Development and validation of a simple and fast method for simultaneous determination of sunset yellow and azorubine in carbonated orange flavor soft drinks samples by high-performance liquid chromatography

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Abstract: *An efficient method was developed for the simultaneous determination of sunset yellow and azorubine in carbonated orange flavor soft drink samples by high performance liquid chromatography (HPLC-UV-Vis). Affecting parameters on separation and detection of the dyes were investigated and optimized. For these dyes good linearity (0.25–50 mg L^{-1}, > r^2=0.99) were obtained. Limits of detection for both of the dyes were 0.1 mg L^{-1}. The recoveries of the dyes ranged from 92 to 102%. Intra and inter-day precision expressed as relative standard deviation (RSD%) at 5.0 and 50 mg L^{-1} levels less than 8.0% were also achieved. This method has been applied successfully in the determination of the sunset yellow and azorubine in soft drink samples. The average sunset yellow concentration of the 42 soft drink samples range between 20.6- 60.2 mg L^{-1}. Also, azorubine was found in the soft drink samples in the range of N.D-4.4 mg L^{-1}. There results confirmed that there are two different formulations in soft drink sample samples. Obtained results showed that levels of the sunset yellow individually or in combination with azorubine in all of the tested samples is lower than EU maximum permitted level (100 mg L^{-1}).*
Keywords: *Synthetic dyes, High performance liquid chromatography, Sunset yellow, Azorubine, Carbonated orange flavor soft drinks.*

I. Introduction

Carbonated soft drinks are very popular beverages. Although their regular intake has been associated with human health problems [1-3], carbonated soft drinks remain one of the most frequently consumed ready-to-drink beverages in the world. They present particular characteristics such as high sugar content and acidity, and are available on the market in several flavors. In Iran, the most popular carbonated soft drinks are orange or cola-based.

Synthetic dyes are usually added to foodstuffs and soft drinks not only to improve appearance, colour and texture but also to maintain the natural colour during process or storage [4]. Compared to natural dyes, synthetic dyes show several advantages such as high stability to light, oxygen and pH, colour uniformity, low microbiological contamination, relatively lower production costs, etc [5] .

Sunset yellow (SY, E-110) and azorubine (E-122) are two highly used synthetic dyes which are added to many food products [6]. The presence and content of these dyes must be controlled due to their potential harmfulness to human beings [7].

An extensive review on the genotoxicity of food, drug, and cosmetic azo dyes and other dyes had been presented in the literature [8]. The European Union [9] has issued comprehensive schemes that regulating the use of food colours and their allowed levels in all food products. The maximum level of SY, and azorubine dyes should not be more than 100 mg L^{-1} (individually or in combination) in nonalcoholic beverages with added juices and/or flavours [6]. Accordingly, monitoring of synthetic food dyes in high consumption products such as beverages becomes an essential task. Thus, it is necessary to develop accurate and reliable analytical methods for the confirmative determination of synthetic food dyes in soft drinks to ensure food safety and consumer health.

A large number of analytical methods for food colors have been proposed, such as thin layer chromatography (TLC) [10], voltammetry [11], polarography [12], spectrophotometry [13,14], capillary electrophoresis (CE) [15,16], ion chromatography [17], and high-performance liquid chromatography (HPLC) [18-24]. Voltammetry, polarography, and spectrophotometry are simple and rapid methods, however, they are not suitable for determining multiple food color mixtures in the food matrix. Therefore, HPLC is extensively used for determination of multiple food color mixtures in the food matrix.

Usually in formulation of orange flavored soft drinks sunset yellow is used but in some formulation combination of sunset yellow and azorubine is also used in order to increase intensity of orange color. Therefore, the aim of this study was to develop and validate a fast, reliable, selective and economic HPLC method for routine analysis of sunset yellow and azorubine dyes in orange flavored soft drinks samples in food analysis laboratories.

