THE LIQUID-CHROMATOGRAPHIC QUANTIFICATION OF SOME SYNTHETIC COLORANTS IN SOFT DRINKS

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Abstract: *A simple, selective, and precise reversed-phase HPLC method with "diode array" detection has been developed, enabling separation and quantification, in a single run of tartrazine (E 102), amaranth (E 123), erythrosine (E 127) and indigotine (E 132) in soft drinks. HPLC was performed with a Surveyor Thermo Electron system and the reversed phase chromatographic separation was achieved at 30 °C, using a DS Hypersil C18.5 μm column and a mobile phase made of 100 mM sodium acetate buffer and acetonitrile. Through optimization studies they were established: the gradient program of the elution, the injected volume 5 μL, the flow rate of the mobile phase 1mL/min, the duration of the analysis being 30 min. The synthetic colorants were detected by absorbance at λ = 430 nm for tartarzine, λ = 520 nm for amaranth and erythrosine and λ = 608 nm for indigotine. The method was validated in terms of sensitivity, linearity range, reproducibility, repeatability, analytical recovery and robustness.*

Keywords: *tartrazine, amaranth, erythrosine, indigotine, HPLC, "diode array" detection, soft drinks*

INTRODUCTION

Colorings have neither nutritional value, nor flavor, yet they are very important in terms of marketability and acceptability. Reasons for food coloring are: to make up the color loss due to light, air, temperature, moisture and storage; to correct natural variations in color; to enhance the natural colors associated with a given product; to provide a colorful identity to the colorless or dull looking foods; to provide a colorful appearance to "fun foods" [1]. Today all food color additives are carefully regulated by authorities to ensure that foods are safe to eat and accurately labeled.

Artificial colorings must be approved by the Food and Drug Administration (FDA) and produced under controlled situations. They are often preferred over natural colorings because they are more stable, add no flavor, are less expensive, blend more easily and can be used in smaller amounts because they are more intense. In the past, artificial colorings had been linked with hyperactivity in children. But a 1980 report of the Nutrition Foundation concluded that there is no evidence that artificial food colorings produce hyperactivity or learning disabilities in children. They did acknowledge, however, that dietary restrictions may have a placebo effect in that adults may perceive a change in the child's behavior when diet changes are made [1].

Safety data, such as the acceptable daily intake, based on toxicological studies on experimental animals and human clinical studies, have been repeatedly determined and evaluated by Food and Agricultural Organization (FAO) and World Health Organization (WHO) [2].

The following azo dyes are permitted in the European Union (E numbers in parentheses): Tartrazine (E 102), Sunset Yellow FCF (E 110), Azorubine (E 122), Amaranth (E 123), Cochineal Red (E 124), Red 2G (E 128), Allura Red AC (E 129), Brilliant Black BN (E 151), Brown FK (E 154) and Brown HT (E 155). The group of triarylmethane colorants is represented by Patent Blue V (E 131), Brilliant Blue FCF (E 133), and Green S (E 142). These dyes contain three substituted aryl residues covalently linked to the central carbon atom. Other classes of food colorants are chinophthalon- (Quinoline Yellow, E 104), xanthene- (Erythrosine BS, E 127), and indigo dyes (Indigotine, E 132) [3, 4].

Tartrazine (E 102) is one of the most used synthetic colorants in the food industry. Tartrazine is used to colour drinks, sweets, jams, cereals, snack foods, canned fish, packaged soups. Tartrazine is known to provoke asthma attacks and urticaria in children. It is also linked to thyroid tumours, chromosomal damage, urticaria (hives) and hyperactivity. Tartrazine sensitivity is also linked to aspirin sensitivity. Because of these harmful effects it is banned in Norway and Austria. The acceptable daily intake (ADI) for tartrazine is 7.5 mg/kg body weight.

Amaranth is a reddish brown odorless mono azo dye and it is one of the most widely tested dyes, yet a consistent characterization of its genotoxicity has not emerged, and its status as a carcinogen remains equivocal [5]. The acceptable daily intake (ADI) for amaranth is 0.75 mg/kg body weight.

