ACADEMIA ROMÂNĂ
Revue Roumaine de Chimie
http://web.icf.ro/rrch/

Rev. Roum. Chim.,
2014, *59*(9), 719-725

DEVELOPMENT AND VALIDATION OF AN HPLC-UV METHOD FOR DETERMINATION OF SYNTHETIC FOOD COLORANTS

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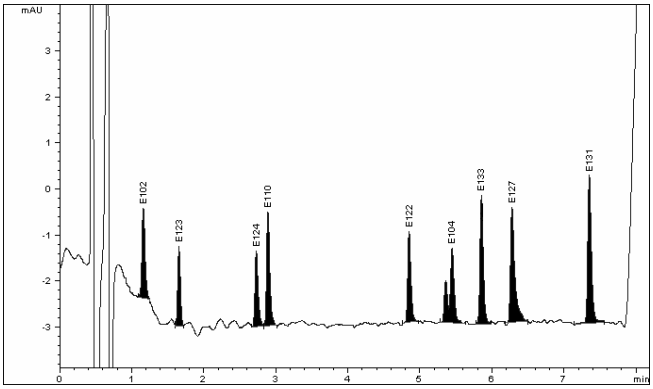
Received November 15, 2013

Liquid chromatography with UV detection has been used to develop a determination method for nine water-soluble synthetic colorants (E102, E123, E124, E110, E122, E104, E133, E127, E131). The colors were separated in 8 minutes on a C18 analytical column, using a mobile phase mixture of 100 mM ammonium acetate/acetonitrile gradient elution. The analytical characteristics of the separation were evaluated. Good linearity (R^2=0.9982-0.9997) and adequate limits of quantification were achieved.



INTRODUCTION

Natural or synthetic food colorants are often added to foodstuffs and soft drinks in order to maintain the natural color during process or storage and to create the desired colored appearance. However, synthetic dyes have more advantages than natural dyes such as low price and high stability to light, oxygen and pH, color uniformity, low microbiological contamination. At present, synthetic dye is widely used to make food more attractive and appetizing. Due to its toxicity, especially when consumed in excess, synthetic dyes is strictly controlled by laws, regulations and acceptable daily intake values for food safety.[1]

Table 1

Maximum acceptable daily intake values for studied synthetic food colorants

Colorant	Maximum acceptable intake values (mg/kg)
E102	10
E123	30
E124	10
E110	40
E122	70
E104	100
E133	10
E127	10
E131	10

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Most synthetic colorants are acidic, water soluble substances, which are usually used as the sodium salts. They can be divided into the azo, triarylmethane, chinophthalon (quinoline yellow E 104), xanthene (erythrosine E 127) and indigo (E 132) colorant classes.[2] Azo colors are characterized by at least one or two azo groups in the molecule connecting two or three aromatic ring systems. Permitted azo dyes are: tartrazine (E102), sunset yellow (E110), azorubine (E122), amaranth (E123), cochineal red (E124), red 2G (E128), allura red (E129), brilliant black BN (E151), brown FK (E154) and brown HT (E155). The triarylmethane colorants contain three substituted aryl residues covalently linked to the central carbon atom. The resulting patent blue V (E131), brilliant blue FCF (E133) and green S (E142) are permitted food colorants.

The chromophore groups in synthetic dyes (Fig. 1) can be analyzed with several methods such as visible spectrophotometry,[3] thin layer chromatography,[4] high performance liquid chromatography,[5,6] capillary electrophoresis[7,8] and ion chromatography.[9] Among the methods mentioned above, HPLC provided the highest sensitivity and the separation of synthetic dyes was performed on a reversed phase C18 column.

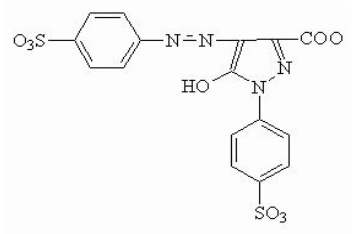
This technique ensures separation of any mixtures to component substances whereas appropriate detector allow to identification and quantitative determination of them. The basis of separation is differences of affinity components to two phases, stationary phase and mobile phase. Dyes have different adsorption affinity to stationary phase. That appears from differences of their mass, structural space and presence of functional groups in each dye's molecule. The most popular kind of HPLC technique is reversed phase high performance liquid chromatography (RP HPLC). In reversed phase system, stationary phase is slightly polar or non-polar, while mobile phase has stronger polarity (*e.g.* tetrahydrofuran, acetonitrile, methanol, water). Application of the appropriate conditions allows analyzing most of the food dyes.