II.   Material and methods

2.1. Chemicals and reagents

HPLC-grade methanol was obtained from Merck Company (Darmstadt, Germany). Analytical grade ammonium acetate and acetic acid were purchased from Merck Company (Darmstadt, Germany). Water was purified using a Milli-Q Ultrapure water purification system (Millipore, Bedford, MA, USA). Azorubine and Sunset Yellow FCF (SY) were purchased from Sigma-aldrich (Steinheim, Germany). The structures, names and color index (C.I.) numbers of the dyes used in this study are shown in Fig. 1. All of the stock solutions (1000 mg L^{-1}) were prepared in water. Working solutions (20 mg L^{-1}) were prepared freshly mixing the stock solutions and diluting with water. Ammonium acetate buffer pH = 6.7 with concentration of 100 mM was prepared by dissolving proper amount of salt in deionzed water by adjusting with dropwise addition of acetic acid followed by filtration.
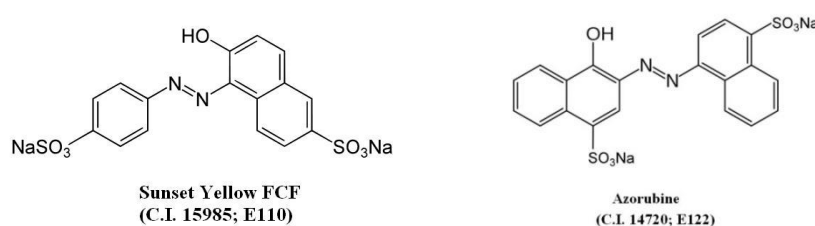

Fig. 1. Chemical structure, name, color index and Enumber of the dyes used.

2.2. Apparatus

The chromatographic analysis was carried out in a high-performance liquid chromatography from Knauer of Germany model EuroChrom consisting of a degasser, quaternary pump (model K1100), manual sample injector with 20 μL loop size and UV detector (model K2600) was controlled by EZChrom software. The HPLC operating mode was gradient, the injection volume was 20 μL and the column temperature was adjusted at room temperature. The chromatography column was a Supelcosil LC-18: 25cm × 4.6 mm, 5μm (Supelco, Bellefonte, PA, USA). Sample data collection was optimized to 8 min per sample with UV detection at wavelength of 500 nm. Mobile phase used was combination of methanol and ammonium acetate buffer (100 mM) pH = 6.7 (22:78, v/v%) in isocratic mode. The mobile phase was filtered through a 0.45 μm pore size filter (Merck Millipore, Billerica, Massachusetts, USA) and degassed by vacuum prior to use. Moreover, the mobile phase flow rate was set to 1.0 mL min^{-1}. All of the pH measurements were performed with a WTW Inolab pH meter (Weilheim, Germany).

III.   Results and Discussion

3.1. Choosing the best conditions for detection and separation of the dyes

It is well known that the detection wavelength is one of the most important factors affecting the sensitivity of the method. So, in order to achieve the highest sensitivity in determination of the dyes, the detection of them must be done in their λ_{max}. λ_{max} for SY and Az is reported 485 nm and 515 nm, respectively. So, detection wavelength was evaluated in the rage of 485-515 nm. Results showed that detection sensitivity is good for SY when $\lambda < 500$ nm specially 485 nm (λ_{max}). On the other hand, detection sensitivity is good for Az when $\lambda > 500$ nm specially 515 nm (λ_{max}). So, under this circumstance, detection wavelength of 500 nm was selected in order to obtain good sensitivity for both of the dyes.

Composition of mobile phase is key factor in resolution of chromatographic separation as same as run time of analysis. Mobile phases containing ammonium acetate buffer as buffering compounds are obviously recommended as the most suitable to assure the very good chromatographic separation of the sulphonated azo dyes [23]. Several different mobile phases were tested including methanol–acetate buffer (15:85), methanol–acetate buffer (20:80), methanol–acetate buffer (22:78), methanol–acetate buffer (25:75), methanol–acetate buffer (30:70). Obtained results showed that in low percent of methanol (methanol–acetate buffer (15:85)), azorubine was eluted so late. On the other hand, in high percent of methanol (such as methanol–acetate buffer (30:70)), resolution of SY and Az are not good. So, by evaluating these results, the mobile phase of methanol–acetate buffer (22:78) was selected because of suitable resolution, good peak shape and also short run time (10.0

min). Under optimum condition, the retention times for SY (first peak) and Az (second peak) are about 3.5 and 8.5 min, respectively.

