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Determination of synthetic colorants in cosmetic products by reversed-phase high-performance liquid chromatography coupled with diode-array detector

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ABSTRACT: This study aimed at developing and validating a reversed-phase (RP) high-performance liquid chromatography (HPLC) method, for simultaneous determination of five synthetic dyes called tartrazine (TRZ), sunset yellow (SY), allura red AC (AR), brilliant blue FCF (BB) and erythrosine B (EB) in cosmetic samples. The separation was performed by a C18 reverse phase analytical column (4.6 x 250 mm, 5 µm) at 30°C with gradient elution and the mobile phase contained 20 mM ammonium acetate buffer, acetonitrile and methanol. Flow was 1.0 mL/min. Detection wavelengths of diode array detector (DAD) were set at 420, 480, 510, 634 and 530 nm for TRZ, SY, AR, BB and EB, respectively. The dyes were analysed in 24 min. The limits of detection (LOD) was ≤ 0.18 µg/mL. The recovery was between 88.7 and 103.0%. Precision was ≤ .7.33 (RSD%) and accuracy was ≤ 3.0 (RE%). It was determined that 7 different cosmetic samples analyzed, consisting of soap, shower gel, eyeshadow, mouthwash, and lip pencil contained synthetic dyes at a concentration of 0.29 to 10.81 mg/g.

KEYWORDS: Synthetic colorants; method validation; HPLC-DAD; cosmetic.

1. INTRODUCTION

Colorants, are widely used in foods, cosmetics, beauty supplies, plastics, toys, chemicals, pharmaceuticals, cosmetics and personal care products to increase the appeal of the product to consumers. It is very common to use colorants in consumer products because it makes them more attractive. Coloring agents are added to cosmetic and personal care products formulations to color the product itself or to color the lashes, hair, nails and skin for decorative purposes [1,2].

Nowadays, approximately 10,000 chemical compounds, including organic and inorganic, are being used as coloring agents in the preparation of the main group consumer products [3,4]. These agents are produced from natural substances obtained from plants, animals and minerals [1]. Natural colorants are often unstable, expensive and easily degraded by the influence of light, temperature or changing pH during the manufacturing process and storage. In addition, natural colorants are not soluble but synthetic colorants are soluble in hydro or oil. Also, synthetic dyes have a lot of important advantages such as higher stability to light, oxygen and pH, low microbiological contamination risk, long-lasting and low price and to color uniformity. Therefore, natural dyes have been partially or completely replaced by synthetic counterparts [5–7].

Synthetic colorants, based on their chemical structure, divided in five main classes: Azo, triarylmethane, xanthene, indigo and quinoline classes and usually used as the water-soluble sodium salts [1,8]. Tartrazine (TRZ), sunset yellow (SY), and allura red (AR) are classified as azo group dyes. Brilliant blue (BB) belongs to the triarylmethane group and also erythrosine B (EB) is classified as a xanthene dye.

The chromophore azo groups can be reduced to the suspected carcinogenic amines under certain conditions [3,4,9]. Many studies have been conducted on mutagenic, genotoxic and carcinogenic effects [10–12]. Extensive cytostatic potential, DNA binding properties and reduced mitotic index were observed for TRZ [13]. However in the another study, it was shown that TRZ caused an increase in the mitotic index unrelated to the dose. The genotoxic effects determined were performed on organs such as the colon and stomach of the

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mouse [14]. TRZ exposure has been shown to cause decreased sperm count and sperm abnormalities [15]. It has been reported by Kemal and El-lethey TRZ exposure causes increased activity, anxiety and anti-social behaviour [16]. In another study, it observed that AR has a direct genotoxic property [17]. Column-specific DNA damage in mice was determined for this dye [18]. The European Food Safety Authority (EFSA) banned AR use in animal feed [19]. It was proven that AR has behavioural effects on humans and animals [20]. The consumption of EB, which could be a significant risk factor in human breast carcinogenesis, and reduced mobility of sperm in mice. The mutagenic effect of EB has been shown in different studies [21-22]. The *In vitro* genotoxic effect of EB was evaluated [23] and results were proven that, EB is genotoxic.

Also another study aimed to determine the genotoxicity and cytotoxicity of EB and the result showed that EB has cytostatic and genotoxic properties [24]. Sasaki et al. demonstrated that EB caused a DNA damage effect [14]. In an *in vitro* study a high degree of cytostatic and cytotoxic properties were observed [13]. According to another study, results conducted chronically with EB, showed an increase of some tissue and organ weights [25]. In addition, some studies proved that EB has potentially toxic effects on the reproductive process [26,27].

Synthetic colorants are added to color the cosmetic material or to color the eyelashes, skin, nails, hair, and even decorative powders for cosmetic and personal care product formulations. People are exposed to dermatologically colored agents except oral products. It poses a great challenge to develop reliable analytical methodologies for the quantification of colorant reagents, depending on the variety of chemical structures and the complexity of the matrices containing them [1,3,4,28]. Due to their toxicity, especially when consumed in excess, synthetic dyes are strictly controlled by laws, regulations and acceptable daily intake values for food safety [9–11].

