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# Design of Experiments Based Validated HPTLC Method for Quantification of Oxybenzone and Avobenzone in Personal Care Products

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**ABSTRACT**
A rapid, simple and economic High Performance Thin Layer Chromatography (HPTLC) method for the simultaneous quantification of Oxybenzone and Avobenzone in various personal care products available in local markets in and around Chennai is developed and validated. Central Composite design (CCD) with three factors and one response was employed to study the robustness of the developed method. The best chromatographic separation was achieved employing Toluene: Acetonitrile: Ethyl acetate (4: 5: 1 v/v/v) as solvent system. Densitometric analysis was carried out at 332 nm. The developed method is examined for validation parameters like linearity, specificity, precision, accuracy and robustness. The method was found linear over a wide range of 2.5 – 200 ng/band with a regression coefficient of 0.9998 and 0.9991 for Oxybenzone and Avobenzone respectively. All the validation parameters were found to be within the acceptance limit. The proposed and developed HPTLC method can be applied for routine analysis of Oxybenzone and Avobenzone, in the presence of other excipients in cosmetic products and can also be extended to analysis in pharmaceuticals and food products.
**Keywords:** central composite design, oxybenzone, avobenzone, personal care products

## INTRODUCTION

Personal care products are those, which are used by most of the people to give a pleasant look and appearance to face or body and they have become the need of society. The importance of these products has gained more interest among people, as they wanted to stay young and attractive.

The general properties of Oxybenzone and Avobenzone are listed in **Table 1**.


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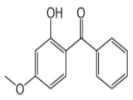
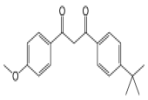
**Table 1.** General properties of Oxybenzone [1] and Avobenzone [2]

| Ingredient name | IUPAC name | Molecular Structure | Molecular Formula | Molecular weight | Appearance | $\lambda_{max}$ | Maximum allowable limit in cosmetic product |
|---|---|---|---|---|---|---|---|
| **Oxybenzone** | (2-Hydroxy-4-methoxyphenyl)-phenylmethanone |  | $C_{14}H_{12}O_3$ | 228.25 g /mol | White powder | 288 nm | 10 %w/w |
| **Avobenzone** | 1-(4-Methoxyphenyl)-3-(4-*tert*-butylphenyl)propane-1,3-dione |  | $C_{20}H_{22}O_3$ | 310.39 g/mol | Colorless crystals | 358 nm | 5 %w/w |

There are many ingredients loading in the personal care products. Oxybenzone and Avobenzone are one among them, which are used as product stabilizers and UV protectants in many of the sunscreen and skin care products [3]. They act by absorbing UV radiation powered by the sun before it gets the opportunity to damage the skin. But recent study shreds of evidence that Avobenzone, which when exposed to sunlight, releases free radicals thereby causes cancer and Oxybenzone are reported more toxic compared to Avobenzone and both are found to have issues related to infertility and birth defects [4, 5]. On the other hand, none of the personal care products contains a proper label claim of these ingredients in their cover; hence, the need for quantifying these harmful ingredients in personal care products has been put forth.

HPTLC, now-a-days is employed widely for the quantification of drugs because of its low maintenance cost, less analysis time, minimal consumption of mobile phase per sample, need for simple and easy sample clean-up procedures. Added to the above, it facilitates automated application of sample and scanning of the plate. Moreover, it is flexible enough to analyze different kinds of samples [6].

The design of experiments (DoE) is based on the principles of use of experimental design. Experimental design procedures are very useful in pharmaceutical development including formulation development, analytical method optimization and validation. They are more efficient than the traditional one-variable-at-a-time approach [7, 8].

The literatures collected reveals that there are very few analytical methods reported for the estimation of Oxybenzone and Avobenzone alone and together with other drugs or excipients by HPLC, UPLC, Capillary electrophoresis and GC-MS in cosmetics, pharmaceuticals and environmental samples [9-20].

