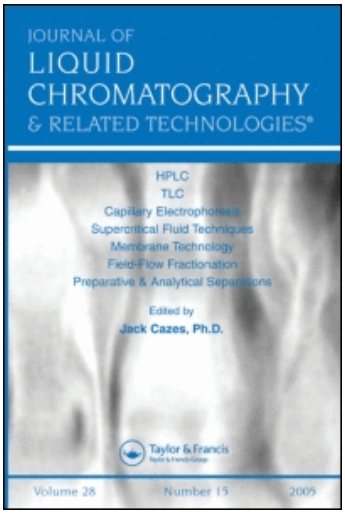

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Chemometrically Assisted Development of IP-RP-HPLC and Spectrophotometric Methods for the Identification and Determination of Synthetic Dyes in Commercial Soft Drinks

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To cite this Article Gianotti, V. , Angioi, S. , Gosetti, F. , Marengo, E. and Gennaro, M. C.(2005) 'Chemometrically Assisted Development of IP-RP-HPLC and Spectrophotometric Methods for the Identification and Determination of Synthetic Dyes in Commercial Soft Drinks', Journal of Liquid Chromatography & Related Technologies, 28: 6, 923 — 937
To link to this Article: DOI: 10.1081/JLC-200051484
URL: http://dx.doi.org/10.1081/JLC-200051484

Journal of Liquid Chromatography & Related Technologies®, 28: 923–937, 2005

ISSN 1082-6076 print/1520-572X online
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Chemometrically Assisted Development of IP-RP-HPLC and Spectrophotometric Methods for the Identification and Determination of Synthetic Dyes in Commercial Soft Drinks

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Abstract: Two independent methods were developed to identify and determinate synthetic dyes and to follow their degradation processes. The IP-RP-HPLC method was optimised by the experimental design technique and the results obtained were compared with ones obtained by direct/derivative spectrophotometry.
The dyes considered in this study that are representative of soft drink components, are: Tartrazine (E102), Quinoline Yellow (E104), Sunset Yellow (E110), Carmoisine (E122), Amaranth (E123), New Coccine (E124), Patent Blue Violet (E131), and Brillant Blue FCF (E133).

Keywords: Synthetic dyes, Experimental design, Dyes in soft drinks

INTRODUCTION

A recent interest of the food chemistry devoted to the safeguard of human health concerns synthetic dyes which are commonly added to a great number of foodstuffs and largely preferred to natural colours, essentially because of their greater stability along the production industrial process.

This interest is more than justified, since dye doses in human consumption is evaluated at as large as about 10 g/year. The toxicity risk generally is not

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due to the dye itself, whose use is regulated by EEC directives,[1] but it might derive from impurities present in compounds used in the synthesis or from side-products formed in the synthesis process itself.[2–4]

Recently, very alarming sources of such an undesired product have been envisaged in the degradation reactions that can naturally occur in the commercial products, due to unsuitable conservation conditions. This is the case of soft drinks that, in summer, are often exposed to strong conditions of temperature and sun light.[5]

As concerns the use of dyes in food and drinks, only in 1995 did the European legislation standadized the normative admitting, for example, in Europe, the use of Amaranth (E123) before being forbidden in Italy,[6] as well as in USA, by the FDA.[7] A number of directives reports a "positive" list of the permitted colorants and of their maximum allowed concentration. The 78/25/EEC[8] directive also considers the presence of organic impurities in food dyes and allows 0.5% as their maximum content.

To develop a contribution in this field, we considered some dyes that are the most commonly used in mixtures, and in commercial soft drinks, namely: Tartrazine (E102), Quinoline Yellow (E104), Sunset Yellow (E110), Carmoisine (E122), Amaranth (E123), New Coccine (E124), Patent Blue Violet (E131), and Brillant Blue FCF (E133).

For these colorants, a chromatographic multiresidue method was developed. Most of the works present in literature for the determination of food dyes are based on spectrophotometric techniques[9–14] or liquid chromatographic methods.[15–18] The spectrophotometric methods make use of the derivative approach in combination with multivariate calibration.[9–15] When more than four dyes are simultaneously present, the chromatographic methods are more efficient. When the dyes residues are characterised by polar properties, ion exchange and ion pair chromatography are the most suitable techniques.[9–14] Recently capillary zone electrophoresis was also used for the simultaneous determination of seven dyes in food and beverages.[19]

In the present paper, a chromatographic method is developed for the separation of the eight dyes and the operative conditions are optimised by chemometric techniques of experimental design.

