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# Liquid Chromatographic Determination of Natural and Synthetic Colorants in Lyophilized Foods Using an Automatic Solid-Phase Extraction System

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Five synthetic and five natural colorants were determined in lyophilized dairy products and fatty foods using an automatic method based on lixiviation and a solid-phase extraction process that includes cotton and RP-$C_{18}$ columns for the sequential retention of synthetic colorants and natural colorants, respectively. The lyophilization of the sample coupled with the separation procedure provides clean extracts despite the complexity of the matrices studied. In addition, the lyophilization process preserves the sample for at least 2 months without changes in the concentrations of the colorants. Identification and determination of synthetic and natural colorants were carried out using a liquid chromatograph equipped with a diode array detector. The detection limits achieved for all of the colorants (0.03−75 $\mu$g/g of lyophilized sample) allowed their determination within the limits established by the European Union, with good precision (≈4.5%). In addition, colorants spiked to different foods provided average recoveries (spiked at three concentration levels in four types of dairy samples and in three types of fatty foods) near 94 ± 4%.

**KEYWORDS: Natural and synthetic colorants; dairy products and fatty foods; solid-phase extraction; liquid chromatography with diode array detection**

## INTRODUCTION

For a consumer, the color of food is one of its most important characteristics. Because it predetermines certain expectations of quality and flavor, a product with an unexpected color will be rejected. It is normal practice to add colorants to enhance, homogenize, or even change the color of food to make it more attractive to consumers (*1*). Food colorants are usually classified as either natural (or nature-identical) or synthetic. Natural colorants generally have a lower tinctorial strength than synthetic colorants and are generally more sensitive to light, temperature, pH, and redox agents (*2*). Both the compounds used as color additives and their concentration limits are subject to regulations in the European Union and the United States (*3*, *4*). Therefore, the need for analytical control of colorants to ensure that banned additives are not present in food and to determine those permitted by regulations is becoming more important.

Simple and rapid methods for the separation and determination of natural and synthetic colorants are required. The preferred choice for the resolution of mixtures of colorants is often liquid chromatography (LC) (*5*−*22*), normally coupled with a diode array detector (DAD) for confirmation (*7*, *11*, *14*, *17*−*22*).

Foods have very complex matrices; for example, samples that contain high concentrations of proteins or high concentrations of fats can complicate the extraction of both natural and synthetic colorants, and some of the particles of the food that pass to the extract can block the sorbent columns of solid-phase extraction and chromatographic systems. To provide clean extracts, these foods require a pretreatment that usually includes extraction or protein precipitation and cleanup processes that are normally tedious and time-consuming. Basically, a homogenized sample (rich in proteins and/or fats) is extracted, once or several times, using a single solvent (*9*, *11*−*13*) or a solvent mixture (*10*, *18*, *21*), or the proteins are eliminated by precipitation by adding a solvent (*6*, *11*, *19*, *20*, *22*) or by enzymatic treatment (*5*, *8*). Usually, additional cleanup steps such as dialysis (*6*, *9*), liquid−liquid extraction (*10*, *12*, *13*, *18*), and/or solid-phase extraction (*5*, *8*, *9*, *19*) are necessary, prior to injection of the sample into the liquid chromatograph, to remove coextractives that could interfere with the colorants during chromatographic separation. The solid-phase extraction (SPE) can be applied on polyamide (*5*) or on RP-$C_{18}$ (*8*, *9*, *19*). Recently, our group developed a screening method that discriminates between synthetic colorants and natural food colorants (*22*). That method uses a continuous-flow system designed to condition dairy samples (previously pretreated manually) in order to remove the sample matrix as well as to preconcentrate colorants before their discrimination/identification in a liquid chromatographic system.

All of the methods that have been developed to date are based on directly extracting colorants from foods. However, pretreating food samples using lyophilization prior to extraction has two principal advantages: the first is in sample preservation because

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lyophilization increases the stability of the samples without loss of analytes; second, the lyophilized sample has a more homogeneous and accessible matrix. In this paper, the SPE system proposed elsewhere (*22*) for dairy sample screening was initially adopted to determine five synthetic and five natural colorants in dairy samples (with high protein content) and fatty foods (with high fat content) without modifying the procedure in any way for the different sample matrices. The proposed method implements a sample lyophilization step prior to automatic lixiviation with a single solvent (1 mol/L of acetic acid/sodium acetate buffer), which is then followed by preconcentration and cleanup on a miniaturized cotton column for synthetic colorants and on an RP-$C_{18}$ column for natural colorants. The method is rapid and does not require either laborious sample manipulation or use of a complicated SPE system.

## MATERIALS AND METHODS

**Apparatus.** A Shimadzu modular liquid chromatographic system (Kyoto, Japan), equipped with an LC-10 AD pump, an SPD-M6A UV−visible diode array detector, equipped with a low-volume flow cell (path length = 10 mm, inner volume = 8 $\mu$L), and a Rheodyne model 7725i injector with a sample loop of 20 $\mu$L was used for the chromatographic analysis of both natural and synthetic colorants. This system was controlled via Class LC-10 software (also from Shimadzu). Separations were done using a Spherisorb ODS-2 RP-$C_{18}$ (5 $\mu$m, 25 cm × 4.6 mm i.d.) column (Alltech, Deerfield, IL) fitted with a Spherisorb ODS-2 (5 $\mu$m, 7.5 × 4.6 mm i.d.) guard column. All analyses were performed at room temperature. The mobile-phase gradient for natural colorants consisted of an initial mixture of 85% methanol and 15% water containing 0.07 g/L ($2 \times 10^{-4}$ mol/L) of cetyl-trimethylammonium bromide (as ion interaction reagent, IIR), adjusted to pH 6.0 with orthophosphoric acid and held for 3 min. Then the mixture was changed to 20% methanol and 80% ethyl acetate and maintained for 10 min. The mobile-phase flow rate was 1.0 mL/min. For synthetic colorants, the optimized mobile phase consisted of a mixture of acetonitrile (ACN) and water containing 0.29 g/L ($8 \times 10^{-4}$ mol/L) of cetyl-trimethylammonium bromide (adjusted to pH 5.5 with orthophosphoric acid). The gradient consisted of an initial ACN concentration of 45% increased to 55% in 6.5 min and then immediately increased to 65% and maintained for 1.5 min, and the flow rate was 2.0 mL/min.

The chromatographic system for both synthetic and natural colorants was conditioned by passing the mobile phase through the column until a stable baseline was obtained (20 min at a flow rate of 1.0 mL/min). This procedure was followed for every new mobile phase used. Detection wavelengths for the diode array detector (DAD) were set at 425, 500, and 610 nm simultaneously for natural colorant analyses and at 400, 530, and 610 nm simultaneously for yellow, red, and green-blue-brown synthetic colorant, respectively, analyses. The spectra (detection wavelengths from 375 to 700 nm) were recorded for all peaks. The identities of the different chromatographic peaks were confirmed by comparing their visible spectral characteristics to standards and retention times. The efficiency of peak separation in samples was checked by the peak purity test carried out at maximum absorbance.