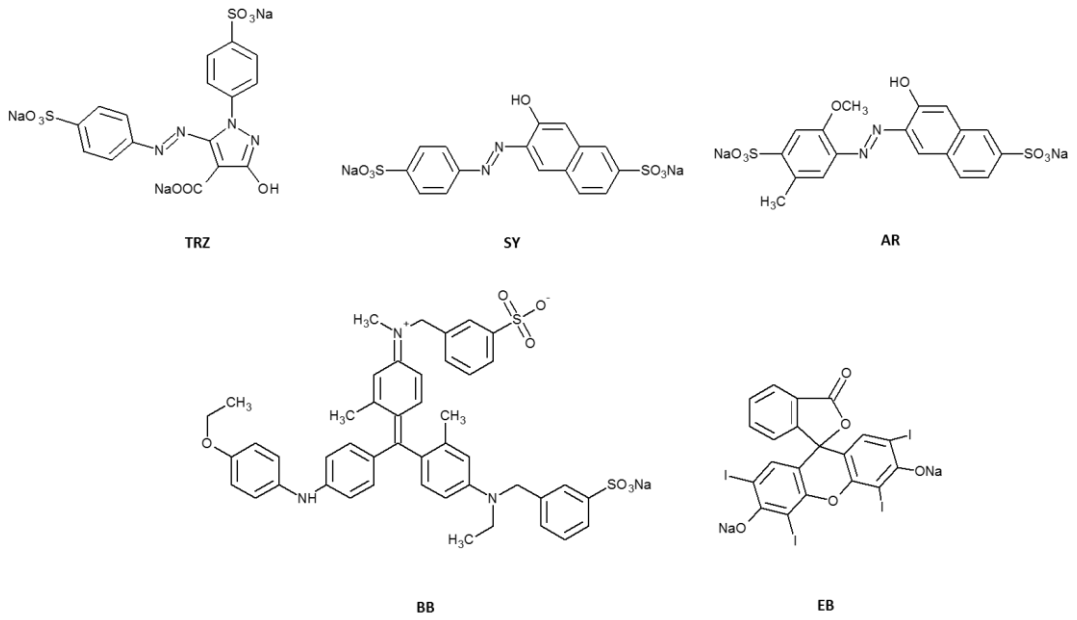

Figure 1. The chemical structures of TRZ, SY, AR, BB and EB.

For this reason, quantitative identification of toxicologically important dyes from real samples by a simple, precise and reliable method is of vital importance. However, due to the often complex matrix of cosmetic products, the sample preparation stage is of great importance. Due to the need for complex techniques, long-term sample preparation processes make routine analysis difficult. At the same time, the analysis of these assays with high-precision analyzers makes regular analysis challenging. For this purpose, various analytical methods such as thin layer chromatography, spectrophotometry, derivative spectrophotometry, fluorimetry, voltammetry, differential pulse polarography, capillary electrophoresis, HPLC and ion chromatography have been used [9,12,31–37].

Spectrophotometric methods have been widely used for determination of these colorants but these methods have involved the relatively low sensitivity values [38]. The separation step is to become a requirement because of the obvious interference from the background caused by other additives [39].

Chromatographic separation is known to give very successful results in the quantitative determination of the amount of target dye compounds. Reverse phase liquid chromatography-based methods are commonly preferred in synthetic dye analysis. Conventionally, because of the ability of the colorants to be absorbed in the UV-Vis spectrum, the preferred detector is DAD as recommended [40]. HPLC techniques are well suited for the analysis of samples containing several colorants. These techniques provide high sensitivity and resolution, and allow simultaneous determination of colorants from the sample [11,33–35].

This paper describes a new, sensitive and reliable RP-HPLC method combined with a diode array detector for the simultaneous determination of TRZ, SY, AR, BB and EB in cosmetic samples. This method allows sample preparation without the need for any extraction process. Our suggested method validated according to ICH guidelines [41]. Finally, our recommended method was used for the quantitative determination of synthetic colorants in five cosmetic products. The chemical structures of TRZ, SY, AR, BB and EB are given at Figure 1.

2. RESULTS AND DISCUSSION

Optimum analytical conditions were set after the optimization procedure was performed for column selection, the content of the mobile phase and wavelength. The best separation performance was obtained from RP C18 Zorbax (USA) analytical column. The mobile phase flow rate and column oven temperature were set to 1.0 mL/min and 30°C, respectively. The analysis was carried out under gradient conditions that were optimized. DAD was set to 420, 480, 510, 634 and 530 nm, with the sequential program for the detection of TRZ, SY, AR, BB and EB, respectively. The retention times of TRZ, SY, AR, BB and EB were 8.5, 12.7, 14.4, 17.8 and 23.1 min, respectively. The method was linear, between 0.5 – 20 µg/mL for all analytes. LOD was found as ≤0.13 µg/mL and LOQ was found as ≤0.40 µg/mL for all compounds investigated. Determination coefficients (R^2) were calculated between 0.9985 and 0.9996. Total run time was 24.0 min.