The chromatographic method is validated by comparing the results obtained with those obtained by the spectrophotometric method. In order to study the possible degradation products, the chromatographic method developed is suitable for mass-spectrometric detection.

To investigate possible natural degradation processes, some commercial soft drinks containing the dyes investigated underwent the natural conditions of light and temperature as those encountered during bad storage.

Theory-Experimental Design and Grid Search Algorithm

In the Factorial Design (FD) each factor is investigated at fixed levels. A full factorial design contains all the possible combinations of the experimental

factors and allows a straightforward calculation of the effects of the factors and of all the possible multi-factor interactions. A two-level full factorial design requires 2^p experiments (where p is the number of the investigated experimental factors).[20]

The star design is an experimental plan with the experiments performed at three levels (along the factor axes and in the centre of the domain), that allows the calculation of a quadratic regression model without interaction terms. The addition of the experiments of the star design to the experiments of a factorial design, provides enough information for the calculation of a model containing the main factors plus interaction and quadratic term, and leads to the so-called Central Composite Design (CDD).[21–23]

The regression models can be used to simulate the experimental responses and to search the optimal settings of the experimental factors. This is done by the grid search algorithm,[20] i.e., by calculating the simulated chromatogram on a grid of points, which span the experimental domain with a given step that can be iteratively reduced as a function of the desired accuracy. The best conditions must guarantee, for the separation of a mixture, the maximum value of the minimum resolution (distance between the nearest peaks) of each simulated chromatogram.

Direct and Derivative Spectrophotometric UV-Visible Methods for Binary Mixture

If the UV-Vis absorbance spectra of two compounds overlap, it is possible to obtain their concentrations by solving a system of two equations, obtained from the application of Lambert-Beer law at two wavelengths. If the absorbance maxima of the two analytes are very close, the use of the derivative approach is preferred.[9] The first derivative absorbance of the spectrum is characterised by zero values of absorbance corresponding to the wavelength of maximum and minimum original spectrum, and the spectra details are magnified. In these conditions, the choice of suitable wavelengths is simpler and more accurate. In the zero-crossing method derivative approach,[9] the choice of λ_1 must give an appreciable derivative absorbance of the first dye, where the second one has a null derivative absorbance. At this λ, are recorded the spectra at different concentrations of the first dye and at constant concentration of the second, and the calibration plot of the first dye is built. In the same way the calibration plot of the second dye at λ_2 is built, at which the first dye has a derivative absorbance close to zero.

EXPERIMENTAL

Apparatus

The analyses were carried out by a Finnigan Mat Spectra System (S. Josè, California, USA) equipped with a Degasator SCM1000, a gradient pump

Spectra System P4000, an Autosampler Spectra System AS3000, interfaced by the module SN4000 to a diode array detector Spectra System UV6000LP.

A Metrohm 654 pH meter (Herisau, Switzerland), equipped with a combined glass-calomel electrode, was employed for pH measurements.

The spectophotometric analyses were carried out with a spectrophometer UNICAM 8700.

Reagents

Ultrapure water from a Millipore Milli-Q system (Milford, MA, USA) was used for the preparation of all the solutions. Dyes E102, E110, E122, E123, E124, and E133 were purchased from Aldrich (Milwakee, WI, USA); dyes E104, E131 were Fluka (Buchs, Switzerland): all of them are analytical grade chemicals. Acetonitrile (ACN), acetic acid, tetrabutylammonium bromide (TBA) were Merck (Darmstadt, Germany) analytical grade chemicals; sodium hydroxide was from Carlo Erba (Milan, Italy). The 1000 mg/L standard solutions of food-dyes were prepared in ultrapure water and kept in dark glass bottles at 4°C.