3.2. Analytical performance
3.2.1. Linear range

The linearity for each compound was checked by analyzing mixed standard solutions of 11 different concentrations (0.1, 0.25, 0.5, 1, 2, 5, 10, 20, 30, 40, and 50 mg L^{-1}). *Table* 1 shows the linear range found for each dyes and the regression coefficient obtained, expressed as r^2. As can be seen, a wide and convenient linear range was obtained for the dyes. Calibration curves of sunset yellow and azorubine are shown in Fig. 2.
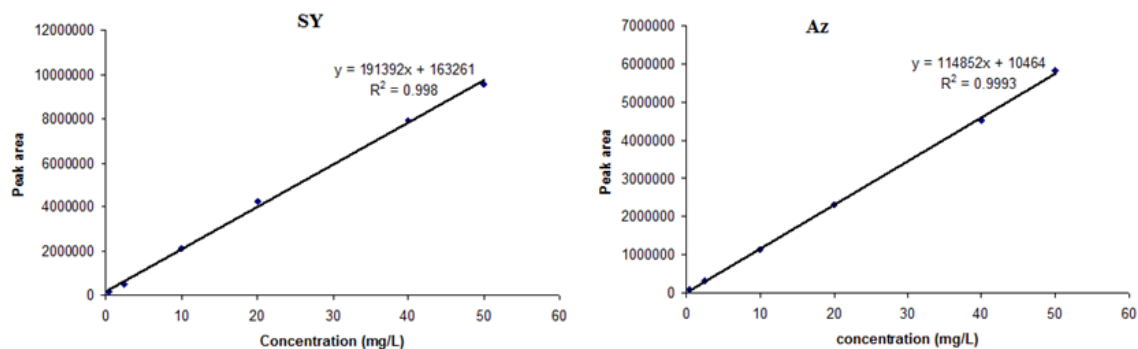

Fig. 2. Calibration curves of sunset yellow and azorubine

Table 1. Analytical characteristics of the HPLC method

Dyes	RSD%						LOQ (mg/L)	LOD (mg/L)	Linear Range (mg/L)	R^2
	Inter-days (n=6)			Intra-day (n=6)						
	0.5[a]	5.0	50	0.5	5.0	50				
SY	8.3	7.3	5.4	5.5	3.9	2.8	0.25	0.1	0.25-50	0.9980
Az	6.5	6.8	4.7	4.8	5.2	3.5	0.25	0.1	0.25-50	0.9993

[a]Concentration unit is (mg L^{-1})

3.2.2. Precision

The method exhibited excellent precision, as shown in *Table* 1. Fortification/recovery experiments resulted in low intra day relative standard deviations (R.S.D.s) for the analytes (n =6, R.S.D.s < 8%) at three different levels (0.5, 5, 50 mg L^{-1}). A comparison of fortification/recovery experiments conducted on six different days (n = 6) at three different levels (0.5, 5, 50 mg L^{-1}) also displayed low inter-day R.S.D.s (<9%), confirming the excellent reproducibility of the method. Moreover, inter-laboratory study was done. Results are summarized in *Table* 2. Obtained results showed that developed method is completely reproducible.