The current research article focuses on determination of robustness of the developed HPTLC method employing CCD. CCD was constructed by employing three chromatographic

parameters such as; solvent phase composition regarding organic phase content, developing distance and band size by optimized experimental domain were selected and varied within a real range and their quantitative effect on response variable, that is, retention factor was determined.

Hence, a modest, rapid, accurate, precise, sensitive, robust and more economical HPTLC method, with simple sample preparation technique has been developed for the simultaneous estimation and quantification of Oxybenzone and Avobenzone in various personal care products, with the aid of CCD design for robustness testing.

## EXPERIMENTAL

### Chemicals and reagents employed

The reference standard of Oxybenzone and Avobenzone were purchased from Sigma-Aldrich (India). All other reagents used in the study were procured from Merck laboratories and S.D. fine chemicals Ltd. Double distilled water is utilized throughout the process of analysis. All the cosmetic products analyzed were purchased from local markets of Chennai.

### Instruments used

The HPTLC instrument used consisted of the following components: Camag HPTLC Sample Applicator – Linomat 5, Twin trough Chamber, Camag HPTLC Scanner and Camag HPTLC Document photo. The other apparatus employed for the study includes - Digital balance Sartorius, Ultra Sonic bath sonicator, Centrifuge, Vortexer. Throughout the analysis, all the dilutions were performed employing Class "A" grade glassware's only. WINCATS 5 (Software for handling HPTLC instrument) was employed. Experimental design, data analysis and desirability function calculations were performed by using Design-Expert® trial version 7.1.6 (Stat-Ease Inc., Minneapolis), MS- Excel 2010.

### Solubility studies

Solubility studies of Oxybenzone and Avobenzone were carried out separately in various solvents like water, 0.1 N NaOH, 0.1 N HCl, methanol, ethanol, ether and acetone. From the above solvents, both Oxybenzone and Avobenzone were found to be freely soluble in Acetonitrile. Hence, acetonitrile was selected for the entire process of solubilization.

### Preparation of stock standard solutions

The stock standard solutions of Oxybenzone and Avobenzone were prepared separately by accurately weighing 10 mg of each and they were kept in a 10 ml standard flask. Half the volume of acetonitrile was added. The solution was sonicated for 15 mins and then the volume was made up to the mark with acetonitrile. The resultant solution was filtered and suitably diluting with acetonitrile to get the working standard stock solution (1 mg/ml). A mixed working standard solution containing 50 µg/ml of each of the Oxybenzone and Avobenzone was prepared.

## Chromatographic development

Based on various trials carried out, the following chromatographic conditions were selected for validation. The optimum separation was achieved using Camag HPTLC instrument. Camag Twin Through glass chamber (20 x 10) was used for development which was saturated for 30 mins with the vapors of Toluene: Acetonitrile: Ethyl acetate as mobile phase in the ratio of (4: 5: 1 v/v/v). Linomat V with Camag 100 µl syringe was used to spot 1 µl of the prepared standard and spiked sample on HPTLC plates (Merk) precoated with Silicagel 60 $F_{256}$ on Aluminium sheets, dried and subjected to development at ambient temperature. The developed plates were scanned using Camag TLC scanner III at 332 nm and quantified employing Wincat Software.

## Extraction of Oxybenzone and Avobenzone from cosmetic products

Twenty different personal care products (mainly including creams and lotions) were procured from the local markets in and around Chennai city. Simple extraction, employing organic solvent like acetonitrile (selected based on solubility of Oxybenzone and Azobenzone) was carried out. Prior to extraction, 15 ml of n-hexane was added to the sample to remove lipids, fats and volatile oils. Individually, 5 g of the sample was weighed and was spiked with a mixed standard solution containing 50 µg/ml of Oxybenzone and Avobenzone. 5 ml of acetonitrile was added and the mixture was vortexed and homogenized under sonication for 20 mins, after which the volume was made up to 10 ml with acetonitrile. The above solution was mixed well and filtered through 0.22 µm Millipore membrane filter. The resultant solution was utilized for further analysis.

