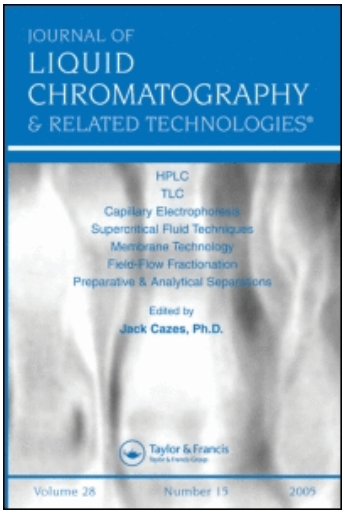

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VALIDATION OF THE METHODOLOGY TO DETERMINE SYNTHETIC DYES IN FOODS AND BEVERAGES BY HPLC

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ABSTRACT

Synthetic dyes are added to foods due to the many changes which alter or destroy the natural colors of the products during processing and/or storage, and principally to increase the acceptance of the product, by the customer. Synthetic dyes show several advantages over natural pigments. The lack of adequate analytical methodology has made it difficult to control the levels of coloring agents in foods. Also, there is no methodology capable of responding to the size and the demand for such analyses by the industrial sector, in several types of food.

This work, proposed and validated a methodology for the simultaneous determination of the eight synthetic colors: Sunset yellow [E-110], yellow n°5 [E-102], bordeaux S [E-123], red n°40 [E-129], red n°17 [E-124], red n°3 [E-127], brilliant blue [E-133] and indigo carmin [E-132], allowed in foods in Brazil, using the technique of high performance liquid chromatography (HPLC).

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The dye was extracted with warm water (40 to 50°C), the solution was centrifuged, and finally filtered through fluoropore filter FHLP 01300 of 0.5 μm. It was then injected into the chromatograph. In the chromatographic process, the use of the column ODS-2 and the mobile phases H_2O/MeOH (70 : 30), plus 0.08 mol/L of ammonia acetate for conditioning and H_2O/MeOH (70 : 30) for the run, allowed for the elution of the synthetic dyes in 20 min, plus 12.5 min to equilibrate the column before another injection.

The detection and recovery limits determined were respectively: 0.5 μg/mL and 96.3% for indigo carmin; 1.30 μg/mL and 98.2% for brilliant blue; 1.02 μg/mL and 102% for bordeaux S; 0.57 μg/mL and 96.8% for red n°17; 1.10 μg/mL and 100.3% for red n°40; 0.51 μg/mL and 96% for red n°3; 0.70 μg/mL and 99.7% for yellow n°5; 1.83 μg/mL and 98.9% for sunset yellow. The test for repeatability showed coefficients of variation below 5% for the colorings. The proposed methodology, validated here, presents efficiency, versatility, speed, and simplicity.

INTRODUCTION

Adding dyes to foods has been a wide spread practice to make the food more attractive. Many substances, like spices and seasonings, have been used as dyes in foods, but have now been replaced by others that also give color.[1]

Interest in the use of synthetic dyes has grown considerably after their discovery in the XVII and XIX centuries and, especially, after influence of color on the consumer acceptance of food was confirmed. However, some abuses have been committed in the use of these additives. They have been used with the intention of masking badly processed or deteriorated foods, some of them being colored with toxic substances.[2]

A lot of industrialized food products have no color, and others lose their natural color during processing and storage. Dyes can supplement or intensify lost color, especially aimed at guaranteeing product acceptance by the consumer.

For many reasons, the food industry has been using synthetic dyes, especially in large scale production, to guarantee food uniformity. Another fact is that synthetic dyes are cheaper when compared to natural ones. They also have a greater coloring range and intensity and are more stable and more available than natural dyes.[3]

Up to 1980, 36 synthetic dye substances and 50 natural substances were used as coloring.[4] Because of the wide range of dye substances, the permitted

ones in each country vary considerably. Since synthetic dyes are being extensively used in foods, it is necessary to control their use and check on possible side effects on human health.

Nowadays, increasing globalization and growth in the importation and exportation of products demands the use of methods, which are more reliable, efficient, and fast in detecting, identifying, and quantifying dyes. It is not enough to prove that a product contains synthetic dyes, it is important to detect each one, or mixtures of them, and quantify each separately. Although, many synthetic dye products are on the market and new ones are released every day, their control has been hampered by the lack of adequate analytical methodologies.

The most widely used techniques employ the classical chromatographic methods, such as paper.[5–9] thin layer,[10–18] and open column chromatography.[19–21] However, quantitative analyses using these techniques are too lengthy and produce inaccurate, imprecise data. Gas chromatography methods present restricted application with synthetic dyes, principally because of their high molecular weights, lack of volatility, and limited heat stability at high temperatures.[22]

The development of new equipment and chromatographic column fillings, and the increasing interest in impurities formed during the synthesis of dyes, has resulted in HPLC being considered as an attractive technique and an important instrument in the control of these additives.[23–30] Recently, new advances in analytical methods for synthetic dyes have appeared in the literature. Capillary electrophoresis[31] is one of these new techniques, but is still very new and sophisticated for routine analyses.