Chromatographic Conditions

An end-capped Lichrospher 100 RP-18 column (250 × 4 mm, 5 μm) (Merck, Darmstadt, Germany) and a (15.0 × 4 mm) Lichrospher RP-18 (5 μm) guard precolumn were used for all the separations. The experiments in the ion-pair mode planned by the experimental design required the preparation of a number of eluents containing different combinations of the values of the three variables considered (pH, ion pair reagent concentration, and acetonitrile concentration). The flow-rate was 1.0 mL/min. A spectrophotometric detection at 290 nm was employed. The samples of drinks were filtered and degassed prior to the injection in the HPLC system. The injection volume is 100 μL in all the chromatographic runs.

Sample Preparation

The commercial soft drinks analysed are: Cedrata, Stappjno, Bravo, Tè verde, Gatorade Blue, Vahinè rosso, Vahinè giallo, Vahinè verde, Gazzosa Abbondio, Energade, Fidel aperitivo, Martini Baby rosso, Aranciata Spumador. These drinks are indicated in the text and in Tables 1 and 5, with letters in a random order.

Duplicate samples of these commercial drinks were bought in stores in Alessandria. One bottle, sealed, was stored in temperature below 30°C and dark conditions, while the other, also sealed, was exposed for two weeks to full sunlight with a strong solar irradiation.

Table 1. Dyes concentrations in commercial soft drinks indicated with A–O obtained by spectrophotometric method

Sample	Dye present	Detection λ (nm)	Found concentrations
A	E104	430	468.18 ± 2.68 mg/L
B	E110	460	18.06 ± 0.32 mg/L
C	E124	500	16.53 ± 0.31 mg/L
D	E131	636	0.24 ± 0.02 mg/L
E	E133	630	5.55 ± 0.50 mg/L
F	E122	550	12.50 ± 0.01 g/L
G	E102	450	9.34 ± 0.09 g/L
H	E102	450	15.10 ± 0.21 mg/L
	E131	636	4.41 ± 0.02 mg/L
I	E104	440	14.86 ± 0.57 mg/L
	E110	500	27.70 ± 0.42 mg/L
L	E102	410	26.29 ± 0.35 mg/L
	E122	550	53.03 ± 0.32 mg/L
M	E102	450	4500.86 ± 7.69 mg/L
	E131	636	2142.78 ± 8.64 mg/L
N	E110	460	26.40 ± 0.25 mg/L
	E123	550	31.60 ± 0.23 mg/L
O	E110	483	18.47 ± 0.31 mg/L
	E122	515	14.50 ± 0.42 mg/L

RESULTS

Analytical Methods

The labels that report the composition of the commercial drinks also indicate the presence of other compounds (like glucose syrup, citric acid, sodium citrate, sodium chloride, mono-potassium phosphate), but do not declare the concentration of the dyes added.

In order to collect information on the dye concentrations, a spectrophotometric and HPLC analysis were performed.

Spectrophometric Analysis

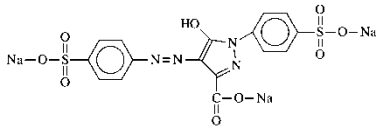
Table 1 shows the concentrations obtained for the mono-dye soft drinks, by the calibration plots constructed at the wavelength that corresponds to the maximum of the absorbance and for concentrations ranging between 1.0 and 10.0 mg/L.

For the analysis of the drinks containing mixtures of two dyes, the direct method was used when the spectra of the two dyes do not overlap and the absorbance maxima are located at different λ. Instead, when the maxima overlap, the derivative approach with the zero crossing method was successfully used. The

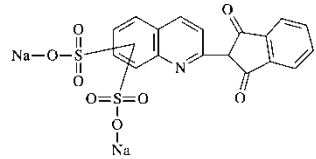
results obtained are given in Table 1. Relatively high concentrations of dyes, as for example E122, E102, and E131, found in some commercial drinks, are shown.

Optimisation of the Chromatographic Separation

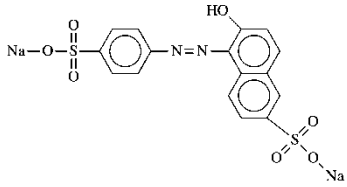
As shown in Figure 1, the molecules of the dyes in the study have different functionalities and are present in ionic form over a wide pH range.