The most important characteristics that must be taken into account during selection of analysis conditions are hydrophobic properties of assaying dyes and presence in their molecules of acidic groups. Hydrophobicity of azodyes is larger than others. It is similar in case of dyes with naphthalene ring in comparison to dyes with benzene ring. In purpose to improve the separation and shorten time of analysis, inorganic electrolyte is added to mobile phase. That kind of modificator often is ammonium acetate buffer in optimal concentration above 0.1 mol/L (L = liter). Examples of chromatographic conditions for HPLC technique (eluent, stationary phase, flow rate and time of analysis) which have been used to determination of dyes are listed in Table 2.

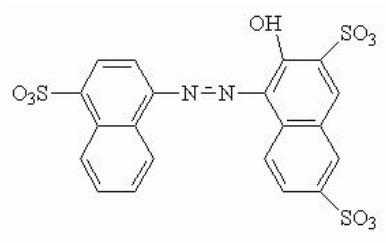
Table 2

Examples of chromatographic systems for HPLC technique

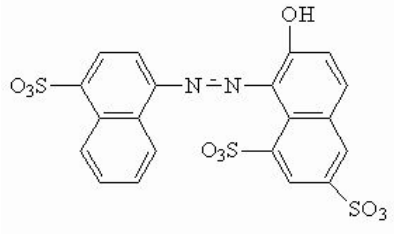
Column	Detector	Mobile Phase	Time of analysis (min)	Flow rate (ml/min)	Ref
C18 (25 cm x 4.6 mm, 5 µm)	UV-DAD	(A) tetrabutylammonium dihydrogen phosphate in Na_2HPO_3 (B) A/methanol (1:4) (gradient elution 45-100% A)	35	1	10
Tracer Analitica ODS (15 cm x 4 mm, 5 µm)	UV-VIS	(A) methanol (B) ammonium acetate 40 mM (pH5) (gradient elution 10-75% A)	20	1	11
C18 (25 cm x 4.6 mm, 5 µm)	UV-DAD	(A) acetonitrile: methanol (20:80) (B) 1% ammonium acetate buffer (pH 7.5) (gradient elution 100-47.5%A)	31	1.5	12
Zorbax (25 cm x 4.6 mm, 5 µm)	UV-DAD	I (A) methanol/(B) ammonium acetate (0.08 mol/L) II (A) methanol/(B) EDTA	5	1	13
Cosmosil C18 (125 x 2 mm, 5 µm)	UV-DAD, ES-MS	(A) triethylamine + acetic acid (pH 6.2) (B) methanol (gradient elution 95-30%-A 35 min, 30-95%-5 min)	40	0.2	14
C18 (25 cm x 4.6 mm, 5 µm)	UV-DAD	(A) 0.02 M ammonium acetate (B) methanol (gradient elution 20-100-20% B)	35	1	15
Lichrospher 100 RP-18 (250 x 4 mm, 5 µm)	UV-DAD	(A) methanol (B) triethylamine 5 mM, acetic acid 5 mM aqueous solution (pH 6.5)	19	0.3	16
BDS Hypersil C18 (250 x 4.6 mm, 5 µm)	UV-DAD	(A) methanol (B) tetrabutyl ammonium hydrogenosulfate (0.005 M, pH 3.5) (gradient 25-100% A)	16	0.8	17