Indigotine is the common name for uncertified FD&C Blue No. 2 and it is a blue color, naturally present in the shrub *Indigofera tinctoria*, though commercially it is produced synthetically. The daily intake for indigotine is up to 5 mg/kg body weight. Indigotine has no dietary restrictions and side effects rarely occur in the concentrations used in foods. Rare allergic reactions have been described, due to coupling of the color to body

proteins. It can also function as a histamine liberator. Indigotine (E 132) is commonly added to tablets and capsules; it is also used in ice cream, sweets, soft drinks, baked goods, confectionary, and biscuits.
Erythrosine is a reddish-pink synthetic dye. It is most popularly used as a food coloring agent and a host of other applications such as printing inks, a dental plaque disclosing agent, a biological stain and a radiopaque medium. It is used in cocktails, tinned fruits, biscuits, chocolate, dressed crabs, garlic sausage, luncheon meat, salmon spread pate, scotch eggs, stuffed olives, sweets, bakery and snack foods.
The use of synthetic colorants in foods is strictly controlled by legislation and harmonized across the European Union by formulating the directive 94/36/EC [2]. Consequently, accurate and reliable methods for the determination of synthetic colorants are required for the assurance of food safety.
Many analytical techniques have been developed for the identification and determination of various synthetic food colorants, such as thin-layer chromatography, adsorptive voltammetry, differential pulse polarography, derivative spectrometry and spectrophotometric methods in combination with chemometrics, but all of them require time-consuming pretreatment or cannot be applied to complex colorant mixtures. Capillary electrophoresis and micellar electrokinetic capillary chromatography have also been used, but they have sensitivity problems as a result of small injection volume [2]. High-performance ion chromatography, reversed-phase liquid chromatography [6-8] and ion-pair liquid chromatography coupled with UV or diode-array detectors are still the most preferred methods, as they provide unrivalled resolution, sensitivity and selectivity. Both isocratic and gradient systems are used, and the latter are preferred for the separation of the more complex mixtures. High-performance liquid chromatography (HPLC) replaces the old chromatographic methods of analysis of food colorants (thin-layer chromatography, paper chromatography), supplying precise and reproducible results [9].
Kirschbaum et al. [7] developed an HPLC method enabling separation and quantification, in a single run, of the 14 food colorants most frequently used, i.e. E numbers 102, 104, 110, 122, 123, 124, 127, 128, 129, 131, 132, 133, 142 and 151.
Bratu et al. [10] elaborated an HPLC method for the determination of some synthetic food colorants, namely: tartrazine, ponceau 4R, allura red, sunset yellow and quinoline yellow. Another method for the determination of food colorants was proposed by Agilent Tehnologies using HPLC and diode-array detection at 190-950 nm [11].
The aim of this work was to develop an HPLC method enabling separation and quantification, in a single run of tartrazine (E 102), amaranth (E 123), erythrosine (E 127) and indigotine (E 132) in soft drinks. A significant set of validation data was performed through recovery and precision studies.

MATERIALS AND METHODS

HPLC was performed by using a Surveyor Thermo Electron system including vacuum degasser, Surveyor Plus LCPMPP pump, Surveyor Plus ASP auto sampler, "diode array" detector with 5 cm flow cell, DS Hypersil C18.5 μm column (250 mm × 4.6 mm) and Chrom Quest 4.2 software.
Certified reference materials have been used such as tartrazine (99.9%, Fluka 86310), amaranth (99.9%, Fluka 06409), erythrosine (90%, Sigma-Aldrich 198269) and

indigotine (98%, Sigma-Aldrich 131164). All the other reagents, acetonitrile (Baker, 8257), sodium acetate (98% Sigma-Aldrich 25023), hydrochloric acid (37%, Sigma-Aldrich 30721), were of analytical purity or for chromatographic use. The water used was ultrapure, Basic TWF. The stock solutions and the corresponding dilutions were made in ultra-pure water and were stored in dark places at low temperature (+4 °C) between experiments.