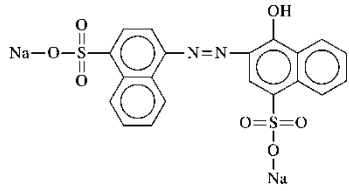
Tartrazine - E102 (C.I. 19140)



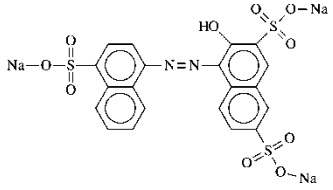
Quinoline Yellow - E104 (C.I. 47005)



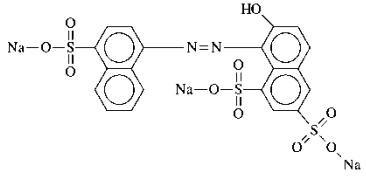
Sunset Yellow - E110 (C.I. 15985)



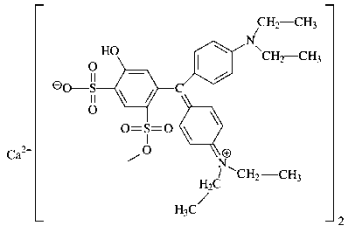
Carmoisine - E122 (C.I. 14720)



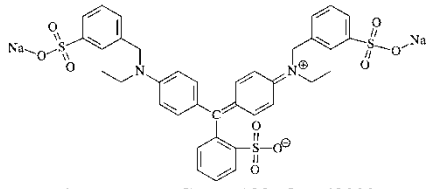
Amaranth - E123 (C.I. 16185)



New Coccine - E124 (C.I. 16255)



Patent Blue Violet - E131 (C.I. 42051)



Brillant Blue FCF - E133 (C.I. 42090)

Figure 1. Chemical structure, commercial name, and color index of dyes used.

Reversed-phase Ion Pair Chromatography (IPC) has gained widespread acceptance as a versatile and efficient method for the separation of ionised or easily ionisable species. The advantage of IPC is, besides its ability to simultaneously separate neutral and ionised molecules, the use of conventional RP columns, which provide higher efficiency and greater versatility than fixed-site ion exchangers, and do not require special or modified equipments. Method development and optimisation in IPC are generally very flexible since retention can be modulated by changes in the composition of the mobile phase. Many variables are, in fact. involved in the retention, such as the chemical properties and the concentration of the ion-pair reagent, the amount of organic modifier, the pH of the mobile phase.

In this work, which makes use of tetrabutylammonium bromide as the ion-pairing agent three variables with potential effect on retention were considered, namely the concentration of the organic modifier, the acetonitrile concentration (c_M), the concentration of the IP reagent (c_{IP}), and the mobile phase pH.

Preliminary analyses indicated the range of these factors: the c_M varied between 40.0% and 60.0%, the c_{IP} between 5.0 and 12.0 mM, and the pH values between 5.00 and 8.00.

A 2-level full factorial design was used first. The experimental response was the retention time of the analytes. Table 2 reports the conditions of all the

Table 2. Experimental conditions for: central experiment replication (1, 6, 11); full factorial design (2–5, 7–10); star design (12–17). Variables: c_m percentage of acetonitrile, C_{IP} concentration of ion pair reagent

n° exp	Design	C_M (%)	C_{IP} (mM)	pH
1	Center repl.	50.0	8.5	6.50
2	Full factorial design	40.0	5.0	5.00
3		60.0	5.0	5.00
4		40.0	12.0	5.00
5		60.0	12.0	5.00
6	Center repl.	50.0	8.5	6.50
7		40.0	5.0	8.00
8		60.0	5.0	8.00
9		40.0	12.0	8.00
10		60.0	12.0	8.00
11	Center repl.	50.0	8.5	6.50
12	Star design	40.0	8.5	6.50
13		60.0	8.5	6.50
14		50.0	5.0	6.50
15		50.0	12.0	6.50
16		50.0	8.5	5.00
17		50.0	8.5	8.00

experiments performed (experiments 2–5 and 7–10). The experiment in the central point was replicated three times along the whole experimentation (experiments 1, 6, 11) to estimate the pure experimental error and to check the column performance stability. In two analytical standards, we found that the dye E104 shows 3 peaks, while the dye E133 shows 2 peaks; for the E104, the presence of isomers is reported,[24] while for the E133, we observed the presence of undocumented impurities.

