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Analytical Methods

Simultaneous determination of synthetic colorants in yogurt by HPLC

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ABSTRACT

This article reports on a method to determine synthetic dyes in yogurts and milk drinks. Initially a method for extraction of artificial dyes was developed to pretreat samples in order to extract most of the artificial colorants. Then, the colorants were analyzed by HPLC–PAD using gradient elutions. The method was linear in the range of 0.5–25 mg L^{-1} colorants ($0.9991 < R^2 < 0.9998$), with LOD of 0.18–4.58 μg L^{-1}. The results recovery test on two levels ranged from 82% to 115%. The reference solution of the analytes remained stable for 3 months. In the yogurt samples analyzed, the dye E122 (1.4–11.75 mg L^{-1}) was found in most samples, followed by dye E123 (3.12 and 12.56 mg L^{-1}). All samples had concentrations below the maximum level allowed for use in yogurt (100 mg/100 g dyes).

1. Introduction

Yogurt is a product obtained by fermenting milk with the lactic acid bacteria *Streptococcus salivarius* spp. *thermophilus* and *Lactobacillus delbrueckii* spp. *bulgaricus* (Trejo, Corzo-Martínez, Wilkinson, Higginbotham, & Harte, 2014). Yogurt can contain a maximum of 30% (w/w) non-dairy ingredients (MAPA, 2007), while milk drink, under Brazilian law, has to contain at least 51% milk (w/w), whey or milk derivatives reconstituted or not, fermented or not, and may have an additive or microorganisms (MAPA, 2005).

The use of food additives is a common practice throughout the world. Food synthetic colorants are the most interesting group of food additives used in the food industry because the color of a product is important for its attractiveness to the consumer (Kucharska & Grabka, 2010). However, their use range and dosage are restricted strictly around the world (Sun, Sun, Li, Zhang, & Yang, 2013).

In Brazil, the use and maximum limit of colorants in foods is controlled by the National Agency of Sanitary Surveillance (ANVISA), based on a legislative resolution (ANVISA, 1999).

Food can be colored with natural or synthetic food colorants. Natural food colorants can be isolated from suitable plants, fungi or insects (Zeng, Wang, Zhang, & Tong, 1993). Synthetic colorants are also widely used for coloration of food not only to improve appearance and color but also to maintain the natural color during processing or storage (Ghoreishi, Behpour, & Golestaneh, 2012; Llamas, Garrido, Di Nezio, & Fernandez-Band, 2009). Synthetic colorants have several advantages, compared to natural colorants, such as high stability with relation to light, oxygen and pH, color uniformity, low microbiological contamination, and relatively lower production costs (Alves, Brum, Andrade, & Netto, 2008).

The following synthetic azo colorants are permitted in food in Brazil: Tartrazine (INS102), Sunset yellow (INS110), Azorubine (INS122), Amaranth (INS123), Ponceau 4R (INS124), and Allura Red (INS129). The permitted food colorants that are not azo are: Erythrozine (INS127), Patent Blue V (INS131), Indigo Carmine (INS132), Brilliant Blue FCF (INS133) e o Fast Green FCF (INS143).

Synthetic colorants are organic compounds with a chemical structure containing double bonds alternating with single bonds, simple or conjugated aromatic rings and azo compounds. The azo-colorants are capable of being reduced by azoreductase enzymes in intestinal bacteria and in liver cells with the release of aromatic amines to the organism (Hildenbrand, Schmahl, Wodarz, Kimmel, & Dartsch, 1999; Rafii, Hall, & Cerniglia, 1997). This may cause frequent headaches in adults while children often become distracted and hyperactive (Hawley & Buckley, 1976). These effects make knowledge about food components an important issue for the detection of and information on the synthetic colorants in foodstuffs and beverages.

