DEVELOPMENT AND EVALUATION OF AN HPLC-DAD METHOD FOR DETERMINATION OF INDIGOTINE IN SOFT DRINKS

Ion Trandafir[*1], Violeta Nour[2], Mira Elena Ionică[2]

[1]University of Craiova, Faculty of Chemistry, Bd. Calea Bucuresti, no.107I, Craiova, Romania
[2]University of Craiova, Faculty of Horticulture, Str. A.I.Cuza nr.13, Craiova, Romania

*Corresponding author: nelutrandafir@yahoo.com

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Abstract: 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. Indigotine is the common name for uncertified FD&C Blue No. 2 and it is a blue food color, water soluble.
In this paper, the operational parameters that shall lead to the development of a new analysis method of indigotine in soft drinks have been studied; the technique used is the liquid chromatography together with diode array detection. It has been tried to validate the analysis method settled, in terms of sensitivity, linearity range, reproducibility, repeatability, and robustness. The indigotine in the sample is separated on a C18 reversed phase chromatography column, diode array detected at 608 nm and quantified with a calibration graph. The method provides stable retention times and a detection limit of 0.03 mg/L indigotine at a signal to noise ratio of 3.

Keywords: *HPLC, diode array detection, synthetic food colorants, indigotine, soft drinks*

INTRODUCTION

Synthetic colorants are a very important class of food additives. They are widely used to compensate for the loss of natural colors of food, which are destroyed during processing and storage, and to provide the desired colored appearance. However, some of these substances pose a potential risk to human health, especially if they are excessively consumed. For this reason, 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) [1]. They are divided into five major colorant classes: the azo compounds (E 102, E 110, E 122, E 123, E 124, E 128 and E 129), the triarylmethane group (E 131, E 133 and E142), the chinophthalon derivative of Quinoline Yellow (E 104), xanthenes as Erythrosine (E 127) and the indigo colorants (Indigo Carmine E 132).

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 and, adsorptive voltammetry, and 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 [3]. High-performance ion chromatography, reversed-phase liquid chromatography [4 – 6] 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.

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, biscuits.

In this work, the operational parameters that shall lead to the development of a new analysis method of indigotine in soft drinks have been studied; the technique used was the liquid chromatography together with diode array detection. It has been tried to validate the analysis method settled, in terms of sensitivity, linearity range, reproducibility, repeatability, recovery (deviation of the answer) and robustness.

EXPERIMENTAL PART

Mainly, the sample is filtered through a membrane filter (0.45 μm) before injection. The indigotine in the sample test solution is separated by reversed phase chromatography on a 250 mm × 4.6 mm i.d., 5 μm particle DS HYPERSIL C18 column, detected by absorbance at the wavelength of 608 nm and quantified with a calibration graph.
Indigotine - certified reference material with 98% purity has been used, produced by Sigma-Aldrich (lot 06505PC). All the other reagents were of analytical purity or for chromatographic use. The stock solutions and the corresponding dilutions were made in ultra-pure water and were stored in dark places between the experiments, at low temperature (+4°C).
HPLC was performed with a Surveyor Thermo Electron system comprising vacuum degasser, Surveyor Plus LCPMPP pump, Surveyor Plus ASP autosampler, diode array detector with 5 cm flow cell and Chrom Quest 4.2 software.
The determinations were made in isocratic conditions, at 30°C, using a mobile phase made of 90% 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 10% acetonitrile. The volume injected was 5 μL and the flow rate of the mobile phase was 1mL/min.

RESULTS AND DISCUSSION

Validation of the method. Determination of the performance parameters for the developed method

To test linearity, standard solutions of 5, 10, 20, 30 and 40 mg/L were prepared and analyzed. The calibration graph is linear, with five calibration levels. Equation of the calibration plot was $y = 1.71439e\text{-}005x - 0.639293$ and the correlation coefficient was $r^2 = 0.999762$.
To test peak area and retention time reproducibility, Chrom Quest software allows the calculation of the relative standard deviation (RSD) for the retention time of indigotine, for all levels of the calibration graph and the calculation of the relative standard deviations (RSD) for peak area at each calibration level.
The relative standard deviation (RSD) for the retention time was of 0.079%, therefore, on standard solutions, the HPLC method developed for the chromatographic separation of the indigotine provides stable retention times. The calculation of peak areas led to a RSD between 0.111% and 0.330%.
In order to establish the method traceability on real samples, a sample of blue soft drink was taken for analysis, sample containing indigotine like food colorant. Applying the developed method, the indigotine in the soft drink sample was determined by giving three replicates. At this sample, five increasing addition levels of indigotine were added. Thus, 1, 2, 5, 8 and 10 mL of standard solution with a concentration of 50 mg/L indigotine were added in the 50 mL volumetric flask and the solution is then diluted to the mark with blue soft drink.
The average concentration of the indigotine in the witness sample was determined (5.7033 mg/L) and the concentrations of indigotine in the addition samples were calculated.