Mainly, the sample is degassed, filtered through a nylon syringe filter (0.45 μm) and adjusted to pH 6.0 by addition of 1N NaOH before injection, in order to ensure the colorants stability. The analytes in the test solution sample are separated by reversed-phase chromatography on a 250 mm × 4.6 mm i.d., 5 μm particle DS Hypersil C18 column, at 30 ^{o}C using a mobile phase made of sodium acetate buffer (100 mM), adjusted to pH 7.0 by addition of 0.1 M HCl and filtered through a polyamide membrane (0.2 μm) and acetonitrile.

As a result of an optimization of the separation, the best results were achieved by use of the gradient program presented in table 1. The colorants were detected by absorbance at 430 nm (tartarzine), 520 nm (amaranth, erythrosine) and 608 nm (indigotine) and were quantified by using a calibration graph.

Table 1. *Gradient program for separation of the food colorants*

Time (min)	Mobile phase A* (%)	Mobile phase B* (%)	Flow rate (mL/min)
0	95	5	1
12	95	5	1
15	70	30	1
22	70	30	1
25	95	5	1
30	95	5	1

* Mobile phase A is 100 mM NaOAc (pH 7.0) and mobile phase B is MeCN.

After a complete series of analysis, the HPLC system was cleaned using an adequate washing method. The mobile phase in this method is made of water and acetonitrile, using a moderate gradient to attain the suitable conditions for the column keeping.

RESULTS AND DISCUSSION

Validation of the method

To test linearity, standard solutions of 10 mg/L, 20 mg/L, 30 mg/L, 40 mg/L and 50 mg/L were prepared and analyzed with three replicates and the results processed with Chrom Quest 4.2 software. The calibration graphs are linear, with five calibration levels. The equations of the calibration graphs, the correlation coefficients and the selected wavelengths for the analytes detection are shown in table 2. The linearity range was 1–50 mg/L (y = peak area in mAU (mili absorbance units)×s; x = concentration in mg/L). To test peak area and retention time reproducibility, Chrom Quest software allows the calculation of the relative standard deviations (RSD) for the retention time of the analytes for all levels of the calibration graph and for peak area at each calibration level.

Table 2. *Equations of calibration graphs and correlation coefficients (r^2) for the four analytes*

Analyte	λ, nm	Retention time, min	Equation of the calibration graph	r^2
Tartrazine	430	3.83	y = 1.56481e-005x – 0.449803	0.999762
Amaranth	520	6.59	y = 1.80516e-005x – 0.285031	0.999745
Erythrosine	520	20.40	y = 9.22750e-006x + 0.269151	0.999760
Indigotine	608	8.41	y = 1.54852e-005x + 1.623580	0.999789

The relative standard deviations (RSD) for the retention time were 0.297% (tartrazine), 0.359% (amaranth), 0.067% (erythrosine) and 0,290% (indigotine) therefore, in standard solutions; the HPLC method developed for the chromatographic separation of the four analytes provides stable retention times. The calculation of peak areas led to RSD between 0.031% (indigotine, 20 mg/L) and 0.508% (indigotine, 30 mg/L). Moreover, the calculated relative standard deviations also prove stability in terms of peak height and asymmetry.

In order to establish the method traceability on real samples, a sample of colorless soft drink available for sale was taken for analysis, at which were added the four synthetic colorants. These additions lead to the obtaining of a mauve soft drink, sensorially acceptable, which was considered as witness. The sample was degassed, filtered and analyzed according to the developed method, in order to determine the analytes concentrations. The mean results obtained for the witness are: 9.841 mg/L tartrazine, 9.356 mg/L amaranth, 9.078 mg/L erythrosine and 10.908 mg/L indigotine.

Five increasing addition levels of all the four analytes were added to this sample. Thus, 1, 2, 5, 8 and 10 mL of the 50 mg/L standard solution were added in the 50 mL volumetric flasks and the solutions were then diluted up to the mark with the witness mauve soft drink. The final concentrations of the five analytes in the addition test solutions were calculated using equation (1):

$$C_n = \left[\frac{(50 - V_n) \cdot C_0}{1000} + \frac{50 \cdot V_n}{1000} \right] \cdot \frac{1000}{50} \qquad (1)$$

where:

C_n is the analyte concentration in the sample with the n level of addition;

V_n is the volume of the 50 mg/L standard solution added at the sample with the *n* level of addition;

C_0 is the analyte concentration in the witness.