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Towards developing this knowledge, many analytical methods have been developed using spectrometric determination (Blanco, Campana, & Barrero, 1996; El-Sheikh & Al-Degs, 2013; Ni, Qi, & Kokot, 2001; Santos, Demiate, & Nagata, 2010), Raman spectroscopic (Uhlemanna et al., 2012), voltammetry (Gan, Sun, Meng, Song, & Zhang, 2013; Ghoreishi et al., 2012; Ni, Bai, & Jin, 1997), capillary electrophoresis (Huang, Chiu, Sue, & Cheng, 2003; López-Montes, Dupont, Desmazières, & Lavédrine, 2013; Prado, Boas, Bronze, & Godoy, 2006), and high performance liquid chromatography with diode array detection (HPLC–DAD) (Kirschbaum, Krause, Pfalzgraf, & Bruckner, 2003) for synthetic colorant determination in foodstuffs and beverages. These techniques require prior sample preparation and can be used anywhere from a simple dilution followed by filtration up to more advanced techniques such as solid phase extraction (SPE) through centrifugation and sonication of samples to extract all the colorant. The complexity of the sample preparation will depend on the type of sample being analyzed.

Procedures to determine colorants in samples of yogurt, however, were not found in the literature. The aim of the present work, then, was to develop an HPLC – DAD method for the separation and quantification, in a single run, of the 10 food synthetic colorants in yogurt. The colorants taken into account were used as permitted as food additives in food: i.e. INS numbers 102, 110, 122, 123, 124, 127, 129, 132, 133 and 143.

2. Experimental

2.1. Instrumentation

Chromatographic analysis was carried out with a Liquid chromatograph from Shimadzu (Kyoto, Japan), containing a Prominence line operating system with a quaternary pump solvent type LC-20AT, automatic injector SIL-20ACHT, UV–Vis detector with a photodiode array SPD-M20A, column oven CTO-20AC, communication module between the microcomputer and HPLC model CBM-20A, DGU-20A5 degasser. The software LC Solution v.1.25SP1 was used for data acquisition and processing.

A manual pipettor Ecopipette CAPP (10–100 mL), and PA162 PACHANE shaker tubes, a centrifugal brand Cientec CT-5000DR and an automatic pipettor Thermo Scientific (100–1000 μL) were used to prepare the reference and sample solutions.

A spectrometer UV/VIS Lambda 25 (Perkim Elmer) was used for the acquisition of synthetic colorants absorption spectrum. The pH of the buffer solution was measured by a pHmeter pH 2100 series OAKTON (Vernon Hills, IL, USA).

2.2. Chemical and reagents

All the chemicals were of an analytical grade and used without further purification. The water was purified with a Milli-Q system (Millipore, USA).

Methanol (HPLC grade) was purchased from Panreac PAI-ACS (Barcelona, Spain), acetonitrile (HPLC grade) was purchased from Burdick & Jackson ACS (Muskegon, MI, USA), and methanol PA from PROQuímios (Rio de Janeiro, Brazil).

The following synthetic food colorants were analyzed: Tartrazine (INS 102), Bordeaux-S (INS 123), Ponceau-4R (INS 124), Erythrosine (INS 127) and Fast Green (INS 143) from Sigma (St. Louis, MO, USA); Twilight Yellow (INS 110) from Aldrich; Azorubine (INS 122) from Fluka (St. Louis, MO, USA); 40 Red (INS 129), Indigo Carmine (INS 132) and Brilliant Blue (INS 133) from Sigma–Aldrich (St. Louis, MO, USA).

A solution of 0.35 mol L^{-1} $K_4Fe(CN)_6{\cdot}3H_2O$ (RK) was prepared by dissolving 15 g solid (Neon, Brazil) in 100 mL water. A 2 mol L^{-1} ammonia solution was prepared by dilution of 10 mL concentrated solution (Vetec, Brazil) in 90 mL water. A solution 1 mol L^{-1} $ZnSO_4{\cdot}7H_2O$ (RZn) was prepared by dissolving 28.7 g of solid (Alphatec, Brazil) in 100 mL water. Solution 0.13 mol L^{-1} CH_3COONH_4 was prepared by dissolving 77.1 mg (Vetec, Brazil) in 100 mL water. Reference solutions and reagents were stored in amber glass bottles, except the solutions of ammonium hydroxide and ammonium acetate. These were prepared daily.

2.3. Determination of the purity of colorants

Some synthetic dyes are sold with impurities making the determination of the purity of these necessary. To determine purity, 100 mg of colorant is dissolved in 100 mL of purified water and this has been taken as the reference solution. The percentage of purity was calculated accordingly (Minioti, Sakellariou, & Thomaidis, 2007). In this equation, the following variables are considered: dilution factor of measured solution from standard solution; absorbance of measure solution with reference to water; concentration of reference solution; and specific absorbance of aqueous solution of the colorant at 1% (w/v).