Table 2. inter-laboratory study results

Laboratory ID	Sample code 1		Sample code 2		Sample code 3	
	SY (mg L^{-1})	Az (mg L^{-1})	SY (mg L^{-1})	Az (mg L^{-1})	SY (mg L^{-1})	Az (mg L^{-1})
Lab 1	57.9	<0.1	26.2	2.5	46.6	<0.1
Lab 2	56.1	<0.1	25.4	2.3	46.9	<0.1
Lab 3	51.7	<0.1	24.4	2.9	43.8	<0.1
Lab 4	52.9	<0.1	24.3	2.9	45.1	<0.1
Lab 5	56.0	<0.1	25.1	2.7	46.0	<0.1
	Statistical Results					
Mean	54.9	-	25.1	2.6$_6$	45.7	-
SD	2.5	-	0.7$_8$	0.2$_6$	1.2	-
RSD%	4.6	-	3.1	9.8	2.7	-

3.2.3. Detection and quantitation limits

Detection limits were calculated by extracting diluted solutions of the analytes (0.01, 0.05, 0.1 and 0.2 mg L^{-1}). The criteria were selected according to IUPAC and ACS definition, as follows:

Detection limit (DL): $A_s - A_b = 3S_b$

Quantitation limit (QL): $A_s - A_b = 10S_b$

were A_s is the average of sample signal (area); A_b is the average of blank signal (area) and S is the standard deviation of blank signal (area). The obtained limits of the method are presented in *Table* 1.

3.2.4. Accuracy

Due to the lack of Certified Reference Materials, accuracy evaluation, in terms of percentage of recovery, was carried out on blank samples spiked with a known amount of analyte (level of 10 mg L^{-1}). Data for these experiments are shown in *Table* 3. Good results were obtained, with average recoveries ranging from 92 to 102% and also between 92 and 101% for sunset yellow and azorubine, respectively. It is interesting to note that, samples did not require matrix matched calibration curves to compensate for difficulties in measuring peak area for the dyes at low concentration, making more rapid analysis possible.

Table 3. Results of food color recovery study

Sample ID	C $_{added}$ (mg L^{-1})	[a]Recovery%	
		Sunset yellow	**Azorubine**
Code 1	10.0	96	97
Code 2	10.0	92	101
Code 3	10.0	98	94
Code 4	10.0	102	95
Code 5	10.0	94	92
Code 6	10.0	98	97
Code 7	10.0	96	94
Code 8	10.0	93	99
Code 9	10.0	97	93
Code 10	10.0	99	96

[a] RSD values for three replicate measurements for all of the tested samples were less than 10%

3.3. Application

The validated method was applied to determine content of sunset yellow and azorubine in different soft drink samples collected locally and the results are summarized in *Table* 4. RSD values for three replicate measurements for all of the tested samples were less than 7.0%. In the analysis of the samples, peak identification was based on the comparison between the retention times of standard compounds and was confirmed by spiking standards to the samples. Quantification was based on the external standard method using calibration curves fitted by linear regression analysis. Under the stated experimental conditions, baseline separation of the two dyes was achieved in less than 10 min. A typical HPLC-UV-Vis chromatogram of a carbonated soft drink sample before (3a) and after spike (3b) 10 mg L^{-1} of the each dyes is shown in Fig. 3

The average sunset yellow concentration of the 42 soft drink samples range between 20.6- 60.2 mg L^{-1}. Also, azorubine was found in the soft drink samples in the range of N.D-4.4 mg L^{-1}. There results confirmed that there are two different formulations in soft drink sample samples. In some samples only sunset yellow has been used to produce orange color but some companies has been used combination of the both sunset yellow and azorubine in their formulation. Moreover, sum of the sunset yellow and azorubine concentration in the tested soft drink sample are in compliance with EU limit (100 mg L^{-1}).
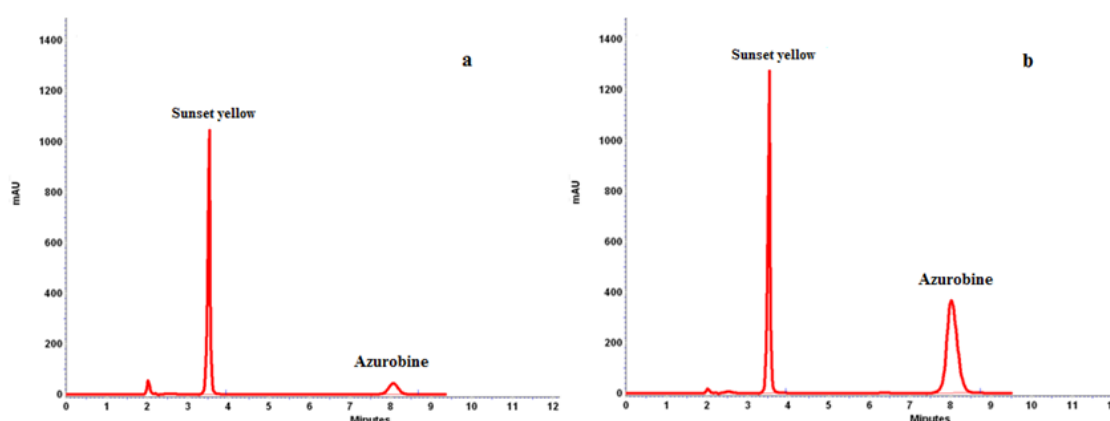