## Method Validation

Once the method was optimized, it was subjected to various validation parameters to establish the method with respect to linearity, limiting values (detection and quantitation limit), precision, accuracy and robustness studies employing design of experiments.

### *Linearity*

The linear response between the peak area and the concentration (ng/band) of Oxybenzone and Avobenzone were evaluated by making five replicate measurements of concentrations 2.5, 5.0, 25, 50, 100, 150 and 200 ng/band for Oxybenzone and Avobenzone individually.

### *Detection Limit (LOD)*

The Detection Limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The detection limit (LOD) may be expressed as

$$\mathbf{LOD = 3.3\,\sigma/Slope}$$

where, $\sigma$ is the standard deviation of the response.

### *Quantitation Limit (LOQ)*

The Quantitation limit of an analytical procedure is the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy. Quantitation Limit (LOQ) may be expressed as

$$\mathbf{LOQ = 10\,\sigma / Slope}$$

where, $\sigma$ is the standard deviation of the response.

### *Precision*

The intra-day and inter-day precision studies (intermediate precision) were carried out by spotting the mixed working standard solution containing 50 µg/ml of Oxybenzone and Avobenzone and estimating the concentration response six times on the same day and three different days.

### *Accuracy (Recovery studies)*

The ingredients of known concentrations (at three concentration levels of 2.5, 50 and 200 µg/ml in triplicate) were added into corresponding blank cosmetic product and 1 µl was spotted for analysis after the same procedure carried out under the extraction of Oxybenzone and Avobenzone from personal care products. The percentage Recovery was calculated from the average peak area ratio of sample to the standard.

### *Robustness*

CCD, as a three level factorial design with k factors, requires 2k factorial runs, 2k axial runs symmetrically spaced at along each variable axis and at least one center point. Twenty experiments with six center points were conducted by selection of three factors, acetonitrile content in mobile phase (A), developing distance (B), band size (C) and retention factor was selected as the response for both the ingredients. The nominal value of all these three factors A, B and C was 5 ml, 8 cm and 5 mm respectively. The data generated were analyzed using Design Expert (Version 7.1.6, Stat-Ease Inc., Minneapolis, MN, USA) statistical software. The significance of the factors was calculated using Fisher's statistical test for Analysis of Variance (ANOVA) model that were estimated. These components were then used to compute an F-ratio that evaluates the effectiveness of the model. If the F-ratio probability is low, the model is considered a better statistical fit for that data. All experiments were performed in randomized order to minimize the bias effects of uncontrolled factors.

## RESULTS AND DISCUSSION

Various solvents in different combinations and ratios were tried for resolving the peaks of Oxybenzone and Avobenzone efficiently. Finally Toluene: Acetonitrile: Ethyl acetate in the ratio of 4: 5: 1 v/v/v, gave a well-defined and good resolved peaks for the Oxybenzone and