The flow system was made using a Gilson Minipuls-3 peristaltic pump (Villiers-le-Bel, France) furnished with poly(vinyl chloride) and Solvaflex pumping tubes, for aqueous and methanolic solutions, respectively, three Rheodyne 5041 injection valves (Cotati, CA), one Rheodyne 5060 rotary valve, PTFE tubing of 0.5 mm i.d., standard connectors, and laboratory-made sorbent columns. The sorbent columns were constructed from PTFE capillaries of 3 mm i.d., hand-packed with 80 mg of cotton or 100 mg of RP-$C_{18}$ for sorption of synthetic or natural colorants, respectively. These columns can be reused for at least 3 months, working daily. To prevent clogging of the sorbent columns, a stainless steel LC filter was fitted at the inlet of the aspiration sample channel.

A Selecta water bath (Barcelona, Spain), a Selecta magnetic stirrer with temperature control, and a Telstar laboratory freeze-dryer type Cryodos-45 (Barcelona, Spain) were also employed.

**Chemicals and Standard Solutions.** Synthetic colorants [tartrazine (E-102), lissamine green B (E-142), brilliant blue FCF (E-133), indigo carmine (E-132), and brilliant black BN (E-151)] and natural colorants [curcumin (E-100), riboflavin (E-101i), *trans*-$\beta$-carotene (E-160a), and carminic acid (E-120)] were supplied by Sigma-Aldrich (Madrid, Spain). Because caramel color (class I; E-150a) was not commercially available, it was obtained by treating glucose as follows: glucose (100 g) in Milli-Q water (10 mL) was heated in a stainless steel vessel up to 170 °C for 170 min with continuous stirring (*23*); color formation was monitored at 610 nm until the color intensity ranged from 0.010 to 0.140 absorbance unit. Color intensity was defined as the absorbance of a 0.1% w/v solution of caramel color solid in water in a 1 cm cell at 610 nm. When the color target was reached, additional water (50 mL) was added to cool the caramel.

Cetyl-trimethylammonium bromide, ascorbic acid, and glucose were also purchased from Sigma-Aldrich. All other reagents (acetic acid, sodium acetate, ammonium hydroxide, ammonium acetate, orthophosphoric acid, sodium hydroxide, petroleum ether, ethanol, *n*-hexane, and HPLC grade acetonitrile, methanol, and ethyl acetate) were supplied from Merck (Darmstadt, Germany). Polygosyl-bonded silica reversed-phase sorbent with octadecyl functional groups (RP-$C_{18}$) was obtained from Sigma-Aldrich. Natural cotton was washed with petroleum ether to eliminate the natural grease of the fiber (*24*).

Stock standard solutions containing 1 mg/mL of individual colorants (tartrazine, lissamine green B, brilliant blue FCF, indigo carmine, brilliant black BN, and caramel) and 0.2 mg/mL of riboflavin were prepared in Milli-Q water and 1 mg/mL of curcumin, *trans*-$\beta$-carotene, and carminic acid in ethanol. All stock solutions were stored in glass-stoppered bottles at 4 °C in the dark. Solutions of variable concentrations were prepared daily by diluting the stock standard solutions in 1 mol/L of the acetic acid/sodium acetate buffer.

**Sample Preparation.** Dairy samples (yogurts, ice creams, milkshakes, and dairy desserts) and fatty samples (sauces, dehydrated soups, and bouillon cubes) were purchased at local markets in Spain. A laboratory sample of ≈100 g was homogenized in a high-speed blender, and fractions of ≈25 g were lyophilized by freeze-drying at 50 mPa for 24−48 h (24 h for the dairy samples and 48 h for sauces). Laboratory-lyophilized samples, dehydrated soups, and bouillon cubes were conserved in glass containers, at −80 °C in the dark, until analysis. Under these conditions, the concentration of colorants remained constant for at least 2 months.

The lyophilized sample was prepared according to the following method. An accurately weighed amount of 0.1−2.5 g of lyophilized sample ($n = 6$) was placed into a 50-mL amber glass bottle (to protect light-sensitive colorants) with 15 mL of 1 mol/L acetic acid/sodium acetate buffer. Because *trans*-$\beta$-carotene oxidizes easily, when this colorant is quantified, optimum results are achieved by adding a reducing agent such as ascorbic acid to the 15 mL of extractant at a concentration of 200 $\mu$g/mL. Once stoppered, the mixture was mechanically shaken for 10 min and allowed to settle. Then, aliquots of the aqueous extract (3 mL) were continuously aspirated and filtered (through a stainless steel LC filter), which filtered the fine particles that were sometimes present in the extract, into the flow system shown in **Figure 1**.

**Analytical Method. Figure 1** shows the continuous-flow system used for the determination of synthetic and natural colorants. In the preconcentration step, 3 mL of a standard solution or a treated sample containing natural and/or synthetic colorants at concentrations of 0.01−200 $\mu$g/mL (omitting caramel; for lyophilized samples, concentrations between 0.1 $\mu$g/g and 3 mg/g) in 1 mol/L acetic acid/sodium acetate, pH 4.7, was passed through the cotton sorbent column. Both the sample and sorbent column were warmed at 40 °C. Synthetic colorants were retained in the cotton column, and the effluent containing natural colorants was passed through an RP-$C_{18}$ column for their retention. A volume of 1.5 mL of water was employed as carrier to complete sample introduction and to remove any potential interfering compound and other organic compounds adsorbed in both columns.

In the elution step for the discrimination of synthetic colorants the selection valve (SV) was in position 1 and the $IV_3$ (75 $\mu$L) loop filled with eluent (1 mol/L $NH_3$). Two sequential injections of 75 $\mu$L of 1 mol/L $NH_3$ (from $IV_3$) were carried by the $N_2$ stream and passed through