The latest trend is to search for methods that simultaneously determine many compounds at low cost and short time, producing qualitative results.[32] HPLC is one of the most appropriate techniques, since it is fast, precise, accurate, and highly sensitive, even for compounds present in very low quantities and within complex matrixes such as food.[33]

The quality of the analytical data is a very important factor to determine the real quantities present in the food, in order to guarantee food safety. In recent years, the AOAC (Association Official Analytical Chemists) has been stimulating analytical quality control using experimental design and various statistical analyses.[34–36] In Brazil, the validation of analytical methods has been a great concern to researchers, but little has been done to validate new methods for synthetic dyes.

The objective of this work was the development and validation of methodology using HPLC, to simultaneously determine eight synthetic dyes: sunset yellow [E-110], yellow n°5 [E-102], bordeaux S [E-123], red n°40 [E-129], red n°17 [E-124], red n°3 [E-127], brilliant blue [E-133] and indigo carmin [E-132] permitted in foods in Brazil.

EXPERIMENTAL

Materials

Six different food products that, according to their labels, claimed the presence of synthetic dyes were analyzed. They were: jelly powder, ready to drink juice powder, soft drinks, isotonic beverages, candies, and chocolate candies. The samples were obtained in supermarkets in Campinas region, Brazil. Two different lots of each product, in duplicate, were analyzed. Each lot consisted of the contents of two packages homogenized together. The samples were separated according to color/taste and manufacturer, giving a total of 45 samples analyzed.

Reagents

The synthetic dye standards were obtained from Importadora Brastóquio Ltda. Chromatographic methanol (ominsolv) and ammonium acetate (analytical grade) were acquired from MERCK, Brazil. The water used to prepare the sample and mobile phases was purified in the Milli-Q system (Millipore). The mobile phases were always filtered with fluoropore FHLP 04500, pore diameter of 0.5 μm and degassed in an ultrasonic bath.

Equipment

After extraction, the samples were centrifuged in a Hitachi centrifuge model Himac CR 21. For the HPLC analyses, an HP liquid chromatography series 1050 with an isocratic pump system, rheodyne injection valve, and 20 μL loop, was used. The separation was carried out using a 150×4.6 mm i.d. Spherisorb ODS-2 column, 5 μm particle size (Sigma-Aldrich, USA), protected by a 30×4.6 mm i.d. Micropore, C_{18} guard column, 5 μm particle size (Varian). The chromatograms were monitored by a diode array detector (DAD), HP series 1050, coupled to a HP ChemStation software, at three different wavelengths. This helped the simultaneous analyses of all types of dye.

Method

Analytical Methodology

For the extraction of jelly and juice powders, 4.0 to 8.0 g of sample were previously blended, and for candies and chocolate candies, 5.0 to 8.0 units of

each color/taste. The dyes were extracted with 20 to 25 mL of heated water (40 to 50°C) and solutions transferred quantitatively to 50 mL volumetric flasks. In the case of chocolate candies with a filling, re-extraction was performed, to guarantee total withdrawal. After completing the flasks to volume, a small portion of 10 mL was taken and centrifuged for 10 min at 15,000 rpm. The supernatant was filtered with a fluoropore, FHLP 01300 (Millipore) membrane (0.5 μm pore) and injected into the chromatograph. Soft drinks and isotonic beverages were simply degassed and filtered.

The column was conditioned with a water/methanol (70 : 30) solution containing 0.08 mol/L of ammonium acetate. The flow rate was 0.5mL/min for 12.5 min. After this period, the same mobile phase, without ammonium acetate, at the same flow rate, separated the dyes by isocratic elution. The eight dyes were separated in 20 min. The column was reconditioned with ammonium acetate in the mobile phase for 12.5 min. The compounds were detected by visible absorption, and the chromatograms were registered simultaneously at three different wavelengths, 595 nm for the blue colorings, 525 nm for the red ones, and 450 nm for the yellow ones.

The identification was performed by comparison of retention times with standards, under the same conditions. Also, co-chromatography and absorption spectra using the diode array detector was performed. Dye quantification was effected using external standard curves with five concentration levels. Each concentration represented the average of three determinations.

The flow chart of the methodology developed for the determination of synthetic dyes present in foods, is shown in Fig. 1.