2.1. Method validation

Method was validated to specificity and selectivity, linearity, sensitivity, repeatability and recovery. The validation protocol was performed according to the ICH guideline [41], taking into account the reproducibility of the method to obtain precise and accurate measurements.

2.1.1. Specificity and selectivity

There were no unwanted peaks observed in all of the chromatograms of blank samples (Figure 2) Neither peaks nor changes in the structure of the baseline chromatogram was detected, especially at retention times of analytes. No structural degradation was observed in the peaks of chromatograms of standard chemicals. In the analysis of samples, peaks were also very stable in terms of retention time and peak structure. This situation was observed in specificity and selectivity of the method and had a very beneficial effect on the reproducibility of the method.

The DAD set at 420, 480, 510, 634 and 530 nm was displaying optimum sensitivity for TRZ, SY, AR, BB and EB. The method demonstrated excellent chromatographic specificity with no interference at the retention times of TRZ, SY, AR, BB and EB (8.5, 12.7, 14.4, 17.8 and 23.1, respectively). The different retention times of the dyes in the HPLC column had a very positive effect on the selectivity and sensitivity values of the method. Chromatograms of dye standards were given in Figure 3, which shows the high resolution with no interference in relatively short separation time (24 min) for 5 dyes. Chromatograms of the synthetic dyes which belong to, TRZ (8.5 min), SY (12.7 min), AR (14.4 min), BB (17.8 min) and EB (23.1 min), according to retention times are shown in Figure 3.

2.1.2. Linearity

Calibration curves of the analytes were plotted with the standard addition method and the response of each calibration point was determined by the response of the 3 individual samples. The calibration points were determined as 0.5, 1, 2, 5, 10 and 20 µg/mL for all colorants. The determination coefficient (R^2) for all dyes was calculated as above 0.998 (n=3). Results are given in Table 1.

2.1.3. Sensitivity

The limit of detection (LOD) and quantification (LOQ) based on the standard deviation of the response and the slopes of the calibration graphs, were calculated according to the ICH recommendations LOD= 3.3σ/S;

LOQ= 10σ/S (σ: The standard deviation of the response; S: calibration curve slope). 0.5 µg/mL concentration of analytes were used to the calculation of LOD and LOQ.

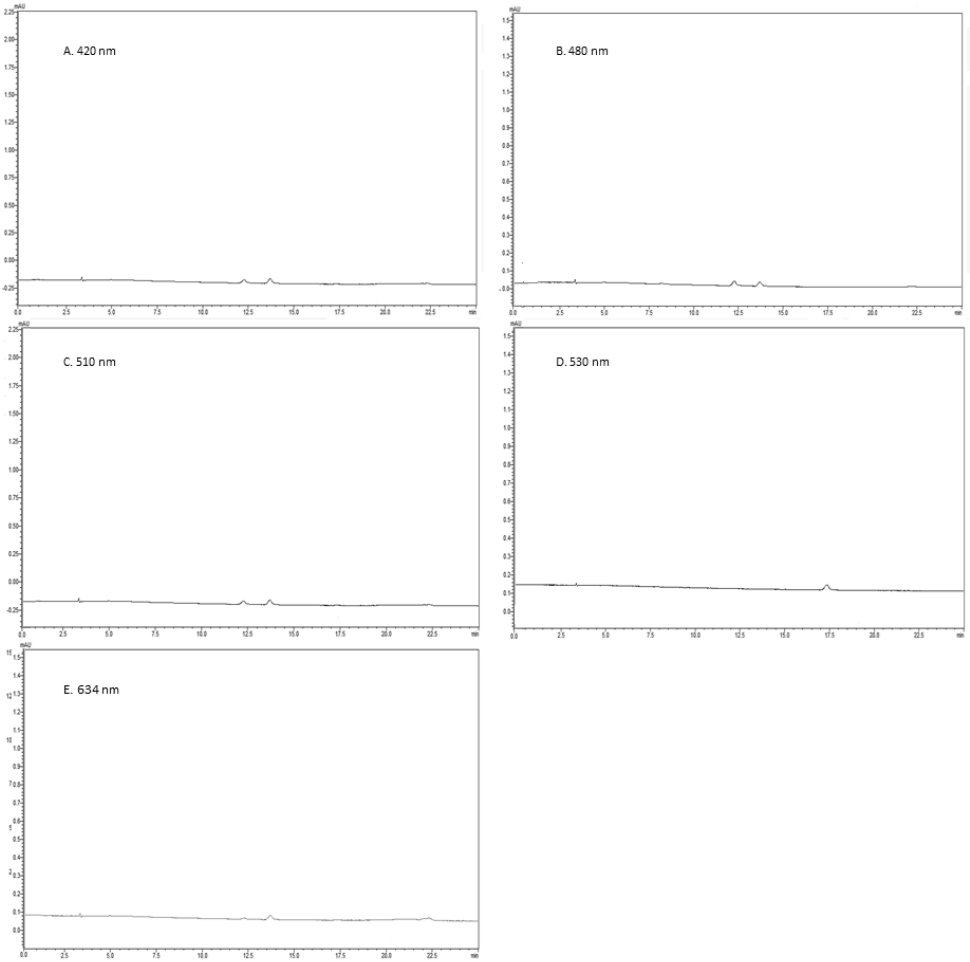

Figure 2. Blank chromatograms of synthetic dyes obtained at their specific wavelengths. A. TRZ, B. SY, C. AR, D. EB and E. BB, according to retention times.