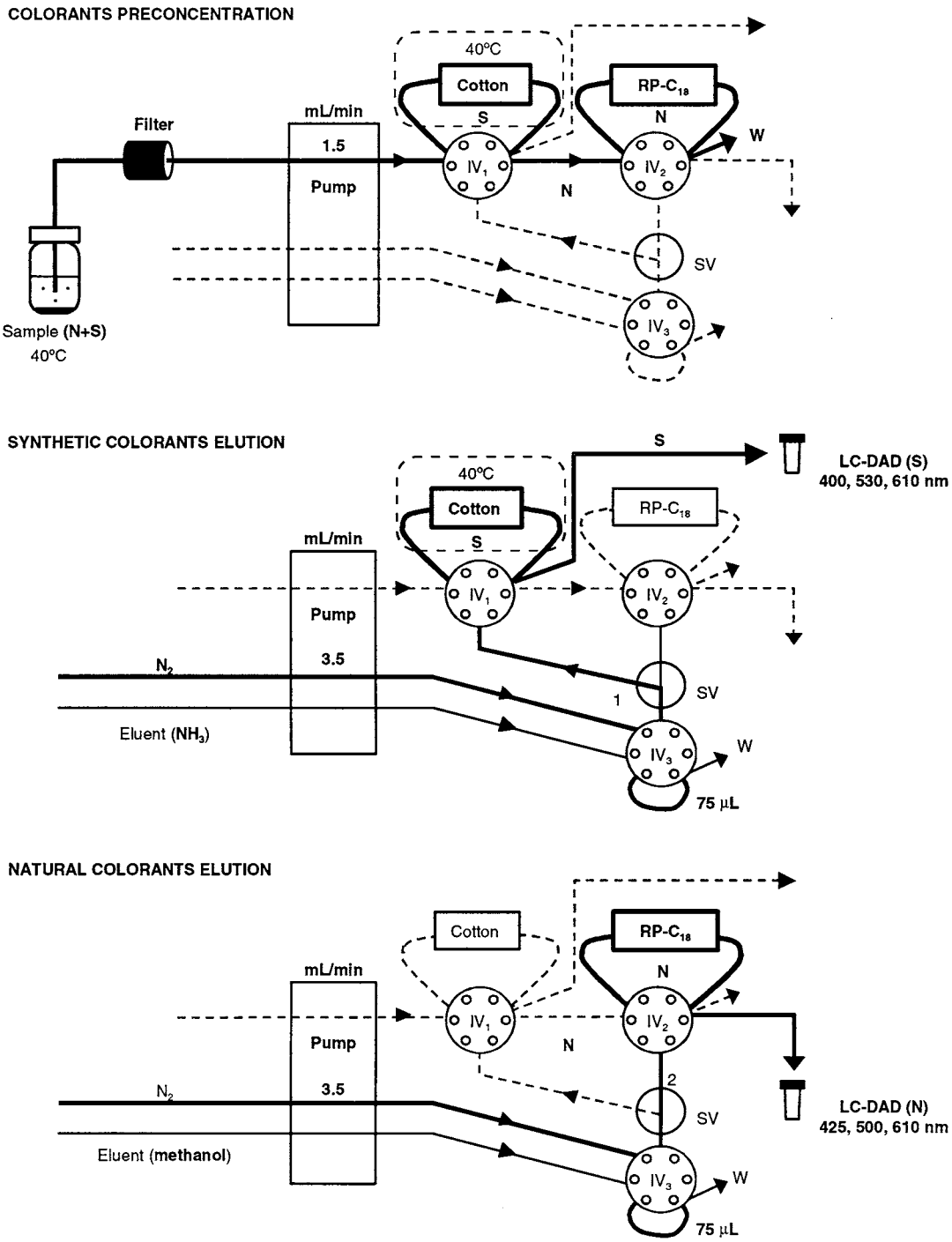


**Figure 1.** Flow injection manifold for the on-line preconcentration of synthetic (S) and natural (N) colorants in dairy and fatty samples and their off-line determination by liquid chromatography. IV, injection valve; W, waste; SV, selecting valve; LC-DAD, liquid chromatograph diode array detector.

the cotton column (in the opposite direction of the sample aspiration) to elute adsorbed synthetic colorants (position in bold lines in **Figure 1**). Only the eluate of the second injection (containing the highest fraction of colorants) was collected into glass vials, and a 20-$\mu$L aliquot was manually injected into the liquid chromatograph. The cotton column was conditioned with 3 mL of 1 mol/L buffer, pH 4.7, and occasionally washed with 3 mL of ethanol (viz. after every 20 real samples). For the discrimination of natural colorants, the SV valve was in position 2 and the $IV_3$ loop filled with the eluent, methanol. Two sequential 75-$\mu$L injections of the eluent were also carried by the $N_2$ stream and passed through the RP-$C_{18}$ column (in the opposite direction of the sample aspiration), to elute adsorbed natural colorants (position in bold lines in **Figure 1**). Again, only the eluate of the second injection was collected into amber glass vials, and a 20-$\mu$L aliquot was manually injected into the liquid chromatograph. The RP-$C_{18}$ column was conditioned with 1.5 mL of methanol and then with 3 mL of 1 mol/L buffer, pH 4.7, and occasionally washed with 3 mL of *n*-hexane (viz. after every 20 real samples). Peak height (as mA units) was used for quantification and a (20-$\mu$L) eluent blank was also used.

**Statistical Analysis.** Analysis of variance was used to evaluate the recovery results obtained from the comparison between nonlyophilized and lyophilized samples and from the different types of analyzed samples, different colorants and the different level of spiked colorants used in the recovery test. Fisher's least-significant difference test (LSD) at the 5% significance level was applied to experimental results to asses intrapair significant differences. Statistical analyses were carried out with the Systat version 10.2 statistical package (Systat Software Inc., Richmond, CA).

**Table 1.** Chemical Variables, Flow Variables, and Chromatographic Conditions Selected for the Proposed Method

| variable | synthetic colorants | natural colorants |
|---|---|---|
| solid-phase extraction system | | |
| sample pH | 4.7 | 4.7 |
| acetic acid/sodium acetate concn (mol/L) | 1.0 | 1.0 |
| eluent | $NH_3$, pH 11.5 | methanol |
| adsorption temp (°C) | 40 | |
| amount of sorbent (mg) | 80 | 100 |
| sample breakthrough vol (mL) | 3.0 | 3.0 |
| sample flow rate (mL/min) | 1.5 | 1.5 |
| eluent vol ($\mu$L) | 75 | 75 |
| $N_2$ flow rate (mL/min) | 3.5 | 3.5 |
| chromatographic conditions | | |
| mobile-phase composition | acetonitrile/water | methanol/water/ethyl acetate |
| IIR concn (mol/L) | $8 \times 10^{-4}$ | $2 \times 10^{-4}$ |
| mobile-phase pH | 5.5 | 6.0 |
| flow rate | 2.0 | 1.0 |
| injection vol ($\mu$L) | 20 | 20 |
| wavelength (nm) | 400, 530, and 610 | 425, 500, and 610 |

## RESULTS AND DISCUSSION

**Lyophilization of the Foods.** Dairy products and fatty foods contain an abundant amount of water (between 55 and 82%), which favors biodegradation and hydrolysis reactions. The lyophilization of samples provides at least three advantages: it facilitates both the storage and conservation of the samples and simplifies the extraction procedure.