Methodology Validation

The detection limits were established using standards, successively diluted to determine the lowest detectable quantity, at approximately two to three times the equipment noise. Higher and lower concentrations of the detection limits were added to candies. The dyes added to the candies were the ones not originally present, in order to determine the detection limits using the extraction method described. The quantification limits were established as twice the detection limits for each dye.[37]

A recovery test with standards was carried out, in duplicate, with candies at two different concentration levels.

The repeatability was determined from 10 duplicates of synthetic dye standard solutions, added to the candies. It was calculated according to the following formula:

$$R = t(2DP)^{1/2}$$

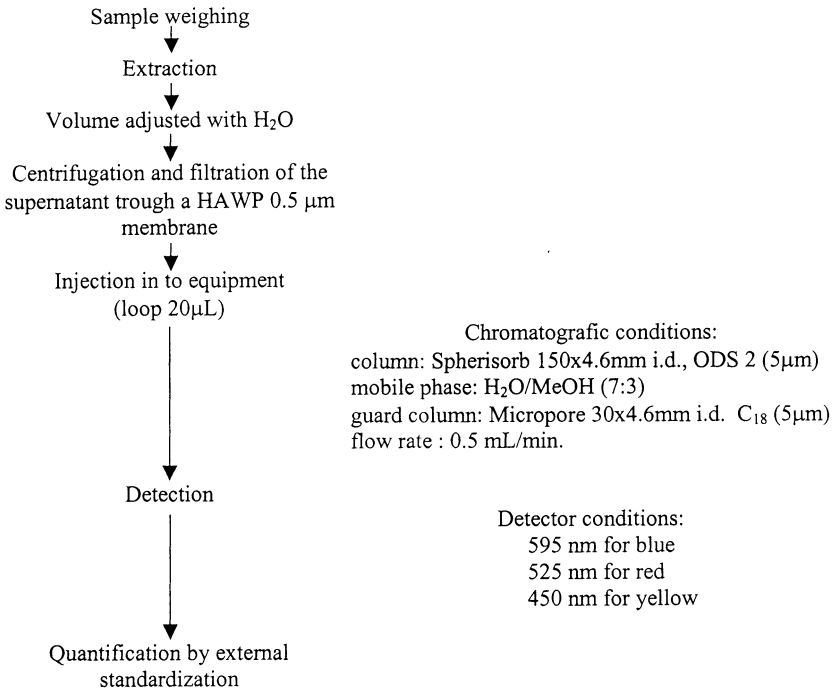

Figure 1. Flow chart of methodology used to determine synthetic dyes.

where: R = repetition, with 90% significance
DP = estimated standard deviation
$t = 2.92$ (according to Caulcutt and Boddy, 1983, Table 1)

The ruggedness study was performed through fractionated factorial planning with eight assays, in order to evaluate how five critical variables

Table 1. Pre-Established (+) and Alternative (−) Conditions to Determine Ruggedness in the Method Developed

Variables	(+)	(−)
A-sample weight	4 g	7 g
B-centrifuge rotation	15.000	10.000
C-centrifuge time	10 min	8 min
D-filter pore diameter	0.5 μm	0.45 μm
E-period between extraction and injection	0 h	24 h

Table 2. Fractionate Factorial Planning—(PROC FACTEX, the SAS System)

	Variables[a]				
Assays	A	B	C	D	E
1	−	−	−	−	+
2	+	−	−	+	−
3	−	+	−	+	−
4	+	+	−	−	+
5	−	−	+	+	+
6	+	−	+	−	−
7	−	+	+	−	−
8	+	+	+	+	+

[a]According to Table 1.

could affect the coloring concentrations. The fractionated factorial planning[38] used was the 2nd (Table 2).

RESULTS AND DISCUSSION

Analytical Step

Besides the advantagses of using an isocratic system, the mobile phase water/methanol (70 : 30) allowed for the eight of the 11 synthetic dyes permitted by the Brazilian legislation to be eluted in a single run of 20 min. Fast conditioning of the column before each injection was significant, since the presence of the buffer improved the selectivity, retention, and symmetry of the peaks. This effect was greater with Sunset yellow [E-110], yellow n°5 [E-102], bordeaux S [E-123], red n°40 [E-129], red n°17 [E-124], red n°3 [E-127], brilliant blue [E-133], and indigo carmin [E-132]. Similar results were obtained by Boley et al.[25] using a buffered mobile phase in a gradient elution system for synthetic dyes.

Preliminary tests were carried out using an ion-pair agent (cetyltrimethylammonium bromide) in the mobile phase, but two days were necessary to condition the system. Although the ion-pair chromatography is the most quoted for synthetic dye analyses,[23–25,28–30,39–41] its use requires long periods of conditioning.