2.4. Preparation of reference solution of colorant

Considering the purity of each synthetic colorant, mixed reference working solutions in concentrations ranging from 0.5 to 25 mg L^{-1} were prepared by mixing the individual standards and diluting them with water. All solutions were stored under refrigeration at 4 °C and remained stable for three months.

The colorants were determined at 427 nm (INS 102), 480 nm (INS 110), 515 nm (INS 122), 520 nm (INS 123), 508 nm (INS 124), 527 nm (INS 127), 505 nm (INS 129), 610 nm (INS 132), 629 nm (INS 133), and 624 nm (INS 143).

2.5. Sample

Fifteen samples of yogurt and fermented milk drinks with different flavors were purchased from commercial markets in Recife (Pernambuco, Brazil). All samples were stored in a refrigerator, at 5 °C, until analysis.

2.6. Extraction of colorants from samples

As a necessary step for the precipitation of proteins, three samples containing 10 g of yogurt or milk drink was blended with 0.5 mL of $K_4Fe(CN)_6{\cdot}3H_2O$ (0.35 mol L^{-1}) and 0.5 mL $ZnSO_4{\cdot}7H_2O$ (1 mol L^{-1}) in a 50 mL Falcon tube. The solution was agitated with a tube shaker (PA162 PACHANE) immediately after each addition and left to stand for 10 min. After this time interval, 10 mL of solution methanol:ammonium hydroxide (2 mol L^{-1}) (80:20) was added, the solution was stirred and centrifuged using a centrifuge (Cientec CT-5000DR) at 6000 rpm and 4 °C for 10 min. The centrifugal step was repeated twice more, each time with an addition of 10 mL methanol:ammonium hydroxide, in order to extract the maximum possible colors from the samples.

After each centrifugation, the fractions were collected and transferred to the same 50 mL volumetric flask and its volume was topped up with purified water. An aliquot of the final solution (1 mL) was filtered through 0.45 μm membrane filters from Millipore (Brazil) using a plastic syringe with a capacity of 10 mL of Plascalpand and transferred to a vial with capacity of 1.5 mL of Uniglas and then injected into the chromatograph. The concentration of each sample was determined by HPLC–PAD.

2.7. Evaluation of extraction percentage of colorants

To determine the percentage of colorant extracted, three samples containing 10 g of yogurt (white, free of colorants), was mixed

with volumes corresponding to each colorant from the reference solution at 5 mg L^{-1}.

The samples were prepared according to item 2.6. After each centrifugation, the fraction was transferred to volumetric flask of 50 mL and its volume was topped up with purified water. The concentration of each fraction was measured by HPLC–PAD and the percentages of extraction were calculated and compared to the value of 5 mg L^{-1}.

2.8. Chromatographic conditions

A NUCLEODUR C18ec column (Macherey–Nagel, Dueren, Germany, 150 mm × 4.6 mm i.d.), with a medium density octadecyl modification, endcapped with spherical shaped 3 μm particles and with a carbon load of 17.5% was used for the separation.

The mobile phase consisted of eluent A, ammonium acetate 1% (pH 7.0, adjusted with ammonium hydroxide); and eluent B, methanol:acetonitrile (80:20). The gradient elution program is shown in Table 1. The injection volume was 20 μL. The flow rate was 1.2 mL min^{-1}, the column temperature was maintained between 38–40 °C and the mobile phase was passed through 0.45 μm membrane filter. The food colorants were determined at 427–624 nm.

3. Results and discussion

3.1. Determination of the purity of colorants

The manufacturing process can reduce the colorant content (Kirschbaum et al., 2003) by formation of inorganic salts as by-products, e.g. NaCl, thus leading to erroneous or unreliable results. For this reason, investigation of the purity of colorants is an important preliminary step to minimize or even eliminate this type of error.

The values of the maximum wavelength, specific absorbance, purity percentage, and concentration for each colorant reference solution are presented in Table 2. The wavelength of absorption maximum was used for the chromatographic analysis with a photodiode array detector.

As can be seen, the purity of colorant standards ranged from 59.0% (INS133) to 87.2% (INS127). Therefore, for posterior studies, colorant standard solutions were prepared in accordance with their purity values.