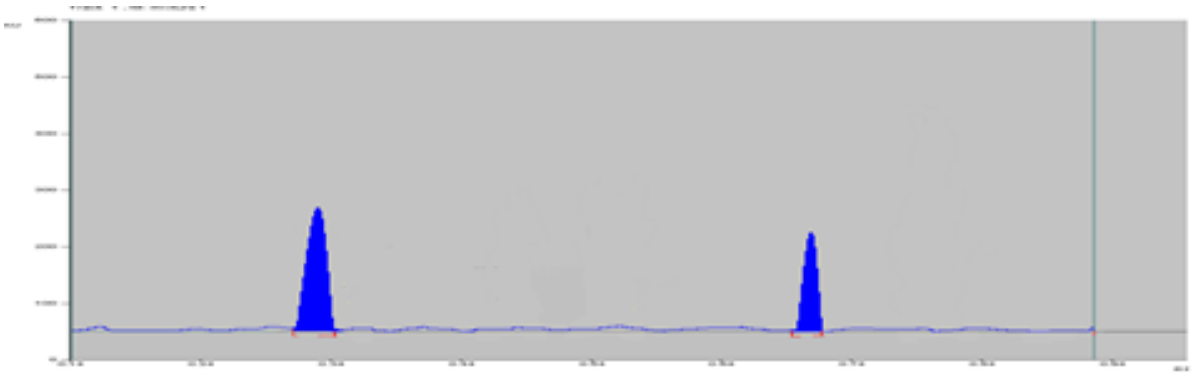


**Figure 1.** Densitogram of mixed standard solution of Oxybenzone and Avobenzone

Avobenzone. Ethyl acetate was added to improve the peak characteristics. An increase in the volume of acetonitrile made bands of Oxybenzone and Avobenzone move towards the solvent front, irrespective of the type. An addition of Toluene made Oxybenzone and Avobenzone run at a slower rate and inclusion of Acetonitrile resulted in the occurrence of well-defined peaks. The selected mobile phase gave an excellent resolution and the Oxybenzone and Avobenzone were eluted at different retention time of about 0.30 ± 0.023 and 0.70 ± 0.018 respectively at the detection wavelength of about 332 nm. (**Figure 1**).

Both the ingredients showed a good correlation coefficient value of about 0.9998 and 0.9991 in the concentration range of 2.5-200 ng/band for Oxybenzone and Avobenzone respectively. LOD and LOQ values were found to be 0.024 and 0.074 ng/band for Oxybenzone and 0.010 and 0.030 ng/band for Avobenzone.

The intra-day and inter-day precision studies (intermediate precision) was carried out, and the percentage Relative Standard Deviation (%RSD) was found to be less than 2% and the results were tabulated in **Table 2**.

**Table 2.** Intraday and Interday precision studies

| Repeatability | Drug | Concentration (µg/ml) | (Mean ± SD)* n= 6 | %RSD |
|---|---|---|---|---|
| **Intra-day** | Oxybenzone | 50 | 99.52 ± 0.23 | 0.31 |
| | Avobenzone | 50 | 98.98 ± 1.14 | 1.21 |
| **Inter-day** | Oxybenzone | 50 | 101.87 ± 1.04 | 1.12 |
| | Avobenzone | 50 | 99.31 ± 0.98 | 0.97 |

* (Mean ± SD) of six determinations

Accuracy of the method was performed by standard addition method, at three levels of concentrations in triplicate. The percentage recovery was calculated and was found within the range of 98.2 - 102.4 and 98.9 - 102.9 %w/w for Oxybenzone and Avobenzone respectively,

which suggests the suitability of the method to perform routine analysis of Oxybenzone and Avobenzone. The results of which are shown in **Table 3**.

**Table 3.** Recovery studies

| Ingredients | Spiked level (µg/ml) | Creams (n=3) | Lotion (n=3) |
|---|---|---|---|
| **Oxybenzone** | 50 | 98.2±2.2<br>101.6±1.3<br>102.4±2.9 | 100.2±2.3<br>101.6±1.3<br>98.2±1.8 |
| **Avobenzone** | 50 | 101.8±1.8<br>102.9±2.3<br>98.9±2.3 | 99.0±2.1<br>102.4±0.9<br>101.6±1.7 |

The data representing system suitability parameters were shown in **Table 4**, indicating acceptable precision concerning repeatability of peak area measurement and sample application.

**Table 4.** Overall results of HPTLC method validation

| Parameter | Results | |
|---|---|---|
| | **Oxybenzone** | **Avobenzone** |
| **Linearity range (ng/band)** | 2.5-200 | 2.5-200 |
| **Retention factor** | 0.30 | 0.70 |
| **Correlation coefficient ($r^2$)** | 0.9998 | 0.9991 |
| **Slope** | 69.292 | 53.661 |
| **Intercept** | 691.13 | 475.07 |
| **Limit of Detection (ng/spot)** | 0.024 | 0.074 |
| **Limit of Quantification (ng/spot)** | 0.010 | 0.030 |
| **Precision (% RSD)** | 0.31 - 1.12 | 0.97 - 1.21 |
| **% Recovery (%w/w)** | 98.2 - 102.4 | 98.9 - 102.9 |

All the experiments in robustness testing were performed employing the levels as depicted in **Table 5**. Response surface plots were constructed to evaluate the effect of all the selected factors on the retention factor of each drug.