The use of DAD coupled to an HP ChemStation software, allowed for the simultaneous chromatographic registration of many wavelengths, providing the

Table 3. Qualitative and Quantitative Composition of Synthetic Dyes[a]

			Synthetic Dyes (mg/100 g)							
Product	Brand	Flavor/ Color	E-102	E-123	E-124	E-110	E-129	E-132	E-133	Total
Drink from powder juice	A	Orange	11 ± 2			4.8 ± 0.6				16.2
		Strawberry		2.5 ± 0.6		16 ± 3				18.3
		Grape	11 ± 1	34 ± 6		0.1 ± 0.1		9 ± 1		54.3
	B	Orange	3.9 ± 0.7			1.7 ± 0.2				5.6
		Strawberry		3 ± 2		2.3 ± 0.5				5.2
		Passion fruit	3.3 ± 0.4			0.8 ± 0.2				4.1
		Pineapple	2 ± 1			0.2 ± 0.1				2.6
		Grape	3 ± 1	3.4 ± 0.2				0.17 ± 0.04		6.97
Soft drink	C	Grape	0.75 ± 0.05	3.6 ± 0.3				0.46 ± 0.00		4.77
	D	Grape	0.73 ± 0.05	3.5 ± 0.2				0.21 ± 0.02		4.4
		Orange	0.46 ± 0.03	0.05 ± 0.00		2.8 ± 0.2				3.34
Chocolate candies	E	Blue							0.43 ± 0.03	0.43
		Yellow	0.92 ± 0.06							0.92
		Red		3.5 ± 0.3	2.0 ± 0.1		1.12 ± 0.08			3.14
		Green	1.5 ± 0.1						0.37 ± 0.03	1.85
		Orange			Trace	2.6 ± 0.2				2.62
		Brown				2.8 ± 0.2	0.79 ± 0.06	0.29 ± 0.02		3.82
		Blue						0.32 ± 0.02		0.32
		Orange				3.1 ± 0.2				3.05
	F	Red		2.9 ± 0.2	1.7 ± 0.1					1.69
		Yellow	2.3 ± 0.2							2.26

		Brown		0.63 ± 0.04	0.36 ± 0.03	3.7 ± 0.3		0.38 ± 0.03	4.47
		Grape		0.23 ± 0.01		0.01 ± 0.00		0.08 ± 0.00	0.32
Isotonic beverages	G	Tangerine		0.01 ± 0.00		0.22 ± 0.01		0.01 ± 0.00	0.24
	H	Passion fruit							
		Watermelon					0.44 ± 0.02		0.44
		Tangerine				0.38 ± 0.02	0.01 ± 0.00		0.39
Jelly	I	Strawberry		4.2 ± 0.2		3.4 ± 0.2			7.6
		Pineapple	1.57 ± 0.01			0.53 ± 0.02			2.1
		Cherry		10.6 ± 0.2					10.6
		Lemon	1.61 ± 0.03					0.35 ± 0.01	1.96
		Grape		3.2 ± 0.4				0.64 ± 0.08	3.84
	J	Strawberry		3.7 ± 0.3		2.74 ± 0.00			6.44
		Pineapple	2.3 ± 0.3			0.46 ± 0.04			2.76
		Cherry		6.6 ± 0.4					6.6
		Lemon	1.56 ± 0.05					0.6 ± 0.01	2.16
		Grape		3.1 ± 0.1				0.49 ± 0.05	3.59
Candies	K	Blue						0.84 ± 0.00	0.84
		Pink					5.9 ± 0.2		5.87
		Yellow	12.0 ± 0.7						12.01
		Orange	0.37 ± 0.00			11.16 ± 0.62			11.53
		Purple					1.74 ± 0.02	0.78 ± 0.00	2.52
	L	Red/White					2.59 ± 0.03		2.59
		Green/Yellow	10.2 ± 0.5				0.41 ± 0.00	0.94 ± 0.01	11.55
		Red/Orange	0.68 ± 0.00			5.9 ± 0.2	3.88 ± 0.08		10.45

[a]Mean and estimate of standard deviation ($n = 4$).

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profiles of the absorption spectrums required for identification and also allowing for lower detection limits, since each dye can be quantified near its wavelength of maximum absorption. This result can also be achieved by changing the wavelength during the chromatographic run.[24] However, favorable conditions must be present in order to effect this shift, which makes the process much slower.