From the experimental data obtained in the experimental design, the regression models calculated by MLR (Multiple Linear Regression) show that the retention of all the analytes is largely affected by c_M and c_{IP}. For all the analytes (except E104) the experiments performed in the central point show large differences between the experimental and the calculated retention times; this deviation is likely to be due to a non linear behaviour of the system. The existence of quadratic significant effects was tested by an F-test that compares the difference between the experimental and the estimated retention time in the central point with its uncertainty.

F is calculated as:

$$F_{(1,\nu,\alpha)} = \frac{(\bar{y}_0 - \bar{y}_F)^2}{s_{pe}^2 \cdot \left(\frac{1}{n_0} + \frac{1}{n_F}\right)}$$

where $\bar{y}_0$ is the average response of the replicated central experiments and $\bar{y}_F$ the average response from the factorial design experiments, which represents

Table 3. Regression models and multiple determination coefficient (R^2)

Dyes	Regression model equations	R^2
E102	$\hat{y} = 5.39 - 7.12c_M + 0.42c_M \cdot c_{IP} + 5.24c_M^2 - 0.92c_{IP}^2$	0.9896
E104 (1)	$\hat{y} = 3.88 - 1.44c_M - 0.85c_{IP} \cdot pH + 0.85c_M \cdot c_{IP} \cdot pH$	0.9414
E104 (2)	$\hat{y} = 4.50 - 1.78c_M - 1.03c_{IP} \cdot pH + 1.03c_M \cdot c_{IP} \cdot pH$	0.9686
E104 (3)	$\hat{y} = 5.13 - 2.21c_M - 1.15c_{IP} \cdot pH + 1.15c_M \cdot c_{IP} \cdot pH$	0.9664
E110	$\hat{y} = 4.57 - 3.42c_M + 1.33c_M^2$	0.9416
E122	$\hat{y} = 7.79 - 9.61c_M + 4.78c_M^2$	0.9267
E123	$\hat{y} = 8.12 - 9.81c_M + 4.62c_M^2$	0.9235
E124	$\hat{y} = 10.40 - 12.39c_M + 2.82c_{IP} - 3.06c_M \cdot c_{IP} - 2.67c_{IP} \cdot pH + 2.67c_M \cdot c_{IP} \cdot pH + 5.17c_M^2$	0.9236
E131	$\hat{y} = 6.45 - 6.65c_M + 3.12c_M^2$	0.9326
E133 (1)	$\hat{y} = 4.94 - 4.54c_M + 0.59c_M \cdot pH + 2.02c_M^2$	0.9366
E133 (2)	$\hat{y} = 5.43 - 4.77c_M + 0.62c_M \cdot pH + 1.83c_M^2$	0.9434

$\hat{y}$ = retention time, c_M = concentration of acetonitrile, c_{IP} = concentration of tetrabutylammonium bromide.

the estimated value in the centre, s_{pe}^2 is the pure experimental error (estimated from the replicated experiments), n_0 is the number of central experiments, and n_F is the number of FD experiments. The F-test suggested the necessity to calculate, for each analyte, a second order model. Six experiments of a star design (reported in Table 2) as experiments 12–17 were added to the FD experiments, to provide a central composite design.

The best regression model for each dye is reported, together with their R^2 values in Table 3. All the models perform in a satisfactory way and can be used to simulate the experimental elutions.

In order to optimise the separation of the eight dyes, a grid search algorithm was employed, searching for the set of the experimental factors values that provide the best resolution. The grid search algorithm allows the