Reference solution stability was determined by comparing the instrumental response generated by reference solution storage with the analytical signal generated by a freshly prepared solution. The aqueous solutions were diluted 1:10 or 1:20, depending on the solution. The absorbance from 370 to 800 nm was measured on the day that the reference solutions were prepared and then every 30 days using an UV–Visible spectrometer. In this work, all solutions of colorants were stored at 4 °C in the dark and were stable for at least 3 months. In the literature (Minioti et al., 2007), it is reported that these solutions have minimal stability of two months when stored under refrigeration in amber vials.

3.2. Extraction of colorants from samples

Four methodologies for sample preparation were tested to precipitate proteins and lipids and maintain the colorants in supernatant. The first test was based on the method of sample preparation for the determination of benzoic and sorbic acids in milk and milk products (ISO 9231, 2008), in order to precipitate proteins present in the samples. In part 1 of this test, 25 mL of NaOH (0.1 mol L^{-1}) and 40 mL of methanol were added to the yogurt samples. This step was excluded from the next tests, because even if the synthetic colorant extraction were successful, the final solution would be too diluted, which could hinder the detection and/or quantification of the analytes in real samples. Another drawback was the turbidity of the supernatant solution and the inefficient precipitation of proteins and lipids, thus proving the inefficiency of test 1.

The second test was conducted to verify if the precipitation of proteins occurred and whether the colorant solubility in the supernatant increased; low volumes of RK and RZN reagents and ten mL of water:ammonium hydroxide (9.1) were added at the pre-treatment and solubility stages, respectively. Although the precipitation was effectively observed, the resulting solution remained turbid, indicating that the colorants had not been totally dissolved and, as a result, the solid material had not completely precipitated.

Another strategy involved a mixture of ammonium hydroxide (ISO 13496, 2000; Zenebon, Pascuet, & Tiglea, 2008) and methanol (Bonan, Fedrizzi, Menotta, & Elisabetta, 2013) solutions in the proportion of 8:2 methanol:ammonium hydroxide (2 mol L^{-1}), to test if the solubility of the synthetic colorant in the supernatant increased. The objective of this test is to verify if the presence of the organic solvent causes the salts (arising from the RK and RZN for example) to migrate to the aqueous phase and agglomerate more easily, anticipating the precipitation of the proteins. When no organic solvent was used, the solution became turbid and there was little precipitate forming material, as in the first test.

The fourth test was performed with addition of methanol to form an extraction mixture with the ammonium hydroxide solution in a ratio of 1:1 (MeOH:NH_4OH (aq)). With this solution the separation between the supernatant and the precipitate increased considerably compared to the second test. Therefore, it was decided to perform a fourth test, increasing the proportion of organic solvent to 8:2 (MeOH:NH_4OH (aq)), which then yielded a much clearer supernatant and totally precipitated solid material.

In these tests, most of the natural colorants were not extracted, with the exception of the Annatto natural colorant (INS 160b) which is soluble in polar solvents, as reported by Tocchini and

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

Time (min)	Mobile phase A (%)*	Mobile phase B (%)*
0	100	0
2	100	0
37	20	80
40	20	80
45	100	0
50	100	0

* Mobile phase A is ammonium acetate 1% (pH 7.0) and mobile phase B is methanol:acetonitrile (80:20).

Table 2
Specific absorbance, maximum wavelength, calculated purity percentage and real concentration of each colorant reference solution.

Colorants	$A^{1\%}_{1cm}$	λ_{max} (nm)	Purity (%)	Real concentration of the reference solution (mg L^{-1})
INS 102	530	427	77.9	790.5
INS 110	555	480	82.7	833.2
INS 122	510	515	75.6	795.3
INS 123	440	520	82.4	840.8
INS 124	430	508	73.3	746.7
INS 127	1100	527	87.2	887.0
INS 129	540	505	85.2	852.7
INS 132	480	610	86.6	868.7
INS 133	1630	629	59.0	592.6
INS 143	1560	624	59.4	607.7

Mercadante (2001), who used methanol and acetone to extract the colorant from plant seeds. Costa and Chaves (2005) tested different types of solvent/extraction solutions (hexane, acetone, $NaHCO_3$ (aq), and NaOH (aq)) to find better extraction efficiency which was obtained with the NaOH (aq) solution. This means that the extraction is similar to that used in the present work, employing methanol in a basic medium. However, except for this colorant, the proposed method is not able to extract natural colorants, being unique to artificial colors. According to Qi, Zeng, Wen, Liang, and Zhang (2011) natural colorants are easily extracted using solvents of low polarity such as hexane.