The 3D response surface plots based on the equation was generated as a function of the significant variables. **Figure 2**, depicts the graphical representation of variations in response, i.e., retention factor, as a function of acetonitrile concentration in mobile phase and developing distance, while the band size is held constant, it was found that as the content of acetonitrile in total mobile phase increases, the retention factor for Oxybenzone and Avobenzone was also found to increase. As can be seen from the 3D response surface plots, an alteration in developing distance and band width doesn't have any significant effect on retention factor. Taking into account the response in the form of retention factor, every parameter was robust, with the exception of acetonitrile content in the mobile phase, where the optimal condition was 5 ml in the total mobile phase content. The only significant factor having influence on robustness study was the content of acetonitrile in mobile phase; hence, control over this parameter is necessary. The model was also validated by analysis of variance (ANOVA) using

**Table 5.** Design of CCD and their response

| Run | A Organic Phase (ml) | B Developing Distance (cm) | C Band Size (mm) | $R_f$ of Oxybenzone | $R_f$ of Avobenzone |
|---|---|---|---|---|---|
| **1** | 3.31 | 8 | 5 | 0.38 | 0.79 |
| **2** | 4 | 9 | 6 | 0.39 | 0.80 |
| **3** | 4 | 9 | 4 | 0.39 | 0.79 |
| **4** | 4 | 7 | 4 | 0.39 | 0.79 |
| **5** | 4 | 7 | 6 | 0.38 | 0.80 |
| **6** | 5 | 9.68 | 5 | 0.31 | 0.69 |
| **7** | 5 | 8 | 5 | 0.30 | 0.70 |
| **8** | 5 | 8 | 5 | 0.29 | 0.71 |
| **9** | 5 | 8 | 5 | 0.30 | 0.69 |
| **10** | 5 | 8 | 5 | 0.30 | 0.72 |
| **11** | 5 | 8 | 5 | 0.32 | 0.71 |
| **12** | 5 | 8 | 3.31 | 0.30 | 0.70 |
| **13** | 5 | 7.31 | 5 | 0.31 | 0.71 |
| **14** | 5 | 8 | 6.68 | 0.32 | 0.71 |
| **15** | 5 | 8 | 5 | 0.29 | 0.70 |
| **16** | 6 | 7 | 6 | 0.22 | 0.62 |
| **17** | 6 | 9 | 6 | 0.23 | 0.61 |
| **18** | 6 | 7 | 4 | 0.21 | 0.62 |
| **19** | 6 | 9 | 4 | 0.24 | 0.63 |
| **20** | 6.68 | 8 | 5 | 0.22 | 0.61 |

Design Expert software 7.1.6. Significant effects had p valve less than 0.05. The high adjusted R-square values and low standard deviation (%CV) indicated a good relationship between the experimental data and those of the fitted models. The final equation, concerning actual components and factors, is shown in **Table 6**.

**Table 6.** Predicted Response Models and Statistical Parameters obtained from ANOVA for CCD

| Response ($R_f$ value) | Type of model | Polynomial equation model for Y | Model P value | %CV | Adequate Precision |
|---|---|---|---|---|---|
| Oxybenzone | Linear | +0.54+0.00123A+0.013B+0.00456C | <0.0001 | 1.04 | 17.92 |
| Avobenzone | Quadratic | +0.76+0.034A+0.012B+0.045C+0.012AB+0.045AC+ 0.065BC-0.00456A2+0.00457B2+0.0562C2 | 0.0043 | 1.12 | 32.13 |

Analysis of marketed products after extraction employing the developed method, showed two significant peaks at $R_f$ value of 0.30 and 0.70 for Oxybenzone and Avobenzone respectively, that was found same as the $R_f$ value of the standard Oxybenzone and Avobenzone. (**Figure 3**)