As shown in Table 1, LOD values were calculated as between 0.13 µg/mL and 0.16 µg/mL in all dyes. Also, it was calculated that LOQ values were between 0.40 µg/mL and 0.53 µg/mL. These sensitivity results were very important for the quantitative determination of low amounts of dyes in cosmetic samples. For example, it was detected 0.29 ± 0.03 µg/g concentration of TRZ in the soap sample was detected as the lowest analyte concentration. Also, AR was determined in mouthwash sample as 0.5 ± 0.005 µg/g concentration.

2.1.4. Accuracy and precision

Accuracy, expressed as the relative error (RE%), was also determined for the same concentrations of analytes. Precision, defined as relative standard deviation (RSD), was determined by five individual replicates at three different concentrations (n=5). It is the degree of veracity while precision is the degree of reproducibility. Table 2 shows the RSD and RE% values of the method that indicates inter- and intra-day of precision and accuracy values of low, medium and high concentrations of analytes. Results are given in Table 2.

The intraday precision was between 0.79 and 4.69 (RSD). The inter-day precision values were between 0.57 and 7.33. It was found that the inter-day precision values generally showed higher deviation values than the intra-day values. This method shows that the accuracy obtained during the intra-day is higher than the accuracy inter-day. This result is an expected situation in validation studies. It has also been observed that the deviation in inter-day precision values is mainly due to the high deviation in the highest concentrations of TRZ, AR and BB.

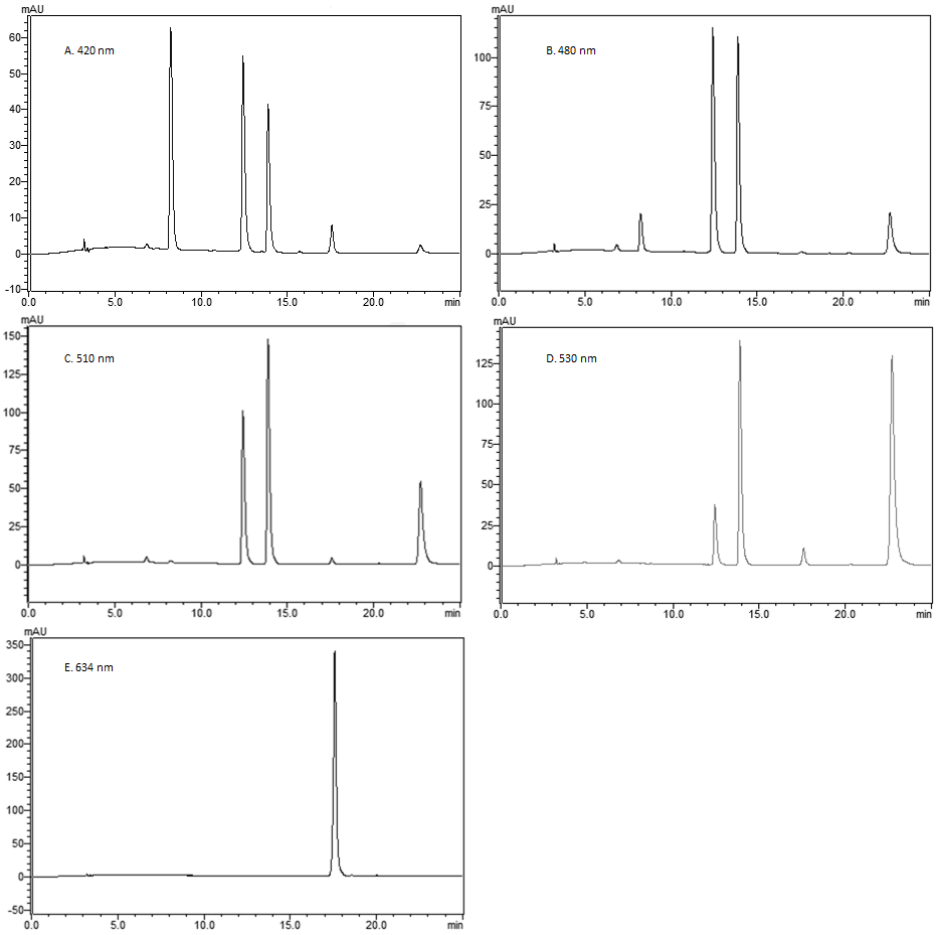

Figure 3. Chromatograms of the synthetic dyes; A. TRZ (8.5 min), B. SY (12.7 min), C. AR (14.4 min), D. EB (23.1 min) and E. BB (17.8 min).