Preliminary experiments were focused on the lyophilization of the samples; pistachio ice cream and peach yogurt were selected as dairy sample tests, and a sauce for chicken was selected for the fatty sample test. Each test sample was homogenized, and then 1 g was treated according to a previous manual method for dairy samples developed by our group (*22*): the sample was directly mixed with 5 mL of 0.1 mol/L ammonium acetate containing 200 $\mu$g/mL of ascorbic acid, magnetically stirred for 5 min, and then centrifuged at 2000 rpm for 10 min to precipitate proteins; next, the supernatant was diluted to 25 mL with 1 mol/L acetic acid/sodium acetate buffer, and aliquots of the sample (3 mL) were aspirated into the flow system shown in **Figure 1**. One gram of each test sample was lyophilized at 50 mPa for 24−48 h (24 h for the dairy samples and 48 h for the fatty sample), and then the remainder of the sample was directly extracted with 25 mL of 1 mol/L acetic acid/sodium acetate buffer (each sample lost water due to the lyophilization process, leaving the following amounts of dehydrated powder: 0.39 g of pistachio ice cream, 0.19 g of peach yogurt, and 0.30 g of sauce for chicken). The results of these experiments were validated by spiking the three homogenized test samples with the 10 colorants studied at concentrations (per gram of nonlyophilized food) of 5 $\mu$g/g of lissamine green B and brilliant blue FCF; 12.5 $\mu$g/g of tartrazine, indigo carmine, and brilliant black BN; 25 $\mu$g/g of carminic acid; 50 $\mu$g/g of riboflavin and *trans*-$\beta$-carotene; 250 $\mu$g/g of curcumin; and 1875 $\mu$g/g of caramel; the recoveries for each colorant were calculated. The results of these experiments (repeated four times) were statistically analyzed and provided the following conclusions: (i) the studied synthetic and natural colorants were not evaporated or destroyed during the lyophilization process; (ii) although the percentages of recovery were similar (≈94%) in the two parallel sets, the precision was slightly higher in lyophilized samples that in nonlyophilized ones; (iii) the direct extraction of the lyophilized samples with 1 mol/L acetic acid/sodium acetate solution provided cleaner extracts than nonlyophilized samples and did not require the addition of ascorbic acid as reducing agent to minimize the oxidation of some colorants; and (iv) there were no significant differences in the recoveries for either of the sample matrices (dairy and fatty foods). Therefore, in further experiments all samples were lyophilized to simplify their pretreatment.

**Solid-Phase Extraction Method.** When a mixture of natural and synthetic colorants is injected into the liquid chromatograph using the optimum conditions for either synthetic or natural colorants, the mixture is not resolved (*22*). Instead, it is necessary to separate each type of colorant in two different extracts (one for natural and the other for synthetic). For this reason, an SPE system was developed elsewhere (*22*) based on the fact that synthetic colorants dye natural materials such as cotton, whereas natural colorants do not. To discriminate colorants, liquid chromatography must be used to separate each compound; synthetic colorants were monitored by simultaneously using 400, 530, and 610 nm wavelengths, whereas natural colorants were monitored at 425, 500, and 610 nm for yellow, red, and green-blue-brown additives, respectively. The most significant variables for the optimized SPE system (*22*) are listed in **Table 1**. Natural colorants were not retained on the cotton column between pH 1 and 10, whereas all synthetic colorants (tartrazine, lissamine green B, brilliant blue FCF, indigo carmine, and brilliant black BN) studied were completely retained between pH 2.0 and 5.0 (adsorption efficiency of ∼100%); the sorbent column must be heated at 40 °C to favor the adsorption of synthetic colorants. Natural colorants (curcumin, riboflavin, *trans*-$\beta$-carotene, carminic acid, and caramel) present in the effluent of the cotton column, as shown in **Figure 1**, were completely retained on the RP-$C_{18}$ column also between pH 2.0 and 5.0 (adsorption efficiency of ∼100%). Therefore, a 1.0 mol/L acetic acid/sodium acetate buffer at pH 4.7 was selected as the medium for sample adsorption, and this solution was employed as extractant of the lyophilized sample. The best efficiency in the elution step was obtained by using ammoniacal and methanolic solutions for synthetic and natural colorants, respectively. The breakthrough volume of the sample, defined as the sample volume at which the analyte begins to elute from the column sorbent, was evaluated in the optimized SPE system in order to find the maximum sample volume that can be used and, therefore, the maximum preconcentration factor possible. The results of this study showed that 3 mL was the breakthrough volume for both synthetic and natural colorants. A summary of the other chemicals and flow conditions studied is given in **Table 1**.

The chromatographic conditions selected for the determination of synthetic and natural colorants are shown in **Table 1**. As can be seen, the composition of the mobile phase varied

**Table 2.** Analytical Figures of Merit of the Proposed Solid-Phase Extraction Method

| | slope | | | linear range[a] | detection limit[a] | |
|---|---|---|---|---|---|---|
| synthetic colorant | 400 nm | 530 nm | 610 nm | ($\mu$g/g lyophilized) | ($\mu$g/g lyophilized) | RSD (%) |
| tartrazine | 31.8 | | | 1.3–120 | 0.3 | 3.6 |
| lissamine green B | 2.3 | 21.2 | 161.0 | 0.3–30 | 0.1 | 4.7 |
| indigo carmine | 1.0 | 20.9 | 48.0 | 0.9–75 | 0.3 | 5.6 |
| brilliant blue FCF | 4.6 | 25.2 | 210.6 | 0.1–30 | 0.03 | 3.7 |
| brilliant black BN | 6.2 | 42.5 | 33.1 | 1.3–120 | 0.4 | 6.4 |

| | slope | | | linear range[a] | detection limit[a] | |
|---|---|---|---|---|---|---|
| natural colorant | 425 nm | 500 nm | 610 nm | ($\mu$g/g lyophilized) | ($\mu$g/g lyophilized) | RSD (%) |
| riboflavin | 10.2 | 5.2 | 1.6 | 3–300 | 0.8 | 3.5 |
| caramel | 0.12 | 0.05 | 0.006 | 220–22000 | 75 | 6.4 |
| carminic acid | 13.4 | 34.7 | 6.1 | 1.5–150 | 0.3 | 3.8 |
| curcumin | 1.4 | | | 30–3000 | 7.5 | 4.5 |
| *trans*-$\beta$-carotene | 4.1 | | | 7.5–750 | 2.2 | 4.1 |

[a] The linear range and detection limit correspond to the most sensitive wavelength.

according to the additive type. Synthetic colorants needed an acetonitrile mobile phase, whereas natural colorants needed methanol and a modifier (ethyl acetate) to control the eluotropic strength. Both mobile phases required an ion interaction reagent (IIR) that modifies the RP-$C_{18}$ stationary phase of the chromatographic column. The lipophilic chain of the IIR is adsorbed onto the RP-$C_{18}$, giving rise to a positively charged primary layer. The anion of the IIR is bound to the RP-$C_{18}$ column through electrostatic forces. The formation of an electrical double layer adsorbed onto the original RP-$C_{18}$ material permits the retention of anionic and/or cationic species (*14*). The IIR concentrations varied depending on whether it was the mobile phase for synthetic ($8 \times 10^{-4}$ mol/L) or natural ($2 \times 10^{-4}$ mol/L) colorants. The concentrations were optimized by taking into account the fact that colorant resolution was not possible at lower IIR concentrations and sensitivity was lower at higher IIR concentrations. Other chromatographic conditions are listed also in **Table 1**.