3.3. Evaluation of extraction percentage of colorants

Three extractions of colorants from the samples were accomplished using the proposed method described in 3.2. The extraction percentages were calculated after each extraction to verify if a substantial part of the analyte had been extracted from the sample. The percentage of extraction was calculated comparing with the sample concentration (5 mg L^{-1}) and are shown in Supplementary Table S1.

The lowest extraction efficiency was observed for the synthetic colorant INS 129 (64.3 ± 3.8%). Even after three extractions, the extraction efficiency for the INS 129 was not as high as for the other colorants as, for example, INS 123 (97.8 ± 1.0%). The use of a methanol–water (95:5) mixture employed by Sun et al. (2013) resulted in extraction efficiency around 80% and 64% for the INS129 and INS127 colorants. Use of methanol as extraction reagent is important because polar compounds are soluble in methanol. In our work, besides the use of methanol, we also used ammonium hydroxide to raise the pH value, in order to deprotonate the colorants and render them more soluble in the aqueous medium. The structures of the colorant are shown in Supplementary Fig. S1.

3.4. Optimization of the separation

A simple method to determine ten food colorants in a single procedure is advantageous for routine analysis. Towards this, gradient elution was tested to attain complete separation of the artificial colorants. Three initial tests were evaluated using the following: phosphate buffer (pH 6.0) and methanol; ammonium acetate buffer (pH 6.5) and methanol; and 1% (m/v) ammonium acetate solution at pH 6.0 and a mixture of methanol:acetonitrile (80:20). The ammonium acetate buffer is known as a modifier for the purification and separation of azo food colorants (**Dinç, Aktas, & Ustundag,** 2005; Garcia-Falcon & Simal-Gandara, 2005; Minioti et al., 2007).

The results obtained in these three tests, however, were unsuccessful. The INS122, INS133, INS143 colorants did not have a satisfactory chromatographic separation with the presence of double peaks in the chromatograms. The INS143 colorant presented a retention time of 41 minutes. Thus, it was necessary to make changes in the composition of the mobile phase to improve these results. The best results were achieved by use of the mobile phase containing an acetonitrile–methanol (20:80) mixture and 1% (m/v) ammonium acetate solution at pH 7.0 (adjusted with ammonium hydroxide solution). Increasing the pH of the aqueous portion of the mobile phase resulted in a poor resolution of the separation of some colorants. A higher concentration of ammonium acetate led to an increase of the retention time, probably due the enhanced interaction of the analyte with the stationary phase.

The elution gradient was established based on Minioti et al. (2007), but some adjustments were made, such as a modification of the pH of the mobile phase to 7.0, because the C-18 column is damaged if a solution with a pH higher than 8.0 (Lanças, 2009) is used. Also, the base used to adjust the pH of ammonium acetate solution was an ammonium hydroxide solution, used to minimize the amount of different ions in the column. Ammonium hydroxide was also used in the sample preparation. These changes did not modify the order of elution and they diminished the time necessary for the chromatographic separation. A chromatogram of the elution of the colorants was obtained with the optimized gradient program (Table 1) by scanning in the wavelength range from 300 to 650 nm, continuously, as shown in Fig. 1.

A total of 60 mL of the solvents methanol, acetonitrile and 1% (m/v) ammonium acetate solution was consumed during the 50 min of the chromatographic run at a gradient program flow of 1.2 mL min^{-1}.

The azo compounds were the first to be eluted from the chromatographic column, followed by non-azo colorants, except for INS 132, which was the third eluted compound. INS 132 is not an azo colorant, but has ketones and amine functions, which confer a polar character onto the compound. The last three synthetic colorants eluted were INS133, INS143 that are derived from triphenylmethane, followed by INS127 that is classified as xanthene (Prado & Godoy, 2003) and have an apolar character. Thus, these compounds interact longer with the column and they are the last to be eluted.