Table 1. Chromatographic characteristics and system suitability parameters of the method for the HPLC-DAD analysis of dyes.

Colorant	λ (nm)	t_R (min)	LOD (µg/mL)	LOQ (µg/mL)	Linear Range	Calibration equation	R^2	k'	N
TRZ	420	8.5	0.18	0.53	0.5 - 20	y=36011.23x + 1798.7	0.9992	1.6	26876
SY	480	12.7	0.13	0.40	0.5 - 20	y=643504x + 2898.6	0.9988	2.8	7942
AR	510	14.4	0.16	0.47	0.5 - 20	y=70737.1x + 12352.6	0.9993	3.3	3691
BB	634	17.8	0.17	0.50	0.5 - 20	y=197946x - 29391.7	0.9996	4.4	7491
EB	530	23.1	0.13	0.40	0.5 - 20	y=113545x - 20867.4	0.9985	5.9	12232

Abbreviations: TRZ: tartrazine, SY: sunset yellow, AR: allura red, BB: brilliant blue, EB: erythrosine B, λ: wavelength, t_R: time of retention, LOD: Limit of detection, LOQ: limit of quantitation, R^2: determination coefficient, k': capacity factor, N: theoretical plate numbers.

The intraday accuracy was between 0.00 and 3.00 (RE%). Inter-day accuracy was between -1.00 and 3.00. Intra-day accuracy values were found to be less deviated than between days. The result obtained is again an expected result in validation studies.

2.1.5. Recovery

Recovery tests were performed at 1 and 2 µg/mL concentrations of the analytes. The known concentrations of the analytes were added to the samples in appropriate conditions and these analytes were calculated quantitatively by the method. The method recovery was calculated by comparing the observed and expected results. Recovery results are given in Table 3.

The average recoveries were calculated as 100.5%, 99.2%, 92.9%, 95.5% and 102.9% for TRZ, SY, AR, BB and EB, respectively. These results were very important for repeatability and sensitivity of the method. The method recovery test was carried out especially in the lowest concentrations of the analytes to demonstrate the method sensitivity.

Table 2. Confidence parameters of method; intra-day and inter-day precision and accuracy for determination of colorants (n=3).

Colorant	Added conc. (µg/mL)	Intra-day			Inter-day		
		Found conc. $\bar{x}$±SD (µg/mL)	Precision (RSD)	Accuracy (RE%)	Found conc. $\bar{x}$±SD (µg/mL)	Precision (RSD)	Accuracy (RE%)
TRZ	2	2.04±0.06	3.1	1.9	2.06±0.04	2.0	3.0
	5	5.12±0.04	0.8	2.5	5.15±0.09	1.7	3.0
	10	9.97±0.3	3.0	0.3	10.02±0.5	5.0	0.2
SY	2	2.1±0.04	2.0	3.0	2.04±0.01	0.6	2.0
	5	5.1±0.2	4.0	2.3	5.11±0.13	2.6	2.2
	10	10.0±0.3	2.9	0.4	9.94±0.28	2.8	-0.6
AR	2	2.1±0.04	2.0	3.0	2.04±0.04	2.0	2.0
	5	5.09±0.06	1.6	1.8	5.05±0.04	0.8	1.0
	10	10.26±0.27	2.6	2.6	9.90±0.32	3.25	-1.0
BB	2	2.03±0.09	4.4	1.4	2.04±0.05	2.5	2.0
	5	5.01±0.06	1.2	0.2	5.05±0.04	0.8	1.0
	10	9.85±0.46	4.7	1.6	10.09±0.74	7.3	0.9
EB	2	2.06±0.02	1.1	3.0	2.02±0.04	3.0	1.0
	5	5.00±0.12	2.5	0.0	5.06±0.16	2.4	1.2
	10	9.94±0.31	3.1	0.6	10.05±0.48	1.4	0.5

Abbreviations: conc: concentration, $\bar{x}$: average of values, SD: standart deviation, RSD: relative standard deviation, RE%: relative error percentage

As the developed method did not involve any extraction method, it is defined as direct analysis. The simple and fast sample preparation phase avoids time loss in the analyzes and allows multiple analyzes to take place within a reasonable time.

Iammarino et al. suggests that in order to investigate 12 food dyes in meat products, a new HPLC method is required. Total analysis time of this method was 43 minutes [12]. The separation was achieved with RP-C18 150 x 4.6 mm analytical column and the quantification of analytes was to be performed with DAD detector set at 520 nm. As a mobile phase, 0.02 M acetate buffer and acetonitrile were applied to the column gradiently. AR and EB were detected with this method at 17 and 23 mins, respectively. The precision values for AR and EB were 11.0 and 15.0%. The result of the recovery test which were performed at 25, 50 and 100 µg/kg concentrations was between 89 – 93% [12]. In a study performed by de Andrade et al., in order to determine concentrations of food dyes in soft drinks, there was two different methods, which are based on TLC and HPLC [10]. Dye identification was performed by the TLC method and quantitation was achieved with the ion pair-HPLC method. Retention times of SY and BB were 14 and 14.5 min, respectively. Analytes were extracted in drinks with the C18 solid phase technique. The correlation coefficients of the method were ≥0.999 and quantitation limits were ≥ 0.012. µg/mL. Methanol, water and ammonium acetate, which are used as a mobile phase, were applied to the RP-C18 column with isocratic elution. Recovery was determined between 81 – 101%. Mathiyalagan et al., developed a new HPLC-UV method. TRZ, SY and BB were determined