To ensure the applicability of the optimized SPE unit to real samples, 1-g aliquots of four test samples [two dairy samples (pistachio ice cream and peach yogurt) and two fatty foods (sauce for chicken and dehydrated onion soup)] were spiked (per gram of nonlyophilized food) with 5 $\mu$g/g of lissamine green B and brilliant blue FCF; 12.5 $\mu$g/g of tartrazine, indigo carmine, and brilliant black BN; 25 $\mu$g/g of carminic acid; 50 $\mu$g/g of riboflavin and *trans*-$\beta$-carotene; 250 $\mu$g/g of curcumin; and 1875 $\mu$g/g of caramel and then lyophilized. The lyophilized samples (0.39 g of pistachio ice cream, 0.19 g of peach yogurt, 0.30 g of sauce for chicken, or 1.00 g of onion soup) were directly extracted with 25 mL of 1 mol/L acetic acid/sodium acetate buffer for 10 min. Three milliliters of the extract was aspirated into the SPE system depicted in **Figure 1**. The peaks corresponding to target compounds were correctly separated in the chromatograms from those corresponding to sample matrices, without overlapping. Therefore, the proposed system can be applied to the determination of synthetic and natural colorants in dairy products and fatty foods. Two important variables affecting the extraction of synthetic and natural colorants from lyophilized samples, the volume of 1 mol/L acetic acid/sodium acetate buffer (extractant) and the extraction time, were studied. For these purposes, several 1-g aliquots of the four lyophilized test samples were fortified with variable amounts of colorants, depending on the extractant volume, so that the concentration in the extract was constant. Next, they were extracted by mechanical shaking for different lengths of time (from 5 to 20 min), with volumes of 1 mol/L acetic acid/sodium acetate buffer from 12 to 25 mL, taking into account that soaking the dry material required at least 3 mL of buffer solution. Three-milliliter aliquots of the extract containing the same amount of colorants were continuously aspirated into the SPE system (3 $\mu$g of lissamine green B and brilliant blue FCF; 7.5 $\mu$g of tartrazine, indigo carmine, and brilliant black BN; 15 $\mu$g of carminic acid; 30 $\mu$g of riboflavin and *trans*-$\beta$-carotene; 150 $\mu$g of curcumin; and 1125 $\mu$g of caramel). Aliquots of 20 $\mu$L were injected into the LC-DAD instrument. In these experiments complete extraction was accomplished after 8 min for volumes $\geq$15 mL. Fifteen milliliters of 1 mol/L acetic acid/sodium acetate buffer was selected as extractant (extraction time = 10 min) by taking into account the additional preconcentration provided by the lower volume.

Finally, to ensure the accuracy of the results, a parameter influencing the recovery of the natural and synthetic colorants was studied: the optimal spiking time (defined as the time of contact between spiked colorants and sample matrix prior to lyophilization, which is necessary to reproduce binding effects that occur in native colorants). Because no significant differences were observed between the recoveries of each synthetic and natural colorant obtained at different spiking times (0, 1, 3, 6, and 24 h), 1 h was established as sufficient.

**Features of the Proposed Automated Method.** The colorants studied showed good liquid chromatographic properties. Analytical curves for synthetic and natural colorant standards were obtained by using a sample volume of 3 mL of 1 mol/L acetic acid/sodium acetate buffer containing variable concentrations (0.01−200 $\mu$g/mL excluding caramel, which was linear up to 1500 $\mu$g/mL) and the SPE system depicted in **Figure 1**. The sensitivity of the method, expressed as the slope of the calibration graphs, and the linear range, calculated using 1 g of lyophilized lemon mousse dessert that had been spiked with all of the colorants at variable concentrations, are summarized in **Table 2**. The precision, expressed as RSD ($n$ = 11), and detection limit (DL) are also shown in **Table 2**. The DL was defined as the minimum concentration providing a chromatographic signal 3 times higher than background noise (at the prevailing retention times). The lowest DLs were obtained for synthetic colorants, being only 0.03 $\mu$g/g for brilliant blue FCF. Caramel had the highest DL but is 20 times less than the recently published results obtained with other methods for the determination of this colorant (*12*). The sensitivity of the method was

**Table 3.** Synthetic and Natural Colorants Found in Lyophilized Dairy and Fatty Foods (SD, $n = 6$)

| dairy sample | water (%) | colorant found | concn[a] (mg/kg) | dairy sample | water (%) | colorant found | concn[a] (mg/kg) |
|---|---|---|---|---|---|---|---|
| yogurts | | | | desserts | | | |
| lemon | 80 | curcumin | 170 (10) | lemon mousse | 82 | riboflavin | 57 (4) |
| banana 1 | 80 | curcumin | 164 (11) | lemon yogurt mousse | 80 | tartrazine | 12.5 (0.8) |
| banana 2 | 80 | *trans*-$\beta$-carotene | 56 (3) | yogurt flavor | 84 | tartrazine | 5.2 (0.3) |
| banana 3 | 80 | curcumin | 228 (16) | | | indigo carmine | 2.0 (0.1) |
| | | carminic acid | 38 (2) | caramel custard | 80 | caramel | 785 (50) |
| pineapple | 80 | *trans*-$\beta$-carotene | 45 (3) | ice creams | | | |
| strawberry | 79 | carminic acid | 21 (1) | lemon 1 | 60 | curcumin | 196 (14) |
| cherry | 80 | carminic acid | 16 (1) | lemon 2 | 65 | tartrazine | 36 (3) |
| vanilla | 80 | *trans*-$\beta$-carotene | 37 (2) | iced lemon fruit | 63 | tartrazine | 6.6 (0.4) |
| peach 1 | 81 | *trans*-$\beta$-carotene | 50 (3) | | | lissamine green B | 1.5 (0.1) |
| peach 2 | 82 | curcumin | 197 (13) | pistachio | 61 | tartrazine | 27 (2) |
| | | carminic acid | 18 (1) | | | indigo carmine | 15 (1) |
| fruit salad | 79 | curcumin | 144 (10) | vanilla 1 | 65 | *trans*-$\beta$-carotene | 44 (3) |
| | | carminic acid | 15 (1) | vanilla 2 | 60 | riboflavin | 49 (3) |
| nuts–oats | 71 | caramel | 635 (40) | | | *trans*-$\beta$-carotene | 31 (2) |
| forest fruits | 79 | carminic acid | 27 (2) | chocolate | 60 | caramel | 705 (50) |
| peach–mango | 82 | *trans*-$\beta$-carotene | 55 (3) | orange | 65 | *trans*-$\beta$-carotene | 53 (4) |
| peach–passion fruit | 78 | *trans*-$\beta$-carotene | 28 (2) | cream–strawberry jam | 62 | carminic acid | 22 (1) |
| strawberry–banana | 81 | curcumin | 142 (9) | | | | |
| | | carminic acid | 23 (1) | vanilla–chocolate–cream | 62 | riboflavin | 65 (4) |
| apricot–mango | 78 | curcumin | 258 (15) | | | *trans*-$\beta$-carotene | 27 (2) |
| | | *trans*-$\beta$-carotene | 44 (3) | fruits | 60 | riboflavin | 49 (3) |
| milkshakes | | | | | | *trans*-$\beta$-carotene | 39 (3) |
| vanilla 1 | 85 | curcumin | 87 (6) | | | carminic acid | 27 (1) |
| | | carminic acid | 23 (1) | | | | |
| vanilla 2 | 73 | riboflavin | 64 (4) | | | | |
| | | *trans*-$\beta$-carotene | 22 (1) | | | | |
| strawberry | 82 | carminic acid | 25 (2) | | | | |