The qualitative and quantitative compositions of the synthetic dyes present in the samples analyzed are shown in Table 3. The Brazilian legislation permits a different maximum of synthetic dyes in the final product.[42] In the juice powders,

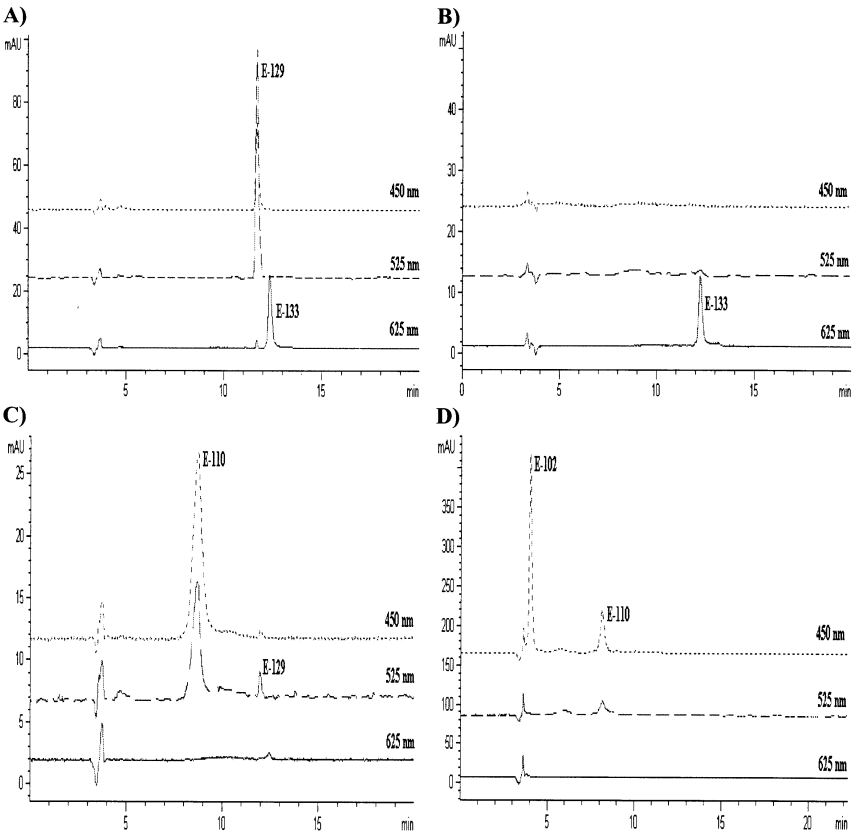

Figure 2. The chromatograms were registered simultaneously at three different wavelengths, 595 nm for the blue colorings, 525 nm for the red ones and 450 nm for the yellow ones. Chromatograms (A) purple candy, brand K, (B) chocolate candy, blue brand, (C) tangerine isotonic beverage brand H, (D) drink from powder juice, brand A. Chromatographic conditions are described in the text.

all the flavors of brand A showed higher concentrations than permitted by the legislation, and in the grape flavor, a mixture of four dyes was detected. All the soft drinks, candies, chocolate candies, isotonic beverages, and jelly powders analyzed, were in accordance with the values established by the legislation. Only one brown chocolate candy sample of brand F presented a mixture of four different dyes and a cherry jelly of brand I, a slightly higher quantity of the allowed limit. For the chewable candies, one of the eight samples showed a concentration higher than the allowed limits. None of the samples showed dyes not permitted by the Brazilian legislation.

All the chromatograms for the isotonic beverage, powder juice, chocolate candies, and candies samples are shown in Fig. 2. There was practically no interference during the detection process.

Methodology Validation

The detection limits found for the synthetic dyes in standard solutions, and in the candies, were the same, and they are presented in Table 4, detection limit signal-to-noise ratio of 3 ($3 \times S/N$). The quantifying limits were considered as double the detection limits. The parameters evaluated agree with those of previous papers.[43–45] No report of these limits was found in the literature on artificial dyes.

The recovery rates for all the eight dyes (Table 5) varied between 96–103% at both levels of candy enrichment. These values indicate an adequate recovery rate. The recovery percentages found in this work are a little higher than those determined by Graichen and Molita[20] and Graichen,[46] and very close to those of Ashkenazi et al.[47] who used open column chromatography to determine food dyes.

Table 6 shows the dye concentrations determined in the candies using factorial combinations of the variables in pre-chromatographic procedures for the evaluation of ruggedness.

Table 4. Detection Limits and Quantification of Synthetic Dyes, According to the Work Conditions of This Study

	Synthetic Dyes							
Limits (μg/mL)	E-132	E-133	E-123	E-124	E-129	E-127	E-102	E-110
Detection	0.51	1.30	1.02	0.57	1.10	0.51	0.70	1.83
Quantification	1.03	2.60	2.04	1.13	2.19	1.03	1.41	3.66

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Table 5. Recovery of Standards Added to Candies in Two Different Concentrations

	Dyes[a]							
Levels (mg/100 mL)	E-132	E-133	E-123	E-124	E-129	E-127	E-102	E-110
I Recovery (%)	3.44	2.93	2.41	2.71	2.52	2.81	2.25	1.84
	96.2	98.0	101.0	96.8	102.0	96.0	99.6	99.0
II Recovery (%)	5.16	4.39	3.61	4.07	3.78	4.21	3.38	2.76
	96.4	98.4	103.0	96.6	100.3	96.0	99.7	98.8

I and II represent the two different concentration levels studied.
[a]Mean of the determination in duplicate.