Some of colorants contain sulfur in their chemical structure, thus creating small peaks in the chromatogram derived from isomers probably in mono, di and tri-sulfo-derivative forms (Bonan et al., 2013; Minioti et al., 2007). This occurred with the INS 133 and INS 143 colorants. As can be observed, the INS 133 (22.35 min) colorant appears with a small shoulder in the signal and the INS 143 (22.85 and 23.02 min) showed two signals. Analysis using a photodiode array detector (PAD) confirmed that these peaks did not represent a co-elution.

3.5. Validation of the method

Linear equations of mixed reference solutions, coefficients of determination (R^2), limits of detection (LOD), limits of quantification (LOQ), resolution (Rs), retention time (tR) and repeatability (intra-assay) RSD (%) of each synthetic colorant studied are presented in Table 3.

Calibration curves were traced for a mixed colorant solution in range of 0.5 to 25 mg L^{-1} of each colorant and the calibration equations were determined using the peak area of the substances. In the case of the INS 143 colorant, the sum of the peak areas of two signals was used.

Linearity was evaluated by the values of the coefficients of determination (R^2) and linear calibration curve for each synthetic colorant, shown in Table 3. As can be seen, linearity was obtained in the range of 0.5 to 25 mg L^{-1}, with the correlation coefficient of 0.9991–0.9998.

The value of the limit of detection (LOD) was calculated as three times the standard deviation of the blank solution divided by the slope of the analytical curve. The limit of quantification (LOQ) was ten times the standard deviation of the blank solution divided by the slope of the analytical curve for each colorant analyzed (Ribani, Bottoli, Collins, Jardim, & Melo, 2004). The LOD and LOQ ranged from 0.18 to 4.58 μg L^{-1} and from 0.54 to 13.9 μg L^{-1}, respectively. These values are consistent with the literature, where some authors Minioti et al. (2007) and Vachirapatama, Mahajaroensiri, and Visessanguan (2008) reported values similar to those found in the present study.

Selectivity was assessed, initially, by calculating the resolution (Rs) of the chromatographic peak, as can be seen in Table 3. According to the literature (Lanças, 2009) the acceptable resolution value for a method of quantification is from 1.5, as indication that most signals are well resolved. The resolution of colorants INS 123