| fatty sample | water (%) | colorant found | concn[a] (mg/kg) | fatty sample | water (%) | colorant found | concn[a] (mg/kg) |
|---|---|---|---|---|---|---|---|
| sauces | | | | dehydrated soups | | | |
| vinaigrette | 72 | tartrazine | 16 (1) | chicken 1 | | riboflavin | 82 (5) |
| mustard 1 | 65 | tartrazine | 12.5 (0.9) | chicken 2 | | riboflavin | 69 (4) |
| mustard 2 | 69 | caramel | 760 (50) | | | caramel | 890 (60) |
| tartar | 65 | *trans*-$\beta$-carotene | 65 (4) | fowl | | caramel | 930 (65) |
| "gaucho" | 56 | *trans*-$\beta$-carotene | 52 (3) | mushrooms | | riboflavin | 75 (5) |
| curry | 59 | *trans*-$\beta$-carotene | 49 (3) | vegetables 1 | | riboflavin | 88 (6) |
| oysters | 61 | caramel | 815 (55) | vegetables 2 | | caramel | 875 (60) |
| guacamole | 55 | *trans*-$\beta$-carotene | 56 (4) | onions | | caramel | 940 (60) |
| | | brilliant blue FCF | 2.8 (0.2) | asparagus | | riboflavin | 68 (4) |
| for chicken | 70 | *trans*-$\beta$-carotene | 60 (4) | leeks | | riboflavin | 80 (5) |
| | | riboflavin | 72 (5) | | | caramel | 965 (60) |
| | | caramel | 950 (55) | beef | | caramel | 890 (60) |
| bouillon cubes | | | | | | | |
| chicken gravy 1 | | caramel | 945 (65) | | | | |
| chicken gravy 2 | | riboflavin | 77 (5) | | | | |
| | | caramel | 940 (65) | | | | |
| beef gravy 1 | | riboflavin | 69 (4) | | | | |
| beef gravy 2 | | curcumin | 284 (18) | | | | |
| | | caramel | 850 (55) | | | | |
| fish gravy | | riboflavin | 59 (4) | | | | |
| | | caramel | 1000 (65) | | | | |

[a] Amount per kilogram of nonlyophilized food.

adequate to determine all colorants at concentrations lower than the limits established by the European Union.

**Analysis of Dairy Products and Fatty Foods.** Different samples with high protein (dairy samples) and fat contents (fatty foods) were analyzed. The labels on the samples indicated what colorants were contained but not the amounts. The positive list of colorant additives limits the concentrations of the synthetic colorants studied (tartrazine, lissamine green B, brilliant blue FCF, indigo carmine, and brilliant black BN) and of two of the natural colorants (curcumin and carminic acid). The limits on these colorants vary depending on the type of food: 150 mg/kg in dairy samples, 500 mg/kg in sauces and bouillon cubes, 300 mg/kg in mustard, and 50 mg/kg in dehydrated soups. The other natural colorants (riboflavin, *trans*-$\beta$-carotene, and caramel) were allowed at quantum satis (*3*). Samples were lyophilized as soon as they were received, and the results of the analysis of 35 dairy samples (yogurts, milkshakes, desserts, and ice creams) and 24 fatty foods (sauces, dehydrated soups, and bouillon cubes) are given in **Table 3**. Samples were quantified using three aliquots of each food sample analyzed in duplicate ($n = 6$). The proposed method allows the individual identification (using each peak's spectrum) and quantification of all colorants present in the sample. In the samples that contained only synthetic or natural colorants, only the effluents corresponding to the cotton or RP-$C_{18}$ columns were analyzed. Only one sample (guacamole sauce) was found to contain mixtures of synthetic and natural colorants. Eight samples were found to have colorant quantities higher than the legal limits. The curcumin concentration was higher than the legal limit (150 mg/kg) in lemon, banana 1 and 3, peach 2, and apricot−mango