The five addition samples and the witness were chromatographically analyzed with three replicates according to the developed method and for indigotine, the samples were treated in a way similar to a six points calibration graph, with the calculated concentrations in the abscissa, as pointed above, and with the peak areas corresponding to the indigotine in the addition samples in the ordinate. The calibration graph achieved is linear, with six calibration levels, the first level being the witness with no addition. The equation of this graph was y = 1.70666e-005x – 0.537028 and the correlation coefficient r^2 = 0.999877.

In figure 1, it is shown the chromatogram achieved for one of the injections of the witness sample. Addition samples have indigotine concentrations within the linearity range of the method (1 – 40 mg/L).

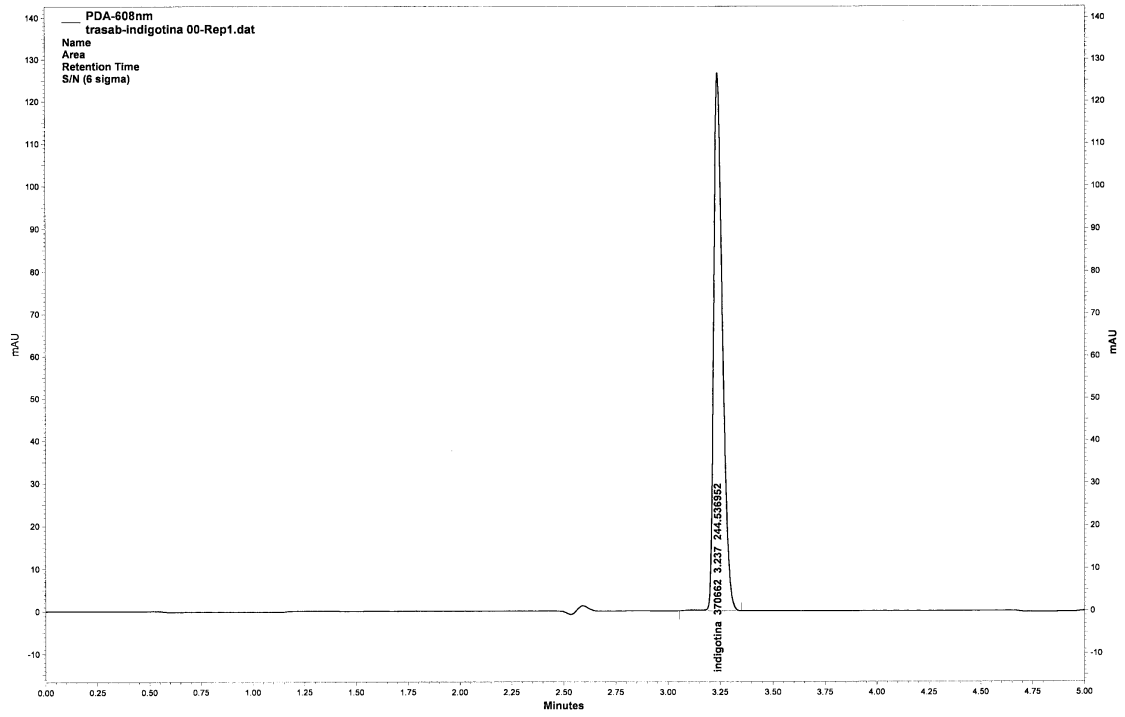

Figure 1. *HPLC of a sample of blue soft drink*

As it proceeded with the determination of the method traceability for standard solutions, we tested the reproducibility of the peak areas and of the retention time and for traceability for real samples. The relative standard deviation (RSD) for the retention time was 0.047%, so, on real samples, the HPLC method developed for the chromatographic separation of the indigotine provides stable retention times. The calculation of the peak areas led to a RSD between 0.036% (the third addition level) and 1.184% (the second addition level), values less than the 2% limit.

In order to determine the standard reproductibility deviation, the standard solution of 20 mg/L indigotine was analyzed by ten replicates. The chromatogram achieved for one of the ten replicates is shown in figure 2.

By the help of Chrom Quest software, there were calculated the relative standard deviations for retention times, asymmetry and peak areas of the indigotine for the ten replicates. For the retention time, RSD = 0.024%, for asymmetry RSD = 0.544% and for

the peak areas, RSD = 0.185%, very good values less than the 2% limit which proves a very good reproducibility of the method developed.
The method repeatability shows the variability noticed inside a laboratory in a short period of time, using a single operator, equipment etc.