The five addition samples and the witness were chromatographically analyzed with three injections, according to the developed method, and for each analyte, samples were treated in a way similar to a six point-calibration graph, with the calculated concentrations on the abscissa axis, as pointed above, and with the peak areas corresponding to the analytes in the addition samples on the ordinate axis. The calibration graphs resulted are linear and have six calibration levels, the first level representing the witness. The equations of these graphs, the correlation coefficients and the wavelengths selected for detection of the analytes are shown in table 3.

Figures 1, 2 and 3 show the chromatograms resulted for one of the injections of the sample with addition (level 2).

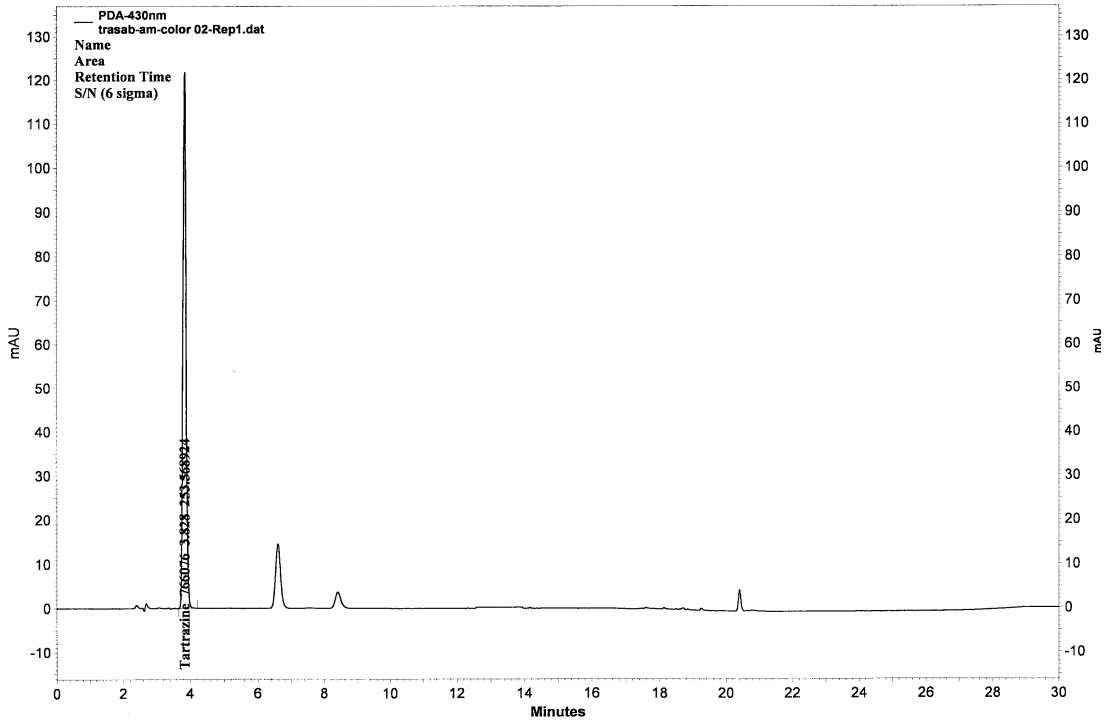

Figure 1. *HPLC at λ = 430 nm of a mauve soft drink with addition (level 2).*

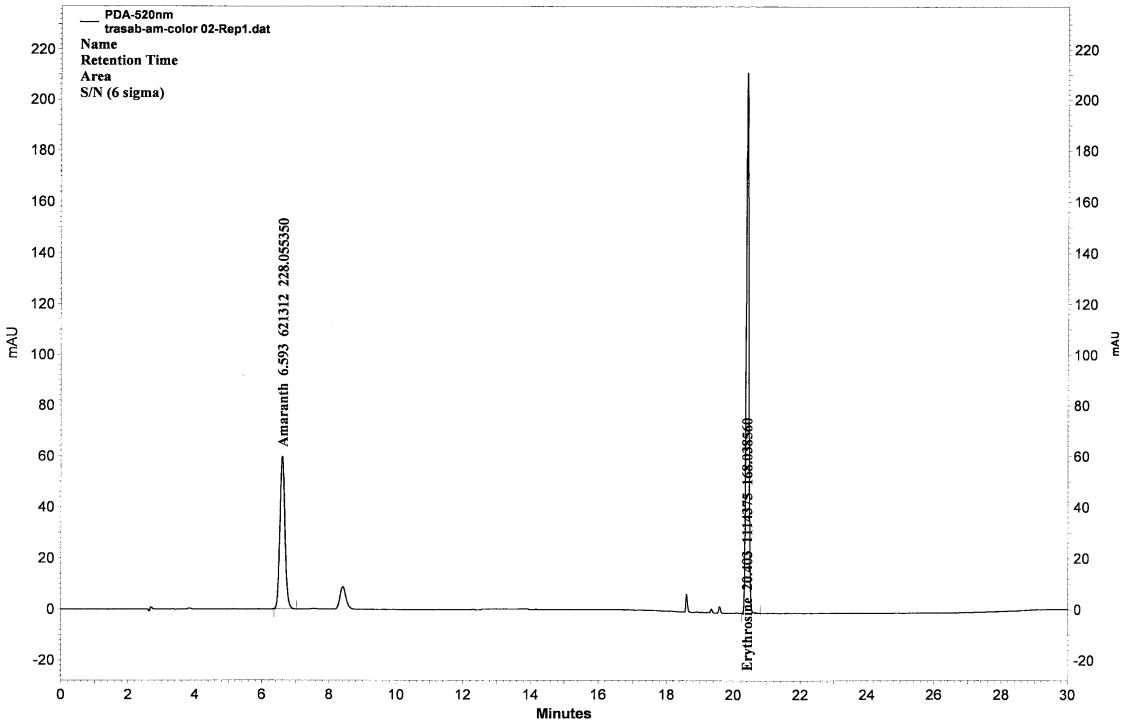

Figure 2. *HPLC at λ = 520 nm of a mauve soft drink with addition (level 2).*

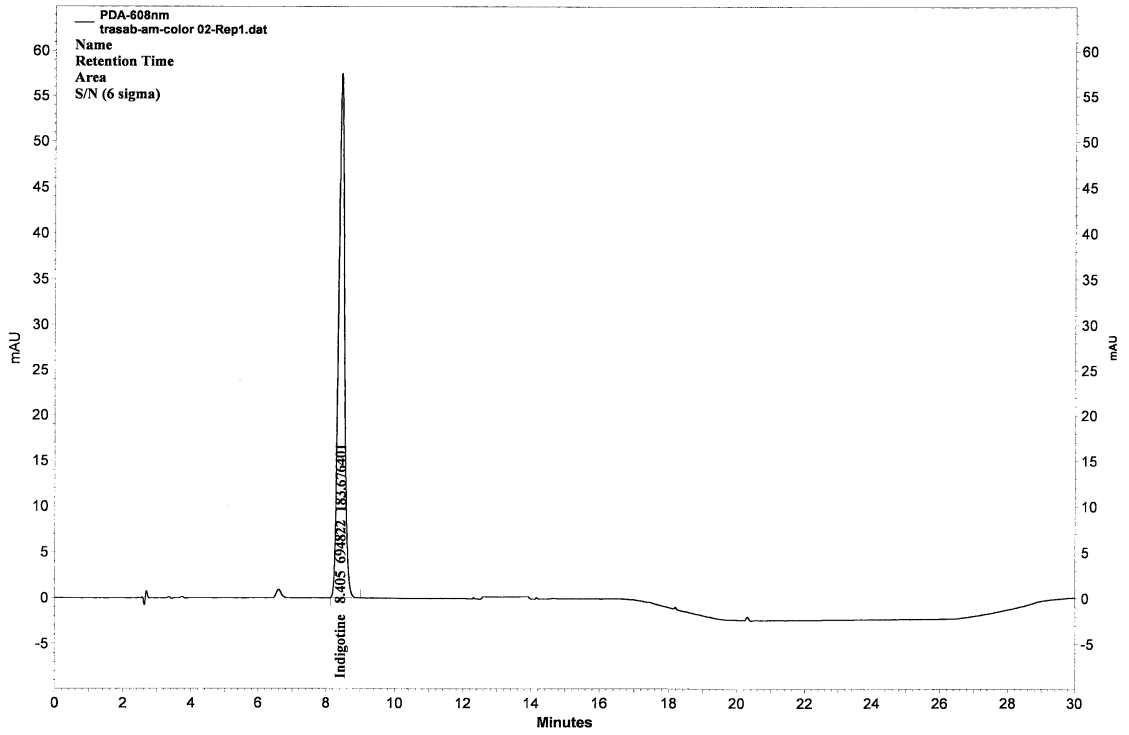

Figure 3. *HPLC at λ = 520 nm of a mauve soft drink with addition (level 2).*

Table 3. *Equations of calibration graphs and correlation coefficients (r^2) of analytes for real samples with addition*