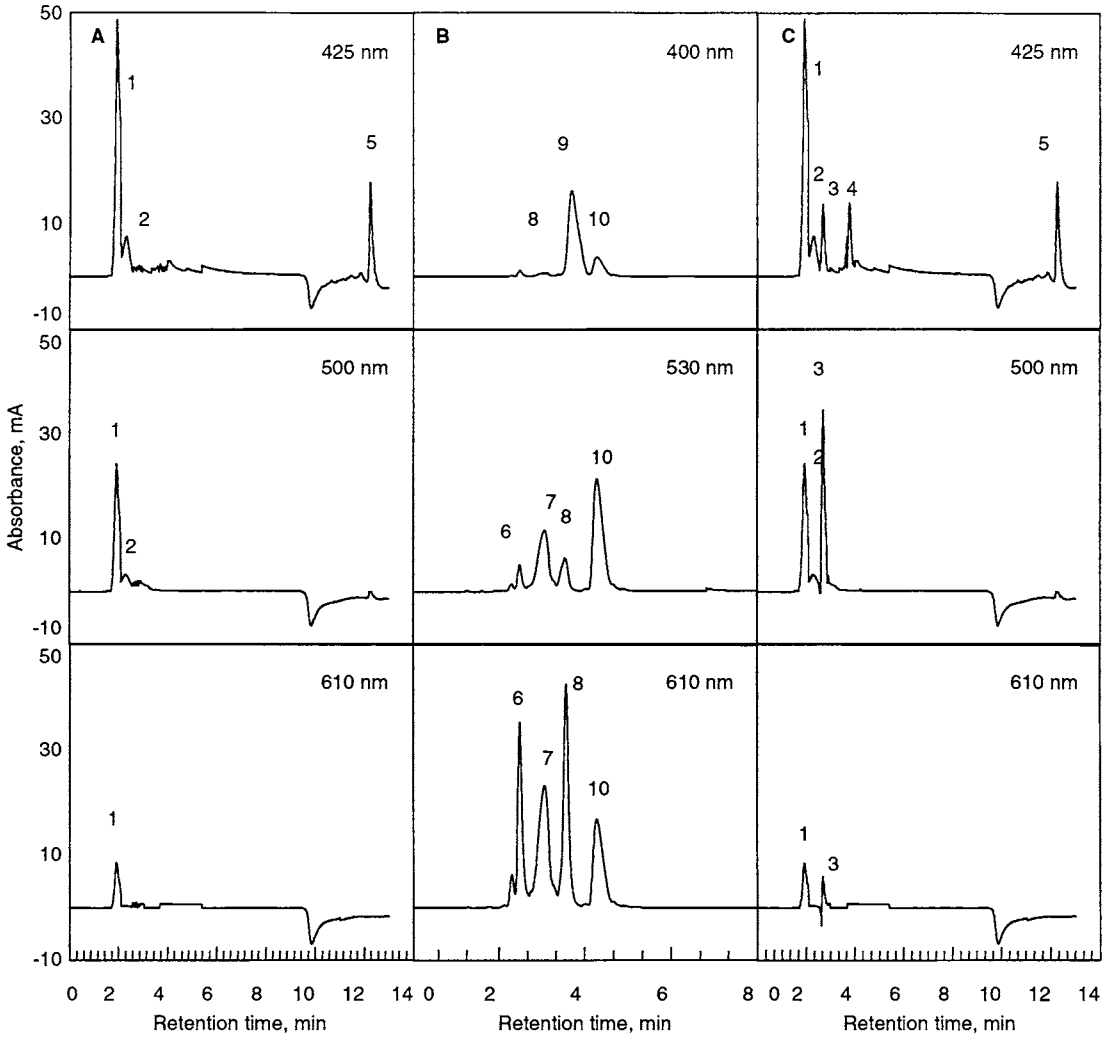


**Figure 2.** Liquid chromatogram for lyophilized sauce for chicken (A) and the same lyophilized sample spiked with five synthetic colorants (B) and with two natural colorants (C). See text for details. Peaks: (1) riboflavin, (2) caramel, (3) carminic acid, (4) curcumin, (5) *trans*-$\beta$-carotene, (6) lissamine green B, (7) indigo carmine, (8) brilliant blue FCF, (9) tartrazine, and (10) brilliant black BN.

**Table 4.** Average Recoveries[a] of Added Colorants at Low, Medium, and High Levels to Dairy and Fatty Foods

| | dairy foods | | | | fatty foods | | |
|---|---|---|---|---|---|---|---|
| sample | yogurt | milkshakes | desserts | ice cream | sauces | dehydrated soups | bouillon cubes |
| tartrazine | 95 (5, $n = 153$) | 96 (5, $n = 27$) | 96 (5, $n = 18$) | 95 (3, $n = 72$) | 96 (4, $n = 63$) | 95 (4, $n = 90$) | 95 (3, $n = 45$) |
| lissamine green B | 95 (5, $n = 153$) | 94 (3, $n = 27$) | 96 (5, $n = 36$) | 95 (4, $n = 90$) | 95 (3, $n = 81$) | 95 (3, $n = 90$) | 95 (4, $n = 45$) |
| indigo carmine | 93 (3, $n = 153$) | 94 (3, $n = 27$) | 95 (3, $n = 27$) | 93 (3, $n = 90$) | 93 (3, $n = 81$) | 93 (4, $n = 90$) | 93 (4, $n = 45$) |
| brilliant blue FCF | 95 (4, $n = 153$) | 95 (4, $n = 27$) | 95 (3, $n = 36$) | 95 (3, $n = 99$) | 96 (4, $n = 72$) | 95 (4, $n = 90$) | 95 (3, $n = 45$) |
| brilliant black BN | 95 (4, $n = 153$) | 95 (4, $n = 27$) | 94 (5, $n = 36$) | 95 (4, $n = 99$) | 95 (3, $n = 81$) | 95 (4, $n = 90$) | 95 (3, $n = 45$) |
| riboflavin | 94 (5, $n = 153$) | 94 (5, $n = 18$) | 95 (4, $n = 27$) | 95 (3, $n = 72$) | 94 (4, $n = 72$) | 94 (5, $n = 36$) | 94 (5, $n = 18$) |
| caramel | 95 (5, $n = 144$) | 95 (4, $n = 27$) | 94 (4, $n = 27$) | 95 (5, $n = 90$) | 95 (3, $n = 54$) | 95 (4, $n = 36$) | 94 (4, $n = 9$) |
| carminic acid | 95 (3, $n = 90$) | 95 (4, $n = 9$) | 94 (3, $n = 36$) | 95 (5, $n = 81$) | 95 (3, $n = 81$) | 95 (4, $n = 90$) | 95 (4, $n = 45$) |
| curcumin | 93 (4, $n = 90$) | 94 (5, $n = 18$) | 94 (3, $n = 36$) | 93 (4, $n = 90$) | 93 (5, $n = 81$) | 93 (5, $n = 90$) | 94 (5, $n = 36$) |
| *trans*-$\beta$-carotene | 91 (6, $n = 90$) | 91 (6, $n = 18$) | 91 (5, $n = 36$) | 91 (5, $n = 54$) | 92 (6, $n = 36$) | 93 (6, $n = 90$) | 93 (5, $n = 45$) |

[a] Standard deviations and replicates ($n$, three spiked concentrations from three replicates for the different samples) are given in parentheses. For experimental details, see text.

yogurts and in lemon 1 ice cream. Two yogurt samples, fruit salad and strawberry−banana, were found to exceed the legal limit when the quantities of both colorants used (curcumin and carminic acid) were added together, although the individual colorant quantities were lower than the legal limit.

The chromatograms of the different dairy and fatty foods assayed show the high extraction efficiency of colorants (due to lyophilization and the sorbent preconcentration/cleanup system) and also the high selectivity of the proposed method (no interferences from the matrices studied were found). For example, **Figure 2** shows the chromatograms obtained in the analysis of a sauce for chicken and the same sample spiked with all of the colorants that the sample did not contain at the following concentrations: 3 $\mu$g of lissamine green B and brilliant

blue FCF; 7.5 μg of tartrazine, indigo carmine, and brilliant black BN; 15 μg of carminic acid; and 150 μg of curcumin per gram of lyophilized sauce.