Fig. 3. HPLC-UV-vis chromatogram (λ = 500 nm) sample of soft drink 15 (a) nonspiked and (b) spike of 10 mg L^{-1} of mixture standard solution of the two dyes.

IV.   Conclusion

Due to the lack of International Standard Method for the determination of the synthetic dyes in foodstuff, in this research we propose an analytical method for the simultaneous determination of the sunset yellow and azorubine in different soft drink samples. Proposed method is simple, sensitive, rapid, reliable and allows good recoveries of

the sunset yellow and azorubine in the tested samples. Analysis of the samples showed that some samples only have sunset yellow. In contrast, some companies are used combination of the both sunset yellow and azorubine in their formulation. Moreover, sum of the sunset yellow and azorubine concentration in the tested soft drink sample are in compliance with EU limit (100 mg L^{-1}).

Table 4. Concentration of the food colorants in the samples

Sample ID	Sunset yellow (mg Kg^{-1})	Azorubine (mg Kg^{-1})	Sample ID	Sunset yellow (mg Kg^{-1})	Azorubine (mg Kg^{-1})
Code 1	44.7	<0.1	Code 22	29.4	1.3
Code 2	38.7	<0.1	Code 23	56.9	<0.1
Code 3	53.7	<0.1	Code 24	20.6	4.4
Code 4	42.1	<0.1	Code 25	38.1	0.8
Code 5	40.0	<0.1	Code 26	27.0	1.4
Code 6	57.9	<0.1	Code 27	28.7	1.0
Code 7	43.7	<0.1	Code 28	29.0	1.0
Code 8	45.8	<0.1	Code 29	25.6	<0.1
Code 9	25.4	1.0	Code 30	26.9	1.7
Code 10	26.2	1.0	Code 31	60.2	<0.1
Code 11	25.1	3.1	Code 32	27.6	1.5
Code 12	26.2	2.5	Code 33	24.8	1.1
Code 13	39.2	<0.1	Code 34	21.2	1.3
Code 14	40.4	<0.1	Code 35	29.9	1.4
Code 15	36.7	<0.1	Code 36	24.4	0.6
Code 16	41.2	<0.1	Code 37	50.8	<0.1
Code 17	46.6	<0.1	Code 38	27.6	1.9
Code 18	25.5	1.3	Code 39	21.4	1.5
Code 19	27.6	1.0	Code 40	29.6	<0.1
Code 20	28.2	1.3	Code 41	38.6	<0.1
Code 21	27.0	1.2	Code 42	29.0	2.3
		Statistical analysis			
		Sunset yellow		Azorubine	
Average		34.5 (mg Kg^{-1})		1.55 (mg Kg^{-1})	
Range		20.6-60.2 (mg Kg^{-1})		4.4 (mg Kg^{-1})-<0.1	
SD		10.8 (mg Kg^{-1})		0.84 (mg Kg^{-1})	

The EU limit is (100 mg L^{-1})

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