Table 6. Synthetic Dyes in Candies, Calculated Using Factorial Combinations for the Validation Ruggedness of the Method

	Synthetic Dyes (mg/100 g)							
Combination[a]	E-102	E-123	E-124	E-110	E-129	E-127	E-132	E-133
1	9.02	6.94	6.01	27.12	3.83	5.41	3.14	3.15
2	10.00	7.06	5.61	27.27	4.49	5.20	3.36	3.40
3	9.48	6.78	6.31	26.57	4.22	5.64	2.27	3.37
4	9.15	6.57	6.21	26.86	4.00	5.47	3.13	3.22
5	9.37	6.72	6.43	24.72	4.07	5.03	3.10	2.86
6	9.47	6.61	6.72	25.17	3.88	5.16	3.22	3.28
7	10.20	6.84	6.20	24.84	4.12	4.99	3.15	3.26
8	9.43	7.08	6.14	25.61	3.93	5.08	3.24	3.24
Mean	9.52	6.82	6.21	26.02	4.07	5.25	3.20	3.22
sd	0.38	0.18	0.30	0.99	0.20	0.22	0.08	0.16
cv (%)	3.94	2.65	4.86	3.79	4.96	4.14	2.56	4.85

[a]According to Table 2, cv: coefficient of variation, sd: standard deviation.

Table 7. Repeatability of Dyes in Candies and in Standard Solution, at Two Different Levels of Concentration[a]

Dyes	Candy Concentrations	Repeatability[a] Candy	Standard Concentration	Repeatability[a] Standard
E-132	0.57 ± 0.00	0.00	3.20 ± 0.08	1.17
E-133	1.14 ± 0.01	0.41	3.22 ± 0.016	1.65
E-123	2.10 ± 0.02	0.58	6.83 ± 0.18	1.75
E-124	13.86 ± 0.96	4.05	6.21 ± 0.30	2.26
E-129	5.87 ± 0.17	1.70	4.07 ± 0.20	1.85
E-127	1.96 ± 0.02	0.58	5.25 ± 0.22	1.94
E-102	8.14 ± 0.33	2.37	9.52 ± 0.38	2.55
E-110	2.39 ± 0.03	0.72	26.02 ± 0.98	4.09

[a]Mean of duplicate determinations according to Caulcutt and Boddy.[37] Significance level of 10%.

In the evaluation of possible interactions during the pre-chromatographic stage, the statistical results show that one of the variables studied, centrifuge rotation time, influenced the analysis of yellow sunset.

Table 7, shows that the methodology presented good levels of reproducibility, with values for the coefficient of variation below 5%.

CONCLUSIONS

The use of a water/methanol buffered mobile phase to condition the column between injection, is essential to obtain good resolution of the dyes without the use of ion-pairs.

The recovery and repeatability data recommend the application of this methodology to foods. The ruggedness analysis of the method showed that the only variable that influenced the determination of yellow sunset was the rotational speed of the centrifuge. The other variables showed no effect on determination of any the dyes.

The new methodology gives satisfactory results when applied to the foods analyzed. However, the data obtained indicated the need for a better control of these additives.

We recommend the methodology presented here for the determination of synthetic dyes by HPLC, because it shows advantages in relation to the routine methods, and can simultaneously determine eight synthetic dyes. It is simple and versatile in the extraction phase, with lower losses and fast performance.