Analyte	λ, nm	Equation of the calibration graph	r^2
Tartrazine	430	y = 1.42591e-005x + 0.492677	0.999876
Amaranth	520	y = 1.77834e-005x – 0.137945	0.999841
Erythrosine	520	y = 8.87109e-006x + 2.514640	0.999156
Indigotine	608	y = 1.67162e-005x – 0.912056	0.999792

Note that addition samples have analytes concentrations within the linearity range of the method (0 – 50 mg/L).

Similar to the determination of the method traceability for standard solutions, we tested the reproducibility of the peak areas and retention time for the traceability on real samples, as well.

The relative standard deviations (RSD) for the retention time were: 0.591% (tartrazine), 0.477% (amaranth), 0.107% (erythrosine) and 0.488% (indigotine), therefore, on real samples, the HPLC method developed for the separation of the four analytes provides stable retention times. The calculation of peak areas led to very good RSD values below 2%, i.e. between 0.026% (tartrazine) and 0.635% (amaranth). Furthermore, the relative standard deviations also prove stability in terms of peak height and asymmetry.

In order to verify the reproducibility, the standard solution of 50 mg/L was analyzed by 10 repeated injections. The relative standard deviations for retention times, peaks areas, peaks heights and peaks asymmetries are presented in table 4 and show very good reproducibility of the method developed.

Table 4. *The relative standard deviations for retention times, peaks areas, peaks heights and peaks asymmetries of tartrazine, amaranth, erytrosine and indigotine at the reproductibility determinations*

Analyte	λ, nm	RSD (%)			
		Retention time	Peaks areas	Peaks asymmetries	Peaks heights
Tartrazine	430	0.497	0.500	0.544	1.452
Amaranth	520	0.876	0.521	0.430	1.017
Erythrosine	520	0.088	0.244	0.243	0.905
Indigotine	608	0.835	0.882	0.446	0.966

The repeatability of the method was verified through the analysis of a mauve soft drink sample with addition, which was fully processed ten times, every time preparing the sample and chromatographically analyzing it, according to the developed method.
By using Chrom Quest software, relative standard deviations were calculated for retention times, heights and peak areas of each analyte for the ten replicates.
The relative standard deviations for retention times, peaks areas, peaks heights and peaks asymmetries are presented in table 5 and show very good repeatability of the method developed.