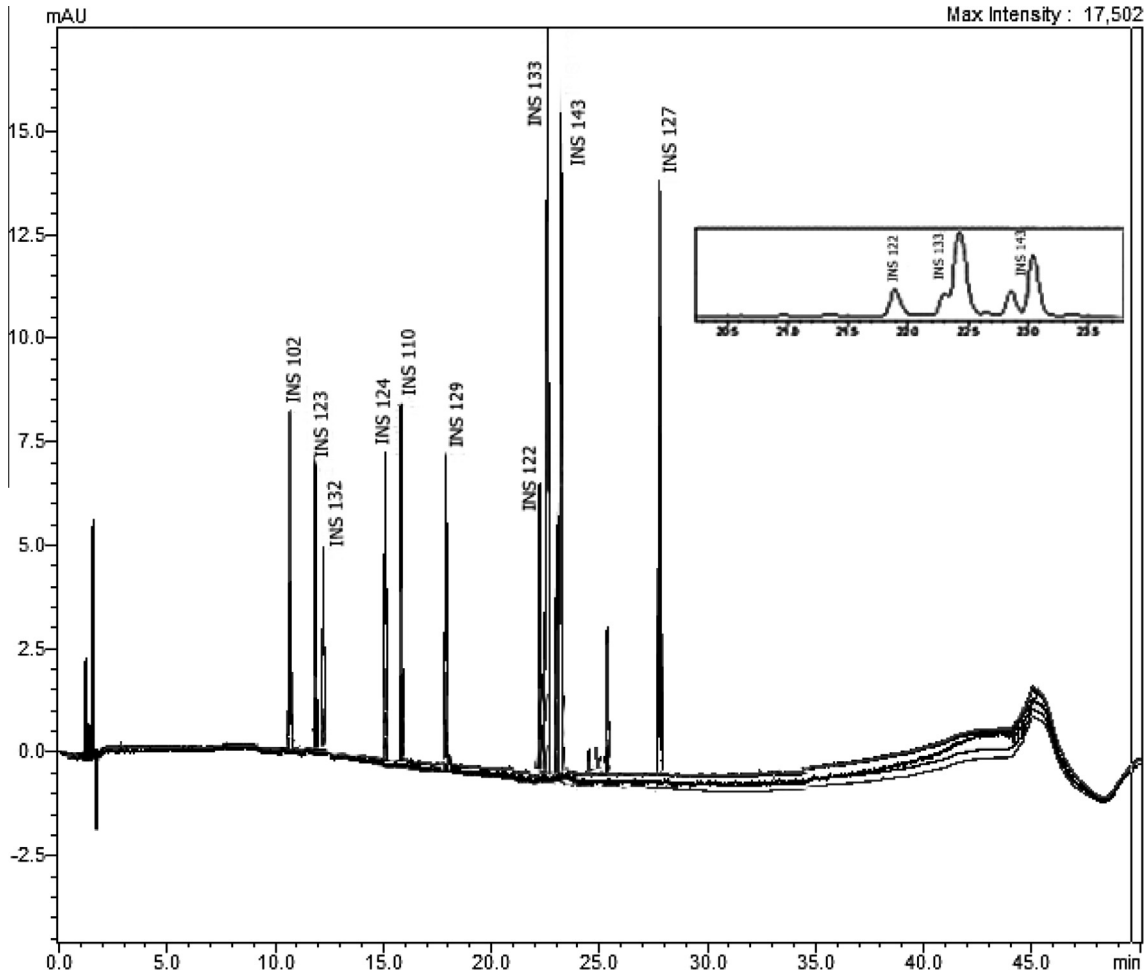

Fig. 1. Chromatogram of a reference mixture solution using the gradient program in Table 1.

Table 3
Linear equations, coefficients of determination (R2), limits of detection (LOD), limits of quantification (LOQ), resolution (Rs), retention time (t_R), repeatability (intra-assay) and RSD (%) of each synthetic colorant studied.

Colorant	Calibration equation	R^2	LOD (μg L^{-1})	LOQ (μg L^{-1})	Rs	t_R (min)	Repeatability (intra-assay) RSD (%)
INS102	Area = 5756.3 + 62743.4 × C	0.9997	1.22	3.71		10.70	1.23
INS110	Area = 5076.4 + 61803.3 × C	0.9996	1.06	3.49	4.2	15.65	0.99
INS122	Area = 3862.3 + 56212.0 × C	0.9993	1.32	4.01	1.4	21.88	1.57
INS123	Area = 9730.9 + 50334.9 × C	0.9998	1.41	4.27	10.8	11.88	1.30
INS124	Area = −252.3 + 53602.1 × C	0.9998	1.20	3.64	4.3	14.80	1.81
INS127	Area = −13284.2 + 1.4 × 10^5 × C	0.9997	0.37	1.12	9.6	27.36	2.83
INS129	Area = 8436.2 + 63810.8 × C	0.9998	4.58	13.9	16.6	17.79	16.20
INS132	Area = -8374.5 + 42846.3 × C	0.9991	1.36	4.13	2.4	12.25	2.58
INS133	Area = 4468.1 + 2.7 × 10^5 × C	0.9996	0.18	0.54	3.0	22.35	1.11
INS143	Area = 12222.8 + 1.7 × 10^5 × C	0.9998	0.29	0.89	16.2	23.02	1.43

n = 6; C: concentration.

and INS 132 was slightly unsatisfactory (Rs = 1.43). However, this value was considered acceptable and there were no difficulties in identifying or quantifying the two colorants. The second method used to determine the selectivity, using a photodiode array detector, is by analyzing the ascending and descending zone of the chromatographic signal and checking to see if there is any change in the absorbance spectrum in the UV–VIS region of the compound interest. In other words, if the total area of the signal has the same absorbance spectrum, this means that is a single compound. The analysis of all signals found non-co-eluting compounds but no interfering species.

The retention time (t_R) of each colorant, in minutes, showed that an excellent separation was achieved within 28 min. This indicates that 50 min is sufficient time for one measure, considering the stabilization time of column and preparation of system for the next analysis.

The repeatability was evaluated to certify the accuracy of the method and was expressed through the residual standard deviation (RSD).

The accuracy and the recovery (Table 4) of the method were verified at two fortification levels (1 and 10 mg L^{-1}) and the RSD calculation ranged from 0.33 to 2.72% (n = 3). The RSD values

Table 4
Average percentage of recovery (%) and RSD values for each concentration level of artificial colorant analyzed.