REFERENCES

1. *IFT-Food Colours*; A Scientific Status Summary by the Institute of Food Technologyst's Expert Panel of Food Safety and Nutrition and the Committee of Public Information, 1986.
2. *IFT-Food Colours*; A Scientific Status Summary by the Institute of Food Technologyst's Expert Panel of Food Safety and Nutrition and the Committee of Public Information, 1980.
3. Sato, G.S.; Chabaribery, D.; Maia, M.L.; Carvalho, F.C.; Neto, A.N.; Marques, S.A. Tendências de mercado para corantes na indüstria de alimentos. Agricultura em São Paulo, SP, **1992**, *39* (1), 1–50.
4. Coulson, J. Synthetic Organic Colours for Food. In *Developments in Food Colours*, 1st Ed.; Walford, J., Ed.; Appl. Sc. Publ. Ltd: London, 1980.
5. Cho, T.H.; Cho, J.H.; Yang, Y.K. Analysis of Artificial Colouring Matter in Sausage and Ham by Paper Chromatography and Spectrophotometry. The Research Reports of Office of Rural Development **1975**, *17* (5), 87–93.
6. Griffiths, M.H.E. Systematic Identification of Food Dyes Using Paper Chromatography Techniques. J. Food Technol. **1966**, *1*, 63–72.
7. Lees, R. Colour-Identification of Food Dyes. In *Laboratory Handbook of Methods of Food Analysis*, 2nd Ed.; Leonard Hill: London, 1971.
8. Pearson, D. General Methods for Additives and Contaminants. In *The Chemical Analyses of Food*, 7th Ed; Churchill Livingstone: Edinburgh, London, New York, 1976.
9. Sramek, J. Paper Chromatography of Dyes–IV. Paper Chromatography of Water-Soluble Dyes. J. Chromatogr. **1964**, *15*, 57–64.
10. Chiang, H.C.; Lin, S.L. Polyamide Kiselguhr Thin-Layer Chromatography of Yellow Food Dyes. J. Chromatogr. **1969**, *44* (1), 203–204.
11. Gilhooley, R.A.; Hoodless, R.A.; Pitman, K.G.; Thonson, J. Separation and Identification of Food Colours, IV. Extraction of Synthetic Water-Soluble Food Colours. J. Chromatogr. **1972**, *72*, 325–331.
12. Grahan, R.J.T.; Nya, A.E. The Partition Chromatography of Food Dyes on Polycarbonate-Coated Foils. J. Chromatogr. **1969**, *43*, 547–559.
13. Clerq, H.; Massart, D.L. The Thin-Layer Chromatography Separation of Water Soluble Food Dyes on Silica Gel Layer. J. Chromatogr. **1974**, *93* (1), 243–247.
14. Hoodless, R.A.; Pitman, K.G.; Stewart, Thonson, J.; Arnold, J.E. Separation and Identification of Food Colours–Identification of Synthetic Water-Soluble Food Colours Using Thin-Layer Chromatography. J. Chromatogr. **1971**, *54*, 393–404.
15. Lepri, L.; Desidere, P.G.; Coas, V. Separation and Identification of Water-Soluble Food Dyes by Ion Exchange and Soap Thin-Layer Chromatography. J. Chromatogr. **1978**, *161*, 279–286.

16. Perry, A.R.; Wooley, D.G. The Identification of Some Water-Soluble Food Colours by Thin-Layer Chromatography. J. Ass. Publ. Analysts **1969**, *7*, 94–98.
17. Ribeiro-Cunha, M.R.; Cardoso, M.G.; Nelson, M.D.L.; Jokl, L. Alguns métodos qualitativos para diferenciar corantes vermelhos e amarelos estruturalmente semelhantes em alimentos. Ciênci. Tecnol. Alimentos **1996**, *16* (1), 6–11.
18. Toledo, M.C.F.; Guerchon, S. Corantes artificiais em alimentos. Ciênci. Tecnol. Alimentos **1990**, *10* (1), 120–136.
19. Dolinsky, M.; Stein, C. Application of a Liquid Anion Exchange Resin to the Separation of FD and C Colours from Foods. J. Assoc. Off. Anal. Chem. **1962**, *45* (3), 767–769.
20. Graichen, C.; Molitor, J.C. Determination of Certifiable FD&C Colors Additives in Food and Drugs. J. Assoc. Off. Anal. Chem. **1963**, *46* (6), 1022–1029.
21. Yanuka, Y.; Shalon, Y.; Weissenberg, E.; Nir-Grosfeld, I. The Isolation and Separation of Dyes from Foodstuffs by Column Chromatography. Analyst **1963**, *88*, 872–876.
22. Terril, J.B.; Jacobs, E.S. Aplication of Gas–Liquid Chromatography to the Analysis of Antraquinone Dyes and Intermediates. J. Chromatogr. Sci. **1970**, *8*, 604–607.
23. Lancaster, F.E.; Lawrence, J.F. Ion-Pair Liquid Chromatography Determination of Uncombined Intermediates in Three Synthetic Food Colors. J. Assoc. Off. Anal. Chem. **1983**, *66* (6), 1424–1428.
24. Lawrence, J.F.; Lancaster, F.E.; Conacher, H.B.S. Separation and Detection of Synthetic Food Colours by Ion-Pair HPLC. J. Chromatogr. **1981**, *210*, 168–172.
25. Boley, N.P.; Bunton, N.G.; Crosby, N.T.; Johnson, A.E.; Roper, P.; Sommers, L. Determination of Synthetic Colours in Food Using High-Performance Liquid Chromatography. Analyst **1980**, *105*, 589–593.
26. Gratzfeld-Hüsgen, A.; Schuster, R. Sensitive Analysis of Synthetic Colors Using HPLC and Diode-Array Detection at 190–950 nm. Application Note. Hewlett Packard: 1995; Publication Number 12-5964-3559E.
27. Greenway, G.M.; Kometa, N.; Macrae, R. The Determination of Food Colours by HPLC with On-Line Dialysis for Sample Preparation. Anal. Methods Sec. **1992**, *43*, 137–140.
28. Mckone, H.T.; Ivie, K. An Introduction to HPLC: Separation of some FD&C Dyes. J. Chem. Educ. **1980**, *57* (4), 321–322.
29. Puttermans, M.L.; Dryon, L.; Massart, D.L. Isolation, Identification, and Determination of Food Dyes Following Ion-Pair Extraction. J. Assoc. Off. Anal. Chem. **1982**, *65* (3), 737–744.