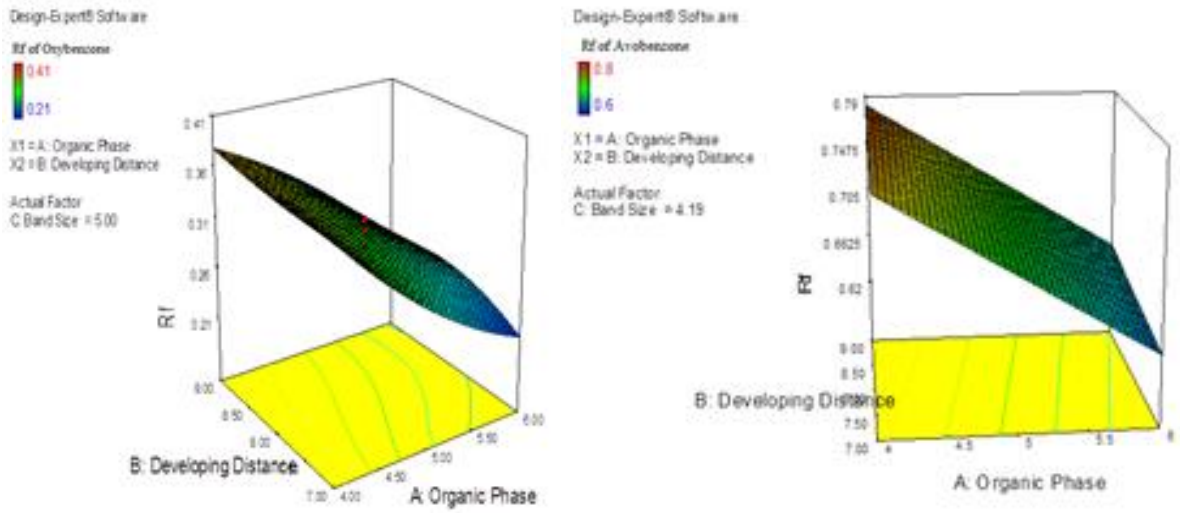



**Figure 2.** Three dimensional (3D) plots of the RSM for $R_f$ value of Oxybenzone (A) and Avobenzone (B)

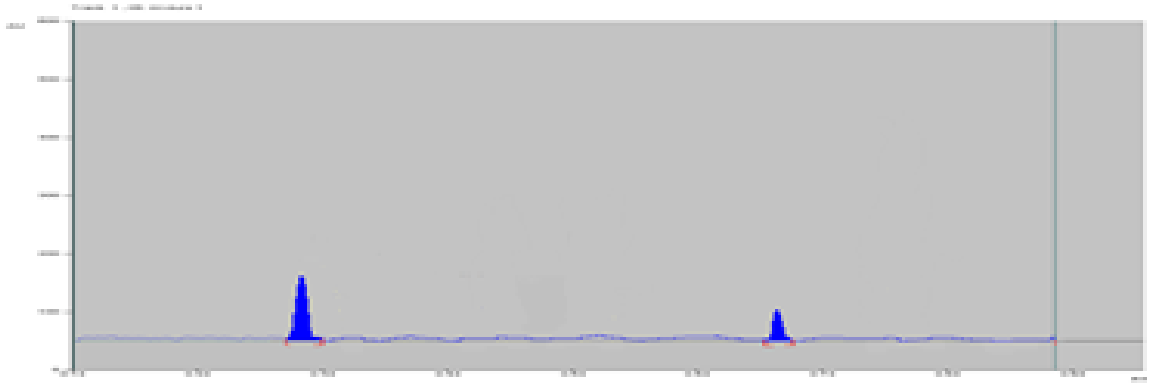


**Figure 3.** Densitogram of extracted sample solution containing Oxybenzone and Avobenzone (cream no. 7)

The content of Oxybenzone and Avobenzone present in 30 different personal care products procured from the local markets were calculated after extraction. The obtained result evidently proves that Oxybenzone and Avobenzone were commonly present in most of the creams and lotions which were selected for analysis. Out of two, Avobenzone was predominately found in most of the sunscreen creams and lotions. The concentration predicted was found to be well below the maximum allowable limit in the cosmetic product (**Figure 4**).