Colorant	1 mg L^{-1}	10 mg L^{-1}
INS 102	98.1 ± 0.3	106.6 ± 1.3
INS 110	105.1 ± 1.6	100.1 ± 1.7
INS 122	101.9 ± 1.6	97.1 ± 1.6
INS 123	103.0 ± 1.6	97.1 ± 1.8
INS 124	100.2 ± 1.7	97.2 ± 1.7
INS 127	99.8 ± 1.5	94.4 ± 1.6
INS 129	97.2 ± 2.5	81.8 ± 2.0
INS 132	114.7 ± 0.3	111.4 ± 2.7
INS 133	101.6 ± 1.6	98.1 ± 1.7
INS 143	101.1 ± 1.7	98.8 ± 1.6

$n = 3$.

Table 5
Artificial colorants identified and quantified in real samples of yogurt (Y) and milk drink (MD).

Sample	Name of colorant declared on the label	Colorant found	Concentration (mg L^{-1})
Y1	INS 110	NI	
MD2	No artificial colorant	NI	
MD3	INS 124	INS 110	0.89 ± 0.27
MD4	No artificial colorant	NI	
MD5	INS 122	INS 122	5.34 ± 0.15
MD6	INS 123	INS 122	1.43 ± 0.05
	INS 124		
Y7	INS 124	NI	
	INS133		
Y8	INS122	INS 122	5.32 ± 0.62
MD9	INS 122	INS 122	11.75 ± 0.21
MD10	INS 122	INS 122	11.72 ± 0.13
Y11	INS 123	INS 123	12.56 ± 0.08
	INS 110		
MD12	INS 123	NI	
	INS 124		
MD13	INS 102	INS 102	75.30 ± 3.85
	INS 110	INS 127	4.16 ± 0.00
MD14	INS 124	INS123	3.12 ± 0.03
		INS124	12.16 ± 0.12
MD15	INS 110	NI	

n = 3.

obtained are below the maximum limit for trace analysis or impurities in samples, which is 20% for chromatographic analyses (Ribani et al., 2004).

The data showed that the recovery for the analytes was in the range of 81.84–114.68% and these results are within the level considered acceptable in the literature. This demonstrates that the approach could ensure simultaneous examination of 10 food colorants with better recovery.

3.6. Application to real samples

The method developed was applied to analyze fifteen samples of yogurt and milk drink and the results are shown in Table 5.

As can be seen, 6 out of the 10 colorants were detected, at least once. The concentrations of colorants in yogurt and milk drink ranged from 0.89 (sample MD3) to 75.30 mg L^{-1} (sample MD13).

The sample MD13 had the highest concentration among the others, containing 75.30 ± 3.85 mg L^{-1} of INS 102 colorant. A curious fact is that some manufacturers report that their product contains a particular colorant although our method identified and quantified a different one and sometimes there was no synthetic colorant. As an example, in the samples Y7, MD12 and MD15 the colorants were not identified or the values were below the limit of detection for all colorants studied in this work.

Among the samples analyzed there were two (samples MD2 and MD4), where the manufacturer stated that no artificial colorant had been used, just natural. Analysis confirmed the absence of artificial colorant or values were below the LOD for the colorant amounts present in the proposed method. In the others, the most commonly used dye was INS 122, having been identified in 33% of samples in a range from 1.43 to 11.75 mg L^{-1}.

4. Conclusions

A method was developed to determine ten artificial food colorants, commercially accepted in Brazil and other countries, in samples of yogurt and milk drinks. For this purpose, initially, a method of sample preparation was developed, which involved a simultaneous extraction step for the ten artificial colorants. The extraction method was simple, rapid and of moderate cost. Although a centrifuge was used to mix the solutions, when compared to the cost of more sophisticated methods of extraction such as solid phase extraction, the method developed in this work was inexpensive.

The method was validated, and the colorant concentration in the samples varied from 0.89 to 75.30 mg L^{-1} for INS 110 and INS 102, respectively. The concentration of certain artificial colorants in real samples was below the upper limit of 1000 mg/100 g. Among the artificial colorants quantified in samples of yogurt and milk drinks, INS 122 was identified in 33% of samples. The method developed proved specific for artificial colorants but not for the natural colorant Annatto.

Conflict of interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.03.050.

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