at 2.2, 4.4, 7.7 mins in this method [9]. The liquid-liquid extraction technique was used to obtane dyes from the samples. Ethanol, hexane, butylated hydroxytoluene, water and ammonia solution were used as extraction solvents. Recovery was found between 90 and 97%. LOQ was ≥15.1 ng/mL and the linearity range was between 10 and 100 ng/mL. The correlation coefficient was ≥0.998. In the method developed by Minioti et al, retention times of TRZ, SY, AR, BB, EB were 11, 16.9, 19.1, 24.8, and 29 minutes, respectively [8]. LOD of the method was between 1.59 and 22.1 ng/mL. The intraday precision was found between 0.37 and 4.8% and the inter-day precision was observed between 0.86 and 10.0%. The recovery values were between 94 and 102%. A study by Vlase et al. showed, a new HPLC-UV method was developed for determination of 9 water soluble dyes [7]. This method's run time was 8 mins and linearity was between 0.9982 and 0.9997. LOQ was ≥ 50.1 ng/mL. Research from Qin et al. demonstrated, the correlation coefficients (R^2) of TRZ, SY and AR were calculated ≥0.9973 and this developed method was linear between 0.45 – 1000.0 ng/mL [11]. The limit of detection was between 0.60 – 0.80 ng/mL. Intra-day and inter-day precision was found as 1.72-3.64 and 1.45 – 1.55%. The analytes were spiked at concentrations of 20, 50 and 200 ng/mL for all 3 dyes. The recovery test was applied at three concentration levels. Recovery was between 80.6 and 116.8%. In the research by Brazeau, the determination of 26 dyes were investigated [35]. TRZ, SY, AR, EB and BB linearity test studies of this method were applied between 0.025 µg/mL and 50 µg/mL concentrations. R^2 values for the calibration curves were above 0.998. LOQ levels were detected as between 0.23 and 0.45 µg/g.

Table 3. The recovery values of all dyes in the cosmetic samples (n=3).

Colorants	Added (µg/mL)	Found[a] (µg/mL)	Recovery%
TRZ	0	nd[b]	-
	1	1.00±0.01	99.7±0.9
	2	2.02±0.01	101.2±0.6
SY	0	nd[b]	-
	1	0.99±0.01	98.7±1.3
	2	1.99±0.01	99.7±0.6
AR	0	nd[b]	-
	1	0.89±0.01	88.7±0.9
	2	1.94±0.05	97.0±2.5
BB	0	0.81±0.05	
	1	1.70±0.01	95.0±1.0
	2	1.91±0.03	96.0±1.0
EB	0	nd[b]	-
	1	1.03±0.03	102.7±3.3
	2	2.07±0.04	103.0±1.9

[a] $\bar{x}$±SD, n=3.;
[b] nd: not detected.

The correlation coefficient value of the proposed method is ≥0.998 which is comparable to the literature. Precision test results were 0.79 and 4.69 (RSD) inra-day and 0.57 and 7.33 for inter-day analysis. The precision values of Iammarino et al. [12], Minioti et al, [8] and Mathiyalagan et al., [9] ranged from 10.0 to 15.0 RSD%. It is clear that the suggested method's accuracy results are comparible with the literature and there are not too many deviations that may result in negative effects on the sample analysis. Our accuracy test results were between -1.00 and 3.00 (RE%) for intra-day and interday. These accuracy values are considered as sufficient for safe analysis. When the literature is examined, it is seen that the recovery values of dye analysis methods vary between 80.6 and 116% [7-10, 12, 35]. The recovery values of our method are between 92.9 and 102.9%. These recovery values indicate that the method can safely perform sample analyzes. The sensitivity values of our method, measured with LOQ values were between 0.13 and 0.16 µg/mL. Although this value is higher than the study reported by Brazeau (35), other studies with higher sensitivity have been observed in the literature. In our study, where the analysis time was 24 minutes, the distinction was very strong. The retention times of the analytes are suitable for use in the laboratory for routine sample monitoring.

2.2. Analysis of the colorants in real samples

The accuracy of the described method was evaluated analyzing spiked samples. For this, dyes in various concentrations were added to the eyeshadow (0.10 g) samples and then the described method was applied. Three parallel analyses were performed for each concentration level. As can be seen from Table 3, the recoveries are found to be satisfactory for cosmetic samples.