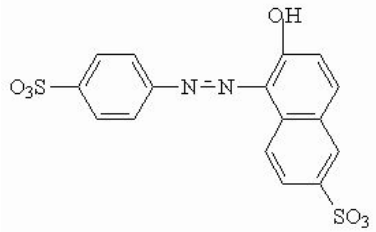
E 102 Tartrazine



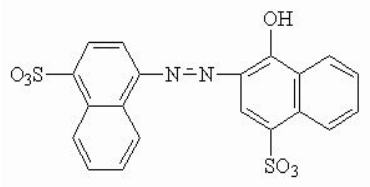
E 123 Amaranth



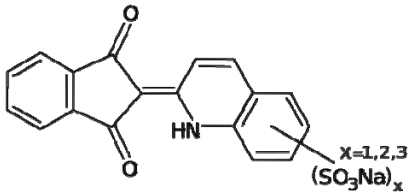
E 124 Ponceau 4R



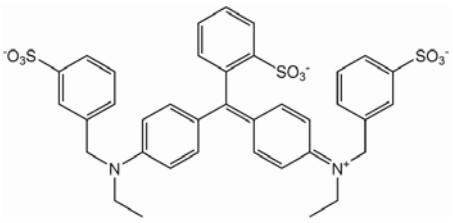
E 110 Sunset Yellow



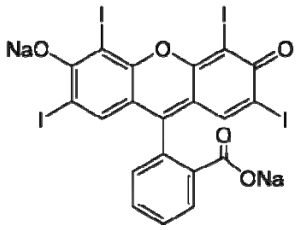
E 122 Azorubine



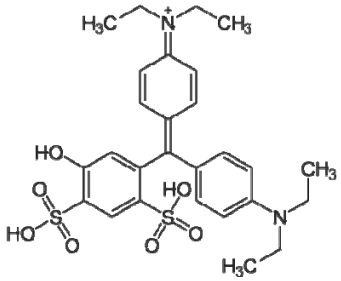
E 104 Quinoline Yellow



E 133 Brilliant Blue



E 127 Erythrosine



E 131 Patent Blue

Fig. 1 – Structure formula of synthetic dyes.

Therefore, the purpose of this study was to develop HPLC-UV method for the separation of nine synthetic dyes in short analysis time and under high sensitivity.

RESULTS AND DISCUSSION

The development of chemical and instrumental methods for the separation, identification and

quantitative analysis of synthetic food colors has become extremely important for the food and beverages industry, academic and governmental institutions to assess the quality and safety of food products. Given the biological variability in the pigment content of the fruit and vegetable extracts used in food and beverages, and variable losses during processing, manufacturers will usually use color additives to ensure that the product will always provide a certain degree of color uniformity. Therefore, it seems necessary the quality control of these products by measuring the level of the synthetic colors added.

A chromatogram obtained from a mixed standard solution of the nine dyes, with detection at the absorption maxima of the dyes is presented in figure. All dyes could be separated and detected within 8 minutes. There are two reasons why in chromatogram are present only ten piks, because for E104 is possible that one of the three isomers is present in a very low ratio compared to the other two, or it is possible to elute together with one of the other two isomers.

As for linearity, the calibration functions were calculated by linear regression. Three replicates for each concentration were analyzed allowing the evaluation of lower and upper quantification limits and correlation coefficients of the studied dyes as shown in Table 3. QL are between 50 and 10000 ng/ml. Correlation coefficients were always larger than 0.998, showing a good relationship between peak area and concentrations in the studied range.

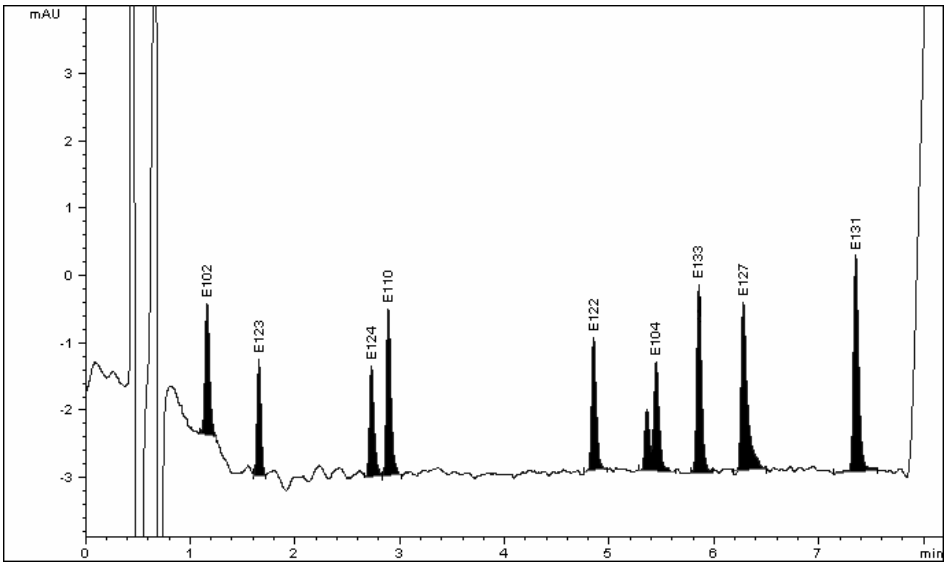


Fig. 2 – Chromatogram obtained from mixed standard solutions at LOQ.