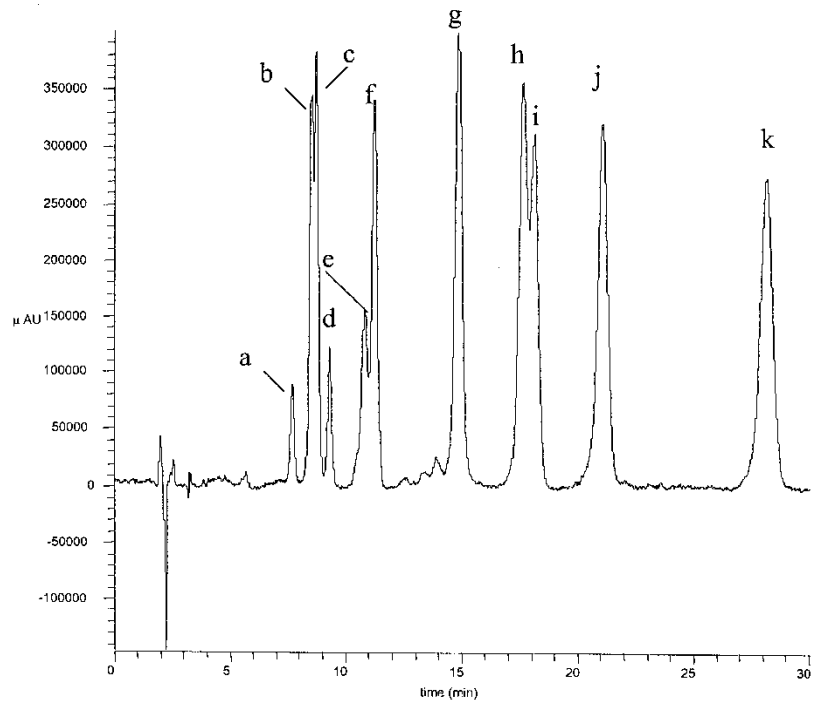


Figure 2. IP-HPLC separation of the mixture of the analytes. Peak identification: (a) E104 (0.8 mg/L) first peak; (b) E104 (0.8 mg/L) second peak; (c) E110 (1.4 mg/L); (d) E104 (0.8 mg/L) third peak; (e) E133 (2.5 mg/L) first peak; (f) E133 (2.5 mg/L) second peak; (g) E131 (2.2 mg/L); (h) E102 (3.0 mg/L); (i) E122 (1.5 mg/L); (j) E123 (1.5 mg/L); (k) E124 (3.5 mg/L). Mobile phase: tetrabutylammonium bromide 5.0 mM, pH 5.6,% ACN 44%, flow-rate 1.0 mL/min. Stationary phase: Merck Lichrospher RP-100 5 μm endcapped, 250 mm × 4 mm, coupled with a Merck Lichrospher RP-18 (5 μm) precolumn. UV detection: $\lambda = 290$ nm.

achievement of the best conditions by iterating the optimisation procedure with progressively shorter search steps.

Figure 2 shows the chromatogram recorded under the obtained optimal condition, which are: $c_M = 44.0\%$, $c_{IP} = 5.0\,mM$, and $pH = 5.60$. The food-dyes that are not baseline separated can be resolved by recording the chromatogram at different wavelengths or by use of a Diode Array Detector (DAD), since at 617 nm only E104 absorbs, while at 423 nm only E102 absorbs, and at 514 nm E110 and E122 can be separated.

In order to express the sensitivity (the peak area for 1.0 mg/L concentration) as given by the slopes of the calibration plots into concentration units in the chromatogram, an area which corresponds to a signal to noise ratio around 3 is identified and used to proportionally transform sensitivity into LOD concentration units (mg/L). LOD values are always lower than 0.28 mg/L. Quantitation limits (LOQs) are evaluated by the calibration plots as the lowest concentration that can be quantified, and are estimated as a signal greater than a signal to noise ratio equal to 10. LOD and LOQ values are reported in Table 4.

A linearity behaviour was always observed in the concentration range between 0.10 and 0.40 mg/L.

Analysis of the Real Samples

From the concentration data of the dyes in soft drinks obtained by the spectrophotometric analysis (Table 1), the suitable dilution factors compatible with the chromatographic analysis are calculated.

The chromatographic method developed is suitable for the rapid screening of a beverage of unknown composition, in order to evaluate the dyes present and their concentrations.

The chromatographic data obtained by the IP-chromatographic analysis, reported in Table 5, are in agreement with the spectrophotometric ones.