Our proposed method was successfully applied to the real cosmetic samples such as soap, shower gels, eye shadows, mouthwash and lip pencil. Different types of samples which had different matrices did not cause any adverse effects on the chromatograms and no interference was observed. 10 mg, 50 mg and 1 g of liquid or solid sample was dissolved in 5 mL of a water-methanol mixture separately and was mixed using a sonicator for 1 hour at 60°C. There was not any extraction application in this method. Also samples were prepared at the end of the sonication process. After the filtration implementation, clear residual was implemented to the HPLC as 20 µL. All of the sample preparation process was simple, time saving, and it did not need any complicated instrument so is reproducible in many laboratories. Total liquid volume used in the sample preparation process was 5 mL of the water-methanol mixture (1:1, v/v). For this reason, it could be evaluated as an environmentally friendly analytic method considering the sample preparation phase. The total analysis time was 30 min as each analysis took 24 min and column reconditioning took 6 min. Also, since the method does not need to any extraction application and the sample was applied to the column directly, the chromatographic analysis time it needs was an acceptable length. Sample chromatograms were given in Figure 4.

When the dye levels in the samples were examined, an unexpected level of SY (10.81 µg / mL ± 0.68) was detected in sample-4, eye-shadow. During the analysis of this sample, it was observed that the analyte peak was very clear and the structure was very sharp. During the analysis, no carry over" which could have a negative effect on the next analysis was not performed. A significant amount of excess TRZ (1.9 µg/mL ± 0.26) was detected in Sample 2, shower gel. The chromatogram and peak structure obtained during the analysis were well suited for repeated analysis.

Table 4. The colorant results obtained in the real cosmetic samples.

Sample number	Sample type	Detected colorants	Content of colorant[a] (mg/g)
1	Soap	TRZ	0.29 ± 0.03
2	Shower gel 1	TRZ	1.9 ± 0.26
3	Shower gel 2	SY	1.2 ± 0.07
4	Eyeshadow 1	SY	10.81 ± 0.68
5	Eyeshadow 2	BB	0.71 ± 0.02
6	Mouthwash	AR	0.5 ± 0.005
7	Lip pencil	EB	1.38 ± 0.12

[a] $\bar{x}$ ± SD, n=3.

3. CONCLUSION

We recommended this optimized and validated reversed-phase HPLC coupled with DAD method to be used in simultaneous analysis of TRZ, SY, AR, BB and EB in cosmetic products. This method has significant advantages such as; direct analysis, environmentally-friendly and low-cost. The application does not require complex tools for the determination of the five dyes. The method can be easily modified and applied for the routine analysis of synthetic dyes in toxicologically reference laboratories and food analysis and control laboratories. Also, this method is suggested for colourant analysis in many types of materials in the toxicological analysis laboratory. Method validation results showed good sensitivity and recovery values. Also, precision and accuracy test results obtained from the repeatability tests were very significant. The method could be used for many kind of cosmetics and it can be adapted for monitoring of other consumer products such as foods and drinks. The method can also be used in environmental pollution studies involving the monitoring of dyes from environmental-biological media. In addition, it may be considered that the method may have limited ability to analyze, especially in cosmetic products containing a much more complex matrix. Here, the absence of any extraction application is the main factor.