Table 3

Chromatographic performance of the method for analysis of synthetic food dyes

Colorant	Wavelength (nm)	Retention time (± S.D.)	Calibration range **LLOQ (ng/ml)**	**ULOQ (ng/ml)**	Equation of calibration plot	R^2
E102	420	1.14 (0.049)	50.1	10020	y = 0.0606(±0.0013)x + 0.1901(±0.3682)	0.9986
E123	520	1.62 (0.047)	50.9	10180	y = 0.0535(±0.0009)x - 0.3175(±0.4762)	0.9988
E124	495	2.67 (0.100)	52.35	10470	y = 0.0446(±0.0012)x + 0.3978(±0.4619)	0.9993
E110	495	2.86 (0.049)	53.25	10650	y = 0.0680(±0.0007)x + 0.4998(±0.2693)	0.9997
E122	520	4.81 (0.052)	50.55	10110	y = 0.0604(±0.0002)x + 0.5487(±0.0595)	0.9997
E104	430	5.41 (0.051)	52.35	10470	y = 0.0824(±0.0009)x + 0.9561(±0.4610)	0.9991
E133	600	5.82 (0.058)	52.4	10480	y = 0.0832(±0.0007)x + 0.3652(±0.1610)	0.9997
E127	527	6.24 (0.036)	53.55	10710	y = 0.1012(±0.0026)x + 2.5516(±0.8955)	0.9982
E131	600	7.33 (0.029)	54.3	10860	y = 0.2765(±0.3038)x + 0.4063(±0.4596)	0.9996

The developed method was evaluated in terms of accuracy and precision, as shown in table below:

Table 4

Intra-day and inter-day accuracy and precision for the measurement of dyes

Colorant	Theoretical concentration (ng/ml)	Intra-day		Inter-day	
		Bias	**CV(%)**	**Bias**	**CV(%)**
E 102	50.1	4.93	3.25	8.85	8.55
	400.8	-8.05	0.92	-4.99	2.28
	6012.0	-1.69	0.13	-1.66	0.25
E 123	50.9	2.42	3.35	2.32	1.72
	407.2	4.97	0.2	2.72	2.27
	6108.0	1.88	0.25	1.73	0.35
E 124	52.4	-1.86	1.9	2.09	3.43
	418.8	1.4	0.86	-0.08	1.52
	6282.0	1.68	0.29	1.54	0.4
E 110	53.3	-2.25	2.67	1.68	4.56
	426.0	0.48	0.35	-0.08	0.88
	6390.0	2.19	0.33	1.94	0.56
E 122	50.6	-1.81	5.44	8.35	3.75
	404.4	-2.46	1.41	-2.11	1.57
	6066.0	1.23	0.21	1.13	0.26
E 104	52.4	5.64	5.39	2.43	3.88
	418.8	1.16	0.72	-2.05	2.41
	6282.0	1.91	0.27	1.82	0.18
E 133	52.4	4.13	2.61	4.80	1.80
	419.2	-1.78	0.55	-2.21	0.77
	6288.0	1.49	0.23	1.37	0.25
E 127	53.6	1.59	2.09	3.77	3.16
	428.4	1.46	2.53	1.37	3.33
	6426.0	5.47	3.73	5.85	4.95
E 131	54.3	3.19	2.16	1.45	4.72
	434.4	-0.36	0.48	-1.31	1.20
	6516.0	2.64	0.42	2.08	0.47

The developed HPLC method was applied to analysis of synthetic dyes in soft drinks, candies and pudding powders. As expected, most of the studied foodstuffs showed two or three dyes in their composition, due to the desired colors of the final product. An example is shown in following figure:

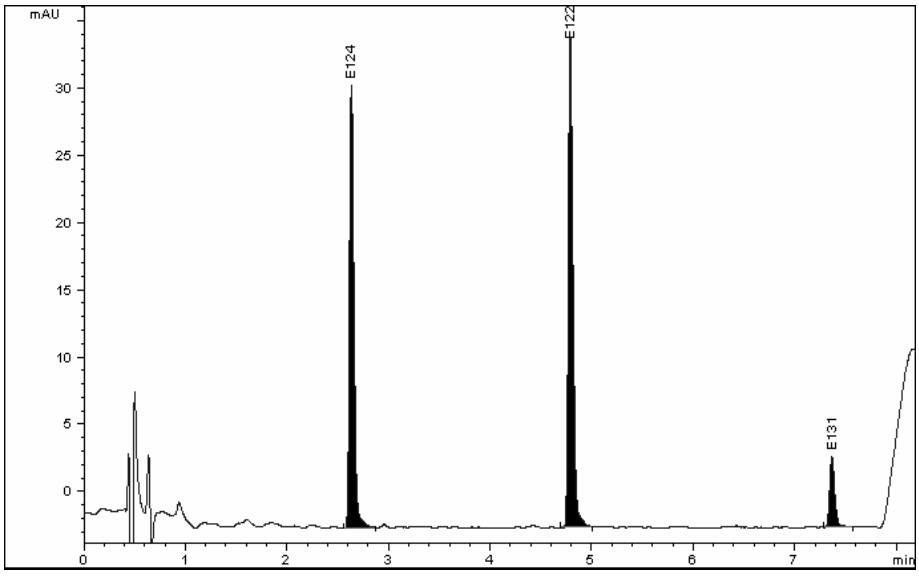


Fig. 3 – Allegria raspberry juice.