Table 4. LOD and LOQ values in the chromatographic method

Dyes	LOD (mg/L)	LOQ (mg/L)
E102	0.28	0.71
E104	0.11	0.32
E110	0.06	0.12
E122	0.03	0.08
E123	0.04	0.10
E124	0.08	0.20
E131	0.07	0.16
E133	0.11	0.22

Table 5. Dye Concentrations in commercial soft drinks indicated with A–O obtained by chromatographic method

Sample	Dye composition	Detection λ (nm)	Dilution factors	Concentrations found
A	E104	400	100	473.21 ± 3.67 mg/L
B	E110	500	50	17.78 ± 0.41 mg/L
C	E124	500	10	16.67 ± 0.31 mg/L
D	E131	600	3	0.23 ± 0.02 mg/L
E	E133	600	5	5.15 ± 0.07 mg/L
F	E122	490	5,000	12.55 ± 0.09 g/L
G	E102	450	7,000	9.30 ± 0.07 g/L
H	E102	450	20	15.10 ± 0.31 mg/L
	E131	600		4.41 ± 0.03 mg/L
I	E104	400	20	18.86 ± 0.81 mg/L
	E110			22.38 ± 0.92 mg/L
L	E102	490	25	25.70 ± 0.41 mg/L
	E122			59.40 ± 0.40 mg/L
M	E102	450	5,000	4.50 ± 0.06 g/L
	E131	600		2.14 ± 0.07 g/L
N	E110	500	40	24.61 ± 0.22 mg/L
	E123			32.88 ± 0.21 mg/L
O	E110	520	20	17.68 ± 0.15 mg/L
	E122			15.00 ± 0.18 mg/L

The deviations are in average within 5%, with the only exception of soft drink **I** for which a deviation around 20% is observed.

Experimental Evidence of Dye Degradation

As mentioned, the soft drinks chosen were subjected, in the original sealed bottle, to conditions able to reproduce possible, not correct, storage conditions as, for example, the exposition to strong solar irradiation and high temperature. It could be noticed that after the first week, the blue drink **E**, containing E133, showed a complete loss of its original colour and in another five days, the colour of the sample **O**, containing E110 and E122, also varied from red to light yellow.

Both the samples underwent spectrophotometric and chromatographic analysis. The chromatograms of the degraded sample and of the undegraded one for drinks **E** and **O** are reported in Figure 3a and Figure 4a. In both beverages is evident the disappearance, after the exposition to summer temperature and sun light, of all the characteristic peaks of the original dyes.