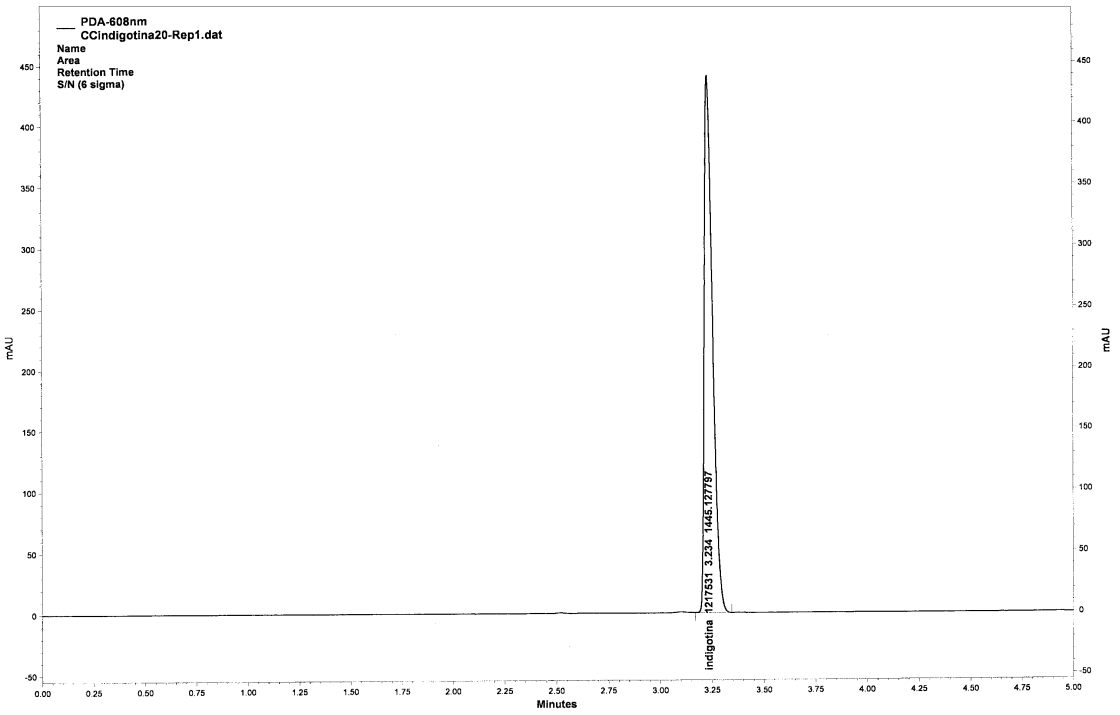

Figure 2. *HPLC of a standard solution of indigotine (c = 20 mg/L)*

For determining the standard repeatability deviation, a sample of blue soft drink with addition (the third level of addition) was integrally processed ten times, according to the method developed. By the help of Chrom Quest software there were calculated the relative standard deviations on retention times, heights and peak areas of the indigotine for the ten replicates.
For the retention time, RSD = 0.027%, for the heights RSD = 1.053% and for the peak areas, the RSD value is 0.735%, which proves a good repeatability of the method developed. The fidelity noticed will be an essential component of the measurement uncertainty of the method developed.
The robustness directly investigates the sensibility of the method toward a certain parameter. This is achieved by a robustness test where the effect of a parameter change is noticed [7]. We choose the sample test injection volume as a parameter, proceeding in the following way: a sample of blue 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: 2.5, 5.0, 7.5 and 10 μL, with 3 replicates for each variant.
The samples were analyzed and the concentrations were calculated using the calibration graph achieved, which was elaborated using a sample test injection volume of 5 μL, but using a multiplier in accordance with the current injection volume. The relative standard

deviation of the four average concentrations was RSD = 0.408%. This value proves a good sensibility of the method toward the injection volume parameter.
The method recovery can be expressed as value observed divided by value expected. It was achieved an average recovery value of 97.20% for an addition level of 5 mg/L and the recovery values had a relative standard deviation of 0.7175%.
The detection limit was 0.03 mg/L indigotine in the analysed sample at a signal to noise ratio of 3.

CONCLUSIONS

A simple, selective and precise HPLC method with diode array detection was developed for the quantitative determination of indigotine in soft drinks. The method provides stable retention times and a detection limit of 0.03 mg/L. The analysis time was less than 5 min.
The indigotine in the degassed and filtered sample is separated by reversed phase chromatography on a 250 mm × 4.6 mm i.d., 5 μm particle DS HYPERSIL C18 column, detected by absorbance at the wavelength of 608 nm and quantified with a calibration graph.
The method was validated in terms of sensitivity, linearity range, reproducibility, repeatability, recovery and robustness. Average recovery of the indigotine was found to be 97.2%.

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