and the true spiked value (recovery), calculated as the quotient found value/true value whereas the precision was calculated as the RSD of the found concentrations. Results have been shown in Table 3. Adequate recoveries (98.4–102.7%) and high precision (< 4.3) of the response were found which indicated the reliability of the concentration of the selected analyte in cosmetic samples obtained by a chromatographic method [18].

3.3.5. Robustness

The robustness of the method was examined by replicate injections ($n = 5$) of standard solutions containing 10 μg/mL with small changes in the chromatographic parameters (SDS concentration, volume percentage of 1-butanol, pH and flow rate). The considered ranges were as follows: SDS concentration (0.14–0.16 M), 1-butanol (6.9%–7.1%), pH (6.9–7.1) and flow rate (0.9–1.1 mL/min) in triplicate and the RSD of the measured retention times and peak areas were then calculated. The small experimental oscillations in the main chromatographic conditions that may happen during routine analysis had no significant influence on the retention time (RSD< 5.9%), efficiency (RSD < 4.7) and capacity factor (RSD < 5.0%) of HQ, RS, CC and DCB (Table 4).

3.4. Analysis of commercial samples

The use of hydroquinone and catechol in cosmetic products is not allowed by European Regulation No. 2013/655. Whereas in India, there is no regulation that monitors these selected skin whitening agents in cosmetics. Therefore cosmetic samples were collected to verify the presence of selected skin whitening compounds. Eighteen samples (branded and non-branded) were collected from the local market of Sagar (M.P.), India, which included thirteen (13) skin whitening face creams and five (05) body lotions. Some of the collected samples were without the name of ingredients printed on the label of the product. MLC-PDA analysis of the above-mentioned samples was performed without any pretreatment (other than filtration) in order to determine the presence of selected compounds. In all the skin whitening products, HQ, RS, CC, and DCB were not mentioned on the product label. Hydroquinone (HQ) was detected at a very high concentration (264.4 μg/mg) in sample no.16, a pharmaceutical cream recommended for the treatment of melasma. Sample no.16 was further diluted 25 times (10.5 μg/mg) to make it plausible (obtained values should be under calibration range and higher than