Table 5. *The relative standard deviations for retention times, peaks areas, peaks heights and peaks asymmetries of tartrazine, amaranth, erytrosine and indigotine at the repeatability determinations*

Analyte	λ, nm	RSD (%)			
		Retention time	Peaks areas	Peaks asymmetries	Peaks heights
Tartrazine	430	0.228	0.215	0.269	1.049
Amaranth	520	0.358	0.349	0.285	0.940
Erythrosine	520	0.071	0.273	0.286	0.430
Indigotine	608	0.289	0.354	0.348	0.506

Robustness directly investigates the sensitivity of the method toward a certain parameter. This requires tests in which the effect of a parameter change is noticed [12]. We choose the sample test injection volume as a parameter, proceeding in the following way: a sample of mauve soft drink was analyzed by the method developed, but using different injection volumes for the chromatographic analysis. Four levels of the sample injection volume were used, i.e. 2.5 μL, 5 μL, 7.5 μL and 10 μL.
With the view to determine the real concentration in the sample, it was applied a multiplier at the results obtained by chromatography, multiplier given by the ratio between the 5 μL volume (volume used for the obtaining of the calibration graphs) and the injection volume used in the analysis. The robustness will be proved through the small relative standard deviations values of the results obtained for the same sample chromatographically analysed with various injection volumes (table 6).
The bias of an analytical method is usually determined by study of relevant reference materials or by spiking studies. Bias may be expressed as analytical recovery (value observed divided by value expected) [12].

The average recovery values resulted were: 1.037 (tartrazine), 1.0332 (amaranth), 1.021 (erythrosine) and 0.9432 (indigotine).

Table 6. *Concentrations of the analytes determined with various injection volumes and relative standard deviations of the obtained results*

Analyte	Injection volume, µL	Mean concentration in the sample, mg/L	Multiplier	Real concentration, mg/L	RSD, %
Tartrazine	2.5	4.958	2.000	9.916	0.173
	5.0	10.031	1.000	10.031	
	7.5	15.301	0.666	10.206	
	10.0	20.606	0.500	10.303	
Amaranth	2.5	5.152	2.000	10.304	0.239
	5.0	9.850	1.000	9.850	
	7.5	14.707	0.666	9.810	
	10.0	19.639	0.500	9.820	
Erythrosine	2.5	5.251	2.000	10.502	0.517
	5.0	9.595	1.000	9.595	
	7.5	14.150	0.666	9.438	
	10.0	18.817	0.500	9.409	
Indigotine	2.5	5.288	2.000	10.576	0.435
	5.0	10.187	1.000	10.187	
	7.5	14.588	0.666	9.725	
	10.0	19.280	0.500	9.640	

The detection limits were determined starting from the principle that a peak, to be detected, must have a signal-to-noise ratio > 3. The detection limits determined for the four analytes and the corresponding values of the signal-to-noise ratio are shown in table 7.

Table 7. *Detection limits for tartrazine, amaranth, erythrosine and indigotine*

Analyte	Concentration mg/L	Signal-to-noise ratio S/N (6 sigma)	Detection limit mg/L
Tartrazine	0.08	6.819	0.08
Amaranth	0.08	4.203	0.08
Erythrosine	0.08	3.945	0.08
Indigotine	0.17	5.311	0.17

CONCLUSIONS

According to the Directive 94/36/EC of the European Union (EU), quantities of synthetic colorants added to foods are restricted by upper limits and, therefore, reliable methods for their quantification have to be established.

The HPLC method developed for the chromatographic separation of tartrazine, amaranth, erythrosine and indigotine in soft drinks enables the quantitative determination of the four colorants within 30 min. The method provides stable retention

times and detection limits ranging from 0.08 mg/L (tartrazine, amaranth, erythrosine) to 0.17 mg/L (indigotine) for a signal-to-noise ratio>3. For determination of the colorants "diode array" detection was employed at the wavelengths 430 nm (tartrazine), 520 nm (amaranth, erythrosine) and 608 nm (indigotine).
The method was validated in terms of sensitivity, linearity range, reproducibility, repeatability, analytical recovery and robustness. Average recovery values were between 0.9432 for indigotine and 1.037 for tartrazine. The proposed HPLC method for the separation and quantification in a single run of tartrazine, amaranth, erythrosine and indigotine in soft drinks was found to be simple, precise, reproductible, sensitive and accurate.

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