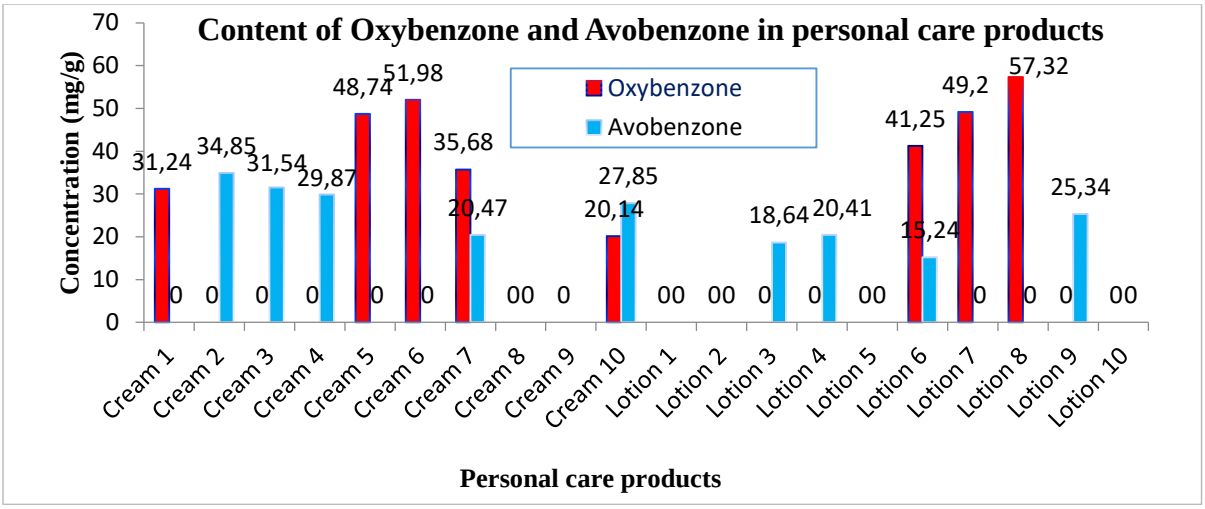



**Figure 4.** Content of Oxybenzone and Avobenzone estimated in personal care products (20 nos.)

## CONCLUSION

The developed method was found to be simple, novel, sensitive, precise, accurate and robust for simultaneous estimation of Oxybenzone and Avobenzone in personal care products. Moreover, the major advantage of HPTLC method is that several components can be run simultaneously, and quantification of compounds can be carried out using minimal analysis time, using small amount of mobile phase and need for minimum sample clean-up procedure unlike HPLC and other advanced analytical techniques with low maintenance cost per analysis adds up credit to the current study. The application of CCD on robustness was to examine the variation of different factors simultaneously on responses. The robustness study carried out employing CCD, evidence that the change in acetonitrile content in total mobile phase appeared to have a significant effect on the response (retention factor), compared to the other two factors such as developing distance and band width selected for the study. Hence, it was important that the significant factor (acetonitrile content in mobile phase) be carefully controlled. It is concluded that use of experimental design and response surface methodology is a flexible procedure and can be used to reduce the number of needed experiments for robustness study for the developed HPTLC method. Finally, the developed and validated method allows easy quantification of Oxybenzone and Avobenzone content in various marketed personal care products, which were found well within the maximum allowable limits for cosmetics. Though found within allowable limits, a careful monitoring on daily exposure to these harmful ingredients have to be taken in account, thereby reducing the risk of cancer, infertility and related adverse effects on reproduction and creating a healthy environment. The method was found to be repeatable and suitable for routine quality control analysis of Oxybenzone and Avobenzone in cosmetic, food and pharmaceutical products.

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