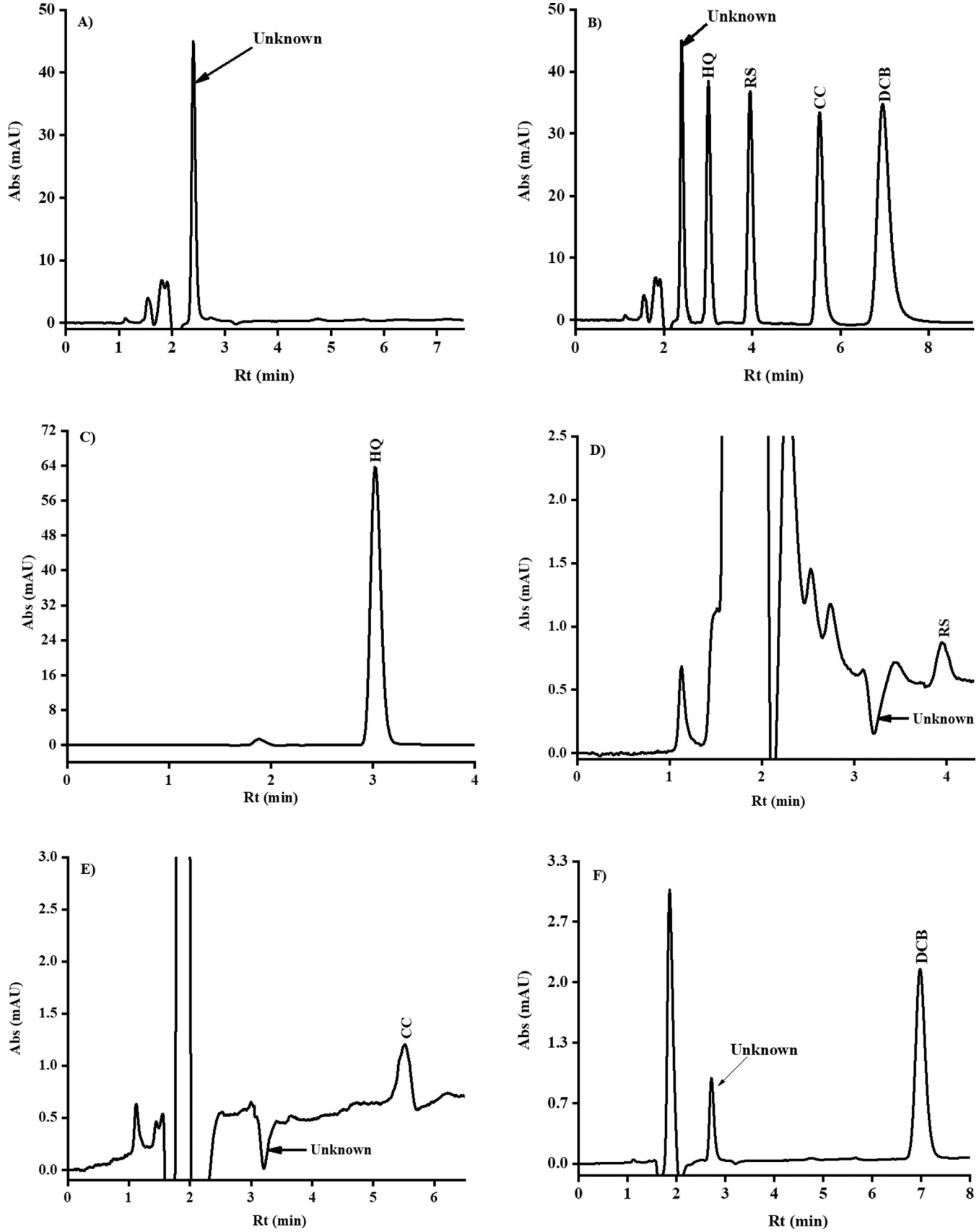

Fig. 2. Chromatograms obtained under the optimal conditions: (A) cream base (blank): (B) cream base (blank) spiked at 5 μg/100 mg (absorbance measured at 246 nm). Real obtained sample no.: 16. (C), 13. (D), 1. (E) and 8. (F), respectively.

Table 3
Intra- (n = 5) and inter-day (n = 6) trueness and precision for the selected skin whitening agents.

Analytes	Added conc. (μg/mL)	Intra-day			Inter-day		
		Found (Mean)	Trueness (%)	Precision (RSD,%)	Found (Mean)	Trueness (%)	Precision (RSD,%)
HQ	0.15	0.14	99.5	4.3	0.15	101.3	1.4
	5	4.98	98.6	3.1	5.10	100.8	1.3
	12	11.9	99.2	0.5	11.9	99.8	2.6
RS	0.12	0.11	98.4	2.8	0.12	99.8	2.5
	5	5.08	101.6	1.9	4.99	100.1	1.7
	10	9.98	99.8	1.7	10.01	99.6	3.3
CC	0.12	0.11	98.8	2.3	0.11	98.6	2.5
	5	4.98	99.6	3.6	5.01	100	3.2
	10	10.1	101.1	0.9	9.98	99.9	1.2
DCB	0.12	0.12	102.7	1.2	0.11	99.8	1.3
	5	5.08	101.7	1.3	4.99	99.2	2.1
	10	9.96	99.6	0.4	10.0	100.2	0.8

Table 4
Robustness study of the selected analytes.

Parameters	Variation	HQ			RS			CC			DCB		
		Rt	N	B/A	Rt	N	B/A	Rt	N	B/A	Rt	N	B/A
Flow Rate	**1.1**	3.4	1700	1.3	4.2	1623	1.8	5.9	1429	1.3	7.4	1653	1.5
	1	3.0	1704	1.3	4.1	1637	1.7	5.7	1428	1.4	7.5	1647	1.5
	0.9	2.8	1723	1.2	3.8	1648	1.8	5.3	1425	1.3	7.2	1648	1.4
	Mean	3.0	1709	1.2	4.0	1636	1.7	5.6	1427	1.3	7.3	1649	1.4
	RSD%	5.9	2.3	3.1	5.1	0.7	2.2	5.2	2.9	2.7	2.8	4.2	2.8
pH	**7.1**	2.9	1743	1.3	4.2	1642	1.7	5.6	1441	1.4	7.4	1635	1.4
	7	3.0	1756	1.2	4.1	1647	1.7	5.7	1436	1.4	7.5	1638	1.4
	6.9	2.7	1798	1.3	4.0	1663	1.7	5.8	1449	1.4	7.4	1642	1.5
	Mean	2.8	1765	1.2	4.1	1650	1.7	5.7	1442	1.4	7.4	1638	1.4
	RSD%	5.2	1.6	2.2	2.5	0.9	2.5	1.4	1.9	2.7	2.2	3.4	5.0
Butanol	**7.1**	2.9	1787	1.3	4.1	1645	1.8	5.9	1420	1.4	7.4	1646	1.5
	7	3.0	1707	1.3	4.1	1640	1.8	5.7	1444	1.4	7.5	1644	1.5
	6.9	3.1	1754	1.4	3.9	1644	1.8	5.6	1422	1.4	7.4	1641	1.5
	Mean	3.0	1749	1.3	4.0	1643	1.8	5.7	1428	1.4	7.4	1643	1.5
	RSD%	2.3	4.7	3.1	2.3	2.4	3.9	2.5	4.4	2.1	3.6	3.1	2.2
SDS	**0.16**	3.1	1733	1.3	4.0	1648	1.6	5.7	1421	1.4	7.6	1642	1.5
	0.15	3.0	1738	1.3	4.1	1645	1.7	5.7	1416	1.4	7.5	1647	1.5
	0.14	2.8	1747	1.3	4.3	1648	1.6	5.7	1437	1.4	7.4	1645	1.5
	Mean	2.9	1739	1.3	4.1	1647	1.6	5.7	1424	1.4	7.5	1644	1.5
	RSD%	3.6	2.4	3.7	3.2	4.5	3.8	2.8	1.7	2.6	1.1	2.1	4.3

Table 5
The mean concentration (n = 3) of selected analytes (μg/100 mg) in the skin whitening cream and body lotion analyzed in this study.