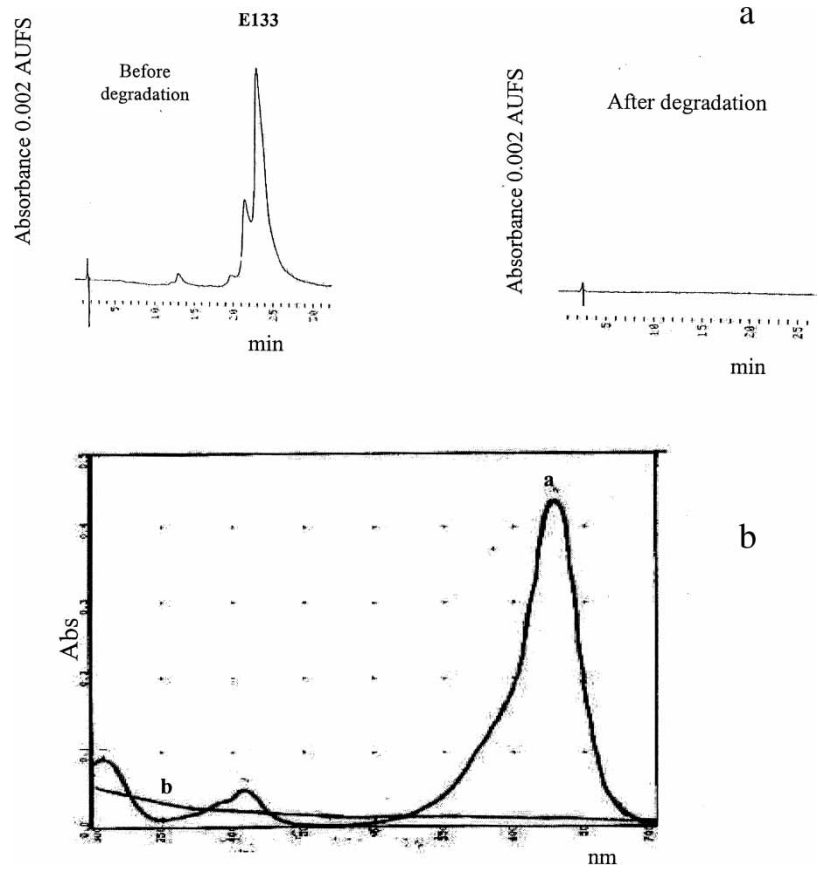


Figure 3. **A** Chromatogram of the drink **E** before and after the degradation process. Chromatographic conditions as in Figure 2. Detection $\lambda = 630$ nm. **B** UV-Vis spectra of the drink **E** before (a) and after (b) degradation.

As concerns the compounds formed for degradation, we can, at present, only say that they do not absorb, not only at the λ of maximum absorbance of the dye (500 nm for both the beverage), but (Figure 3b and Figure 4b) in the whole UV-Vis region between 350 and 700 nm.

Studies are in progress in order to try to identify the degradation products by the use of a mass spectrometric detection.

CONCLUSION

Eight dyes representative of the most commonly used, and reported on the labels of commercial beverages, were selected. The IPC chromatographic

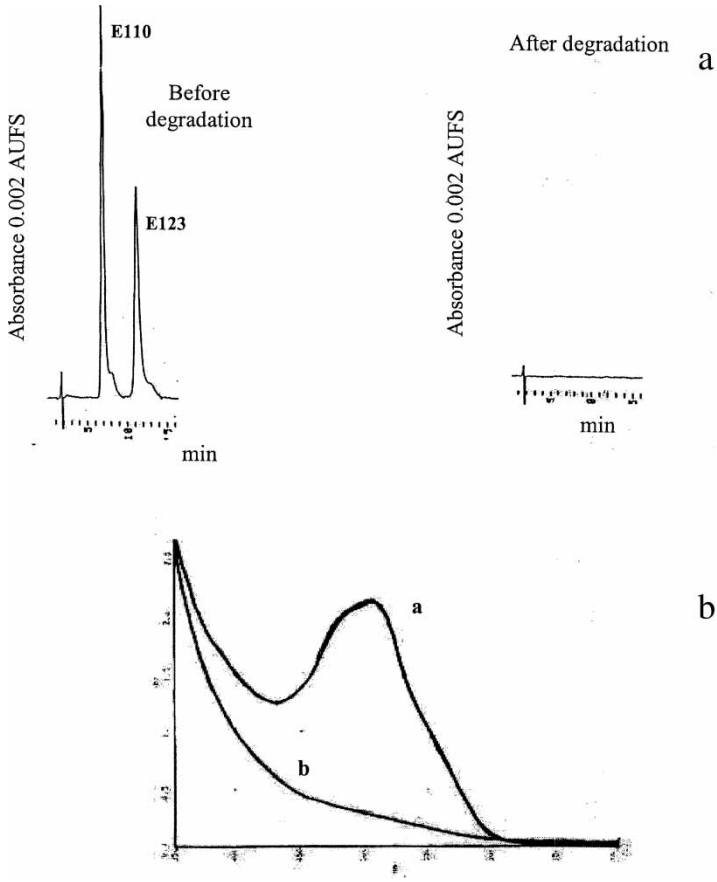


Figure 4. **A** Chromatogram of drink **O** before and after the degradation process. Chromatographic conditions as in Figure 2. Detection $\lambda = 520$ nm. **B** UV-Vis spectra of drink **O** before (a) and after (b) degradation.

separation of these compounds was optimised by a Central Composite Design through the calculation of quadratic regression models and the use of a grid search algorithm. The application of spectrophotometric and chromatographic methods permitted:

i. to state or confirm by means of two independent methods, the dye composition reported on the labels of the commercial beverages;
ii. to identify, beside the dye declared, the presence of other allowed dyes;
iii. to evidence that E110 and E133 may degrade in the sealed commercial products when undergoing drastic but natural conditions of storage.

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Received March 1, 2004
Accepted November 2, 2004
Manuscript 6360