30. White, P.C.; Harbin, A. HPLC of Acidic Dyes on a Dynamically Modyfied Polystyrene–Divinylbenzene Packing Material with Multi-Wavelength Detection and Absorbance Ratio Characterization. Analyst **1989**, *114*, 877–882.
31. Hewlett Packard—Applications of the HP[3d] Capillary Electroforesis System Publication number 12-5963-7140E, 1, 1995, 107 pp.
32. Agostini, T.A.; Godoy, H.T. *Desenvolvimento de Metodologia para Determinação Simultânea, por CALE, das Vitaminas B_1, B_2, B_6, Ácido Nicotínico e Nicotidamida em Alimentos Enriquecidos.* Tese de doutorado apresentada à faculdade de Engenharia de Alimentos, 1997.
33. Macrae, R. *HPLC In Food Analysis*, 2nd Ed.; Academic Press: London, 1990.
34. Ulberth, F. Optimizing Methods by Ruggedness Testing. In *Analytical Quality Assurance and Good Laboratory Practice in Dairy Laboratories.* International Seminar of AOAC. AOAC International, CEC, IDF, VDM, Alemanha, Int. Dairy Federation: Belgica, 1992; 9302, 202–203.
35. Werminont, G.T. *Use Of Statistics to Develop and Evaluate Analytical Methods.* AOAC: Virginia, 1985; 183 pp.
36. Wilrich, P. Role of Statistics in Analytical Quality Assurance. In *Analytical Quality Assurance and Good Laboratory Practice in Dairy Laboratories.* International Seminar of AOAC. AOAC International, CEC, IDF, VDM, Alemanha, Int. Dairy Federation: Belgica, 1992; 9302, 61–79.
37. Caulcutt, R.; Boddy, R. *Statistic For Anaytical Chemists*, 1st. Ed.; Chapman and Hall: London, 1983; 253 pp.
38. Barros Neto, B.; Scarminio, I.S.; Roy, E.B. *Planejamento e Otimização de Experimentos*, 2nd Ed., Campinas SP, editora UNICAMP, 1996.
39. Lancaster, F.E.; Lawrence, J.F. Ion-Pair HPLC Separation and Detection of Subsidiary Dyes in Synthetic Food Colors. J. Assoc. Off. Anal. Chem. **1982**, *65* (6), 1305–1310.
40. Puttermans, M.L.; Dryon, L.; Massart, D.L. Ion-Pair HPLC of Synthetic Water-Soluble Acid Dyes. J. Assoc. Off. Anal. Chem. **1981**, *64*, 1–8.
41. Puttermans, M.L.; Dryon, L.; Massart, D.L. Evaluation of Thin Layer, Paper, and, HPLC for Identification of Dyes Extracted as Ion-Pairs with Tri-*n*-Octylamine. J. Assoc. Off. Anal. Chem. **1982**, *65* (3), 730–736.
42. ANVISA Agência Nacional de Vigilância Sanitária—Reference Eletronic. http://www.anvisa.gov.br. 12/07/2000.
43. Ciola, R. *Fundamentos da Cromatografia a Líquido de Alto Desempenho HPLC*, 1st Ed.; Blücher, E., Ed.; São Paulo, SP, 1998.
44. Ferreira, I.M.P.L.V.O.; Marques, E.M.J.; Ferreira, M.A. Development, Validation, and Application of an HPLC/UV Method for Quantification of Casein in Infant Formulae and Follow-Up Milks. J. Liq. Chrom. & Technol. **2000**, *23* (13), 2057–2065.

45. Kayali-Sayadi, M.N.; Rubio-Barroso, S.; Garcia-Iranzo, R.; Polo-Díez, L.M. Determination of Selected Polycyclic Aromatic Hydrocarbons in Toasted Bread by Supercritical Fluid Extraction and HPLC with Fluorimetric Detection. J. Liq. Chrom. & Technol. **2000**, *23* (12), 1913–1925.
46. Graichen, C. Quantitative Determination of FD&C Colors in Foods. J. Assoc. Off. Anal. Chem **1975**, *58* (2), 278–282.
47. Ashkenazi, P.; Yarnitzky, C.; Cais, M. Determination of Synthetic Food Colours by Means of a Novel Sample Preparation System. Anal. Chim. Acta **1991**, *248*, 289–299.

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