Branded					Non-branded				
Sample No.	HQ	RS	CC	DCB	Sample No.	HQ	RS	CC	DCB
1.	–	–	0.95	–	11.	–	–	0.09**	–
2.	–	–	0.91	2.1**	12.	4.1**	–	–	–
3.	–	–	0.77	–	13.	–	0.13	0.32	5.0**
4.	–	–	–	–	14.	–	–	–	1.5**
5.	–	–	–	–	15.	–	–	–	6.8
6.	1.13	–	–	–	16.	10.5*	–	0.82	–
7.	0.08**	–	0.75	1.6**	17.	–	–	–	–
8.	–	–	–	7.74**	18.	–	–	–	2.1**
9.	–	–	–	–					
10.	–	–	0.91	7.9					

*samples were diluted 25 times.
** Samples were diluted 15 times.
blank fields (-) indicates that selected analytes were not detected in the sample or the content of the analytes was below the LOD of the proposed method.

LOQ) (Fig. 2C). The use of this cream is quite common among women with the problem of dark patches on the face. In the beginning, it is generally prescribed by a physician but later on due to the effectiveness of the cream, the person continues its use even without the prescription of a physician.

In all the samples analyzed resorcinol (RS) was detected only in one product with a concentration of 0.13 μg/mg (Fig. 2D). Though 0.5% concentration of RS is permitted in body lotion and pharmaceutical preparations but its safety is being questioned by the Scientific Committee on Consumer Safety (SCCS) [29]. The most commonly found whitening agent was 3,3′-dichlorobenzidine (DCB) detected in eight products having a concentration in the range of 6.8–116.1 μg/mg (Fig. 2F is 15 times diluted) and catechol (CC) was detected in seven products (Fig. 2E). Samples, which were detected out of the calibration range

were further diluted 15 times (except for sample no.16; diluted 25 times) to make them plausible. The concentration of the skin whitening agents understudy for all positive samples has been summarized in Table 5. Out of eighteen samples, fourteen had at least one of the banned analytes which have been selected for the present study.

4. Conclusion

The present study describes the application of central composite design (CCD) and response surface methodology (RSM) to optimize the micellar liquid chromatographic parameters. The optimized parameters were used to detect and separate selected analytes in minimum chromatographic run time. The proposed design provides a better understanding of possible interactions between independent (SDS, OM, pH) and dependent variables (chromatographic run time). The optimum values of selected independent variables are SDS concentration; 0.15 M, organic modifier concentration; 7% and pH of the mobile phase; 7 which provided a chromatographic run time of 7.5 min. for the simultaneous determination of selected analytes. As well as the experimental and predicted values for all variables were compared and the predicted values from the empirical model matched well with the observed ones within experimental values. Regression analysis with R^2 values and ANOVA test results also indicated an adequate correlation between both variables. Thus, the proposed models provide an efficient, automated and robust method for simultaneously determining selected skin whitening agents in skin whitening cream and body lotion.

The present chromatographic study shows the detection and quantification of selected four skin whitening agents in cosmetic and pharmaceutical preparation. In many of the samples, the detected skin whitening agents were not even mentioned and some of them had a higher concentration range than the amount printed on the wrapper. As already mentioned, although 3,3′-dichlorobenzidine (DCB) is a known carcinogen and its production is discontinued, it was still found in most selected samples (branded and non-branded) in a very high concentration. Due to unawareness among the consumers and lack of strict regulations, the manufacturers inherently add such compounds which may induce serious health issues. The fact can be justified by the increase in the number of cancer patients around the world. Thus it is time to make necessary changes in the legislation so that the use of non permitted skin whitening agents may be reduced.

Authorship contribution statement

Conceptualization, A.D. and D.B., S.C.B; Methodology, A.D. and D.B; Software, D.B.; Validation and Formal Analysis, P.P., and J.P.V.; Investigation, P.P., D.B. and A.D.; Resources, A.D. and D.B.; Data curation, A.D., P.P. and D.B.; Writing - original draft, D.B. and A.D.; Writing - review & editing, A.D. and D.B.; Project administration and Funding Acquisition, S.C.B., A.D. and D.B.

Declaration of Competing Interest

All authors of the present manuscript declare that they have no competing financial interests that could influence the work reported in this paper.

CRediT authorship contribution statement

Priyanka Pahade: Validation, Formal analysis, Investigation, Data curation. **Devasish Bose:** Conceptualization, Methodology, Software, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition. **Juan Peris-Vicente:** Validation, Formal analysis. **Samuel Carda-Broch:** Conceptualization, Project administration, Funding acquisition. **Abhilasha Durgbanshi:** Conceptualization, Methodology, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcoa.2021.100010.

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