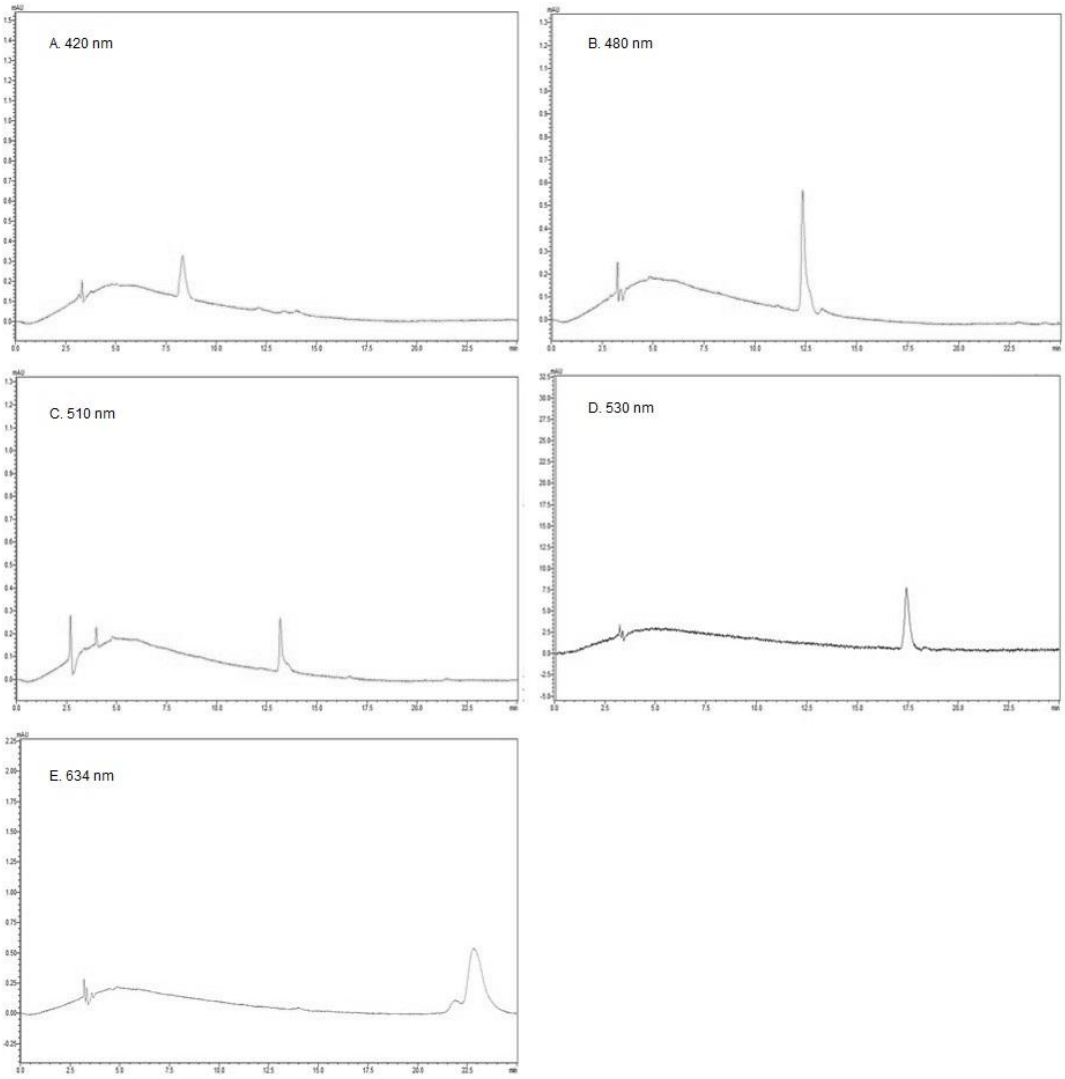

Figure 4. Sample chromatograms which belong to sample of 1(A), 3(B), 5(E), 6(C) and 7(D) which are soap, shower gel, eye shadow, mouthwash and lip pencil, respectively. The numbers 1, 3, 5, 6 and 7 represent the sample numbers given in Table 4.

4. MATERIALS AND METHODS

4.1. Chemicals and reagents

The analytical standard of SY (E110), AR (E129), BB (E133) and EB (E127), obtained from Sigma-Aldrich (Massachusetts, USA) and TRZ (E102) were ordered from Alfa Easer (Kandel, Germany). The HPLC grade acetonitrile and methanol and also the analytical grade ammonium acetate was purchased from Sigma-Aldrich (Missouri, USA). The Elga Purelab Water Purification System (Buckinghamshire, United Kingdom) was used to obtain ultrapure water.

4.2. Instrument and chromatographic conditions

Shimadzu LC-20AD HPLC system (Kyoto, Japan) equipped with a degasser (DGU-20A5R), an automatic liquid sampler (SIL-20ACHT) have a 20 µL sample loop volume, a column oven (CTO-10AS) and a DAD (SPD-M20A) was used for chromatographic separation and quantification. A reverse phase C18 analytical column (250 x 4.6 mm i.d., 5 µm p.s.) Zorbax (USA) was used for separation. Ammonium acetate buffer, acetonitrile and methanol were used as the mobile phase. Mobile phase A was prepared with 20µM ammonium acetate buffer (pH: 6.7) and it was degassed over the 30 minutes by the sonicator that before each analysis. Mobile phase B was prepared with methanol and acetonitrile (1:1, v/v). DAD was set to 420, 480, 510, 634 and 530 nm, with the sequential program for the detection of TRZ, SY, AR, BB and EB, respectively.

The analysis was carried out under gradient condition using 1.0 mL/min flow rate at 30°C. The gradient mobile phase flow program was as follows: The initially mobile phases flow ratio is 97:3, (v/v) for mobile phase A and B. Then mobile phase ratios linearly changed to 40:60, (v/v) over 18 mins. This elution ratio is held over 6 mins. The total run time was 24 minutes.

4.3. Preparation of stock solutions and working standards

The stock solutions of 100 mg/L TRZ, SY, AR, BB and EB were prepared by dissolving in deionized water and stored at +4 °C. The main stocks and working solutions were chemically stable at +4°C for at least 1 month. The calibration standards and quality control samples were prepared by the serial dilution from the working solutions with the mixture of methanol: water (1:1, v/v).

4.4. Collection of cosmetic samples

Cosmetic samples were purchased from the different markets located in Sivas, Turkey and were stored at room temperature until analysis. All of the samples were analyzed before the expiry date of the shelf life.

4.5. Sample preparation

10 mg of lip pencil and eyeshadow, 50 mg of soap, 1 g of shower gel were dissolved in 5 mL of the water-methanol mixture (1:1, v/v) by the ultrasonic bath at 60 °C for 1 hour. The mouthwash was diluted with the water-methanol mixture (1: 1, v / v) by the ½ ratio. The dissolved sample was filtered with a 0.45 µm filter. Finally, 20 µL clear sample was loaded to the HPLC. The analysis of the samples was performed in three replicates.

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