Table 5

Retention time and wavelength for studied dyes

Line	Time (min)	Wavelength (nm)
1	0.00	420
2	1.37	520
3	2.00	495
4	4.00	520
5	5.05	430
6	5.55	600
7	6.00	527
8	7.00	600

EXPERIMENTAL

Reagents

The standard synthetic dyes were provided by Merck (Merck KgaA, Darmstadt, Germany). Methanol, acetonitrile, ammonium acetate were purchased from Merck (Merck). KgaA, Darmstadt, Germany Distilled, deionised water was produced by a Direct Q-5 Millipore (Millipore SA, Molsheim, France) water system.

Standard solutions

Synthetic dyes stock solution were prepared in methanol at concentrations of around 1 mg/ml. Working solutions were prepared by diluting specific volumes of stock solution with water. Calibration standards were prepared at the beginning of each analytical run by serial dilution of water using the working solutions, yielding nine standards with concentration ranged between 50 and 10000 ng/ml. Quality control samples (QC) containing dyes at 100, 1000 and 10000 ng/ml were prepared in order to evaluate the precision and accuracy of the method, as well as the stability.

Liquid Chromatography and UV Conditions

The HPLC system (1100 series, Agilent Technologies, Palo Alto, CA, USA) was coupled to an UV detector, working after the program from table. Data acquisition was performed using Chemstation software (Agilent Technologies, Palo Alto, CA, USA), version B.01.03.

For the separation, a reversed phase Agilent SB C18 analytical column, 100x3mm, 3.5 µm particles was used (Agilent Technologies, USA). The used column is extremely stable at pH between 1 and 8, at temperature up to 90°C, therefore no change in the retention time of the compounds analyzed along the analytical series was observed. Although compounds such as sucrose, preservatives, stabilizers, being polar are not retained on hydrophobic column, after each analytical series column was washed for 30 min with acetonitrile. The column was operated at 45 ºC in a G1316A oven. For the elution a degasser (G1322A), and a binary gradient pump (G1311A) were used. The mobile phase consists in a mixture of 100 mM ammonium acetate (A)/acetonitrile (B) gradient elution (beginning with 4% B, until 7 min 35% B, to 7.2 min 35% B, until 7.21 min 4 %B). The flow rate was 1 mL/min and the injection volume 25 µL.

Sample pretreatment

Juice sample pretreatment consisted in degassing for two minutes in the ultrasonic bath, followed by centrifugation at 12000 rpm (2-16 Sartorius centrifuge, Osterode am Harz, Germany) for 3 min. A 0.2 ml of the supernatant liquid was then transferred into a HPLC autosampler vial for injection of 25µl onto the column. The candy samples were dissolved into 100 ml water and 0.2 ml were transferred into HPLC autosampler vial for injection onto the column.

Method validation

The concentration of the analytes was determined automatically by the instrument data system. The calibration curve model was y = ax + b, linear response, weighting scheme 1/y, where y-peak area and x-concentration. A weighting scheme of 1/y, corresponding to a Poisson model error or standard deviation of the error is constant, can be used in any regression, linear or nonlinear, the response error depends on the measured value. In this case, the total error in estimating chromatographic peak area, due to any factor related to analyst/analysis methodology depends on all these factors in a proportional manner, therefore it was used. The distribution of the residuals (% difference of the back-calculated concentration from the nominal concentration) was investigated. The calibration model was accepted if the residuals were within ±20% at the lower limit of quantification (LOQ) and within ±15% at all other calibration levels and at least 2/3 of the standards meet this criterion, including highest and lowest calibration levels.

The lower limit of quantification was established as the lowest calibration standard with an accuracy and precision less than 20%.

The intra- and inter-day precision (expressed as coefficient of variation, CV%) and accuracy (expressed as relative difference between obtained and theoretical concentration, bias%) of the assay procedure were determined by analyzing on the same day five different samples at each of the lower, medium and higher levels of the considered concentration range and one different sample of each at five different occasions, respectively.

CONCLUSIONS

The versatility of HPLC as an analytical tool makes it an ideal technique for analytical quality control and research and development laboratories in the food and beverage industry. We propose a rapid and interference-free HPLC method. The description of the method is accompanied by a detailed characterization of its performance. Detection limits for the additives in the beverages were found to be satisfactory. The method is

recommended for the determination of those additives in food and soft drinks. The results obtained with the samples confirm that the proposed method works well and is useful for a serious control or screening of the addition of synthetic colors in soft drinks.

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