**Recovery Test.** To validate the proposed method, taking into account that there is no appropriate reference material containing both types of colorants in dairy samples or fatty foods, a recovery test was carried out. Recoveries of analytes were studied in all of the dairy and fatty food samples listed in **Table 3** (yogurts, milkshakes, desserts, ice creams, sauces, dehydrated soups, and bouillon cubes). About 1 g of each food was homogenized and then fortified with the colorants that the sample did not contain at three levels of concentration (per gram of nonlyophilized food): 0.75, 3, and 15 μg/g of lissamine green B and brilliant blue FCF; 3, 7.5, and 45 μg/g of tartrazine, indigo carmine, and brilliant black BN; 4.5, 15, and 75 μg/g of carminic acid; 7.5, 30, and 150 μg/g of riboflavin; 15, 30, and 150 μg/g of *trans*-β-carotene; 75, 150, and 750 μg/g of curcumin; and 375, 1125, and 4500 μg/g of caramel from standard solutions in 1 mol/L acetic acid/sodium acetate buffer (1 mL of buffer containing from 0.75 μg to 4.50 mg of colorants). After the addition, the mixture was slightly shaken and left to stand for 1 h and then lyophilized. The lyophilized samples (with quantities ranging between 0.15 g for the sample that contained the most water, vanilla milkshake, and 1 g for bouillon cubes or dehydrated soups) were directly extracted with 1 mol/L acetic acid/sodium acetate buffer and aspirated into the developed SPE system. All of the colorants were spiked into different samples in triplicate at each of the levels indicated above ($n = 3$). The levels of colorants were quantified by comparison with standard solutions in 1 mol/L acetic acid/sodium acetate buffer, which were passed through the SPE unit under identical conditions. There were some differences in the recoveries of the spiking levels (low, medium, and high). In all samples assayed (35 dairy samples and 24 fatty foods, each one in triplicate, $n = 129-177$ for each spiked level), the amount of colorants recovered increased as the amount spiked increased; the lowest values (92%) corresponded to the amounts spiked at the low level. At higher concentrations, the recoveries were nearly 98% for all of the colorants except indigo carmine, curcumin, and *trans*-β-carotene (95%).

**Table 4** gives the average recovery values obtained from the three replicates of each colorant at the three concentrations spiked with the colorants that the sample did not contain ($n = 9$) in the different types of samples assayed ($n$ ranges between 3 and 17 samples for milkshakes and yogurts, respectively). The recovery averages were similar for all of the additives (94−95%), the lowest being 92% for *trans*-β-carotene. At the same time it was shown that there do not exist any significant statistical differences between the average recovery values of all additives in each type of food, being 94%. This shows that colorant extraction is not influenced by the matrix and is only slightly influenced by the type of additive, probably due to the different stabilities of each colorant. Therefore, the lowest average values are always obtained for *trans*-β-carotene, probably because it partially oxidizes when the sample is treated (lixiviation) with the acetic acid/sodium acetate buffer. Adding ascorbic acid to the extractant could minimize this effect, which increases the *trans*-β-carotene recovery to 94%. However, to simplify the proposed method, the addition of this reducing agent is not necessary because the recovery values obtained were satisfactory (92%). In summary, the proposed method gives acceptable recovery averages for all of the colorants in the different matrices assayed, making perfectly valid its use as a routine analysis method.

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## LITERATURE CITED

(1) Noonan, J. Color additives in Food. In *CRC Handbook of Food Additives*, 2nd ed.; Furia, T. E., Ed.; CRC Press: Boca Raton, FL, 1981; Vol. I, pp 587−615.

(2) Lea, A.; Scotter, M. J. Colours. In *Encyclopaedia of Food Science, Food Technology and Nutrition*; Macrae, R., Robinson, R. K., Sadler, M. J., Eds.; Academic Press: San Diego, CA, 1993; Vol. II, pp 1164−1180.

(3) European Union (EU). *Community Directive 94/36/EEC*; Off. J. Eur. Communities L 237/13; European Community: Brussels, Belgium, 1994.

(4) U.S. Government. *Code of Federal Regulations*, Parts 70−82; Title 21; U.S. Government Printing Office: Washington, DC, 2001.

(5) Jalón, M.; Peña, M. J.; Rivas, J. C. Liquid chromatographic determination of carminic acid in yogurt. *J. Assoc. Off. Anal. Chem.* **1989**, *72*, 231−234.

(6) Greenway, G. M.; Kometa, N.; Macrae, R. Determination of food colours by HPLC with on-line dialysis for sample preparation. *Food Chem.* **1992**, *43*, 137−140.

(7) Gennaro, M. C.; Abrigo, C.; Cipolla, G. High-performance liquid chromatography of food colours and its relevance in forensic chemistry. *J. Chromatogr. A* **1994**, *674*, 281−299.

(8) Hägg, M. Effect of various commercially available enzymes in the liquid chromatographic determination with external standardization of thiamine and riboflavin in foods. *J. AOAC Int.* **1994**, *77*, 681−686.

(9) Greenway, G. M.; Kometa, N. On-line sample preparation for the determination of riboflavin and flavin mononucleotides in foodstuffs. *Analyst* **1994**, *119*, 929−935.

(10) Granelli, K.; Helmersson, S. Rapid high-performance liquid chromatographic method for determination of β-carotene in milk. *J. Chromatogr. A* **1996**, *721*, 355−358.

(11) Carvalho, R. N.; Collins, C. H. HPLC determination of carminic acid in foodstuffs and beverages using diode array and fluorescence detection. *Chromatographia* **1997**, *45*, 63−66.

(12) Coffey, J. S.; Nursten, H. E.; Ames, J. M.; Castle, L. A liquid chromatographic method for the estimation of class III caramel added to foods. *Food Chem.* **1997**, *58*, 259−267.

(13) Weissenberg, M.; Levy, A.; Schaeffler, I.; Menagem, E.; Barzilai, M. Rapid isocratic HPLC analysis of β-carotene in red peppers (*Capsicum annuum*) and food preparations. *Chromatographia* **1997**, *46*, 399−403.

(14) Angelino, S.; Fell, A. F.; Gennaro, M. C. Development of system suitability tests for ion-interaction chromatography of colorants on reversed-phase packing materials. *J. Chromatogr. A* **1998**, *797*, 65−74.

(15) Chen, Q. C.; Mou, S. F.; Hou, X. P.; Riviello, J. M.; Ni, Z. M. Determination of eight synthetic food colorants in drinks by high-performance ion chromatography. *J. Chromatogr. A* **1998**, *827*, 73−81.

(16) Berzas Nevado, J. J.; Guiberteau Cabanillas, C.; Contento Salcedo, A. M. Separation and determination of dyes by ion-pair chromatography. *J. Liq. Chromatogr. Relat. Technol.* **1997**, *20*, 3073−3088.

(17) He, X. G.; Lin, L. Z.; Lian, L. Z.; Lindenmaier, M. Liquid chromatography-electrospray mass spectrometric analysis of curcuminoids and sesquiterpenoids in turmeric (*Curcuma longa*). *J. Chromatogr. A* **1998**, *818*, 127−132.

(18) Oliver, J.; Palou, A.; Pons, A. Semi-quantification of carotenoids by high-performance liquid chromatography: saponification-induced losses in fatty foods. *J. Chromatogr. A* **1998**, *829*, 393−399.

(19) Ishikawa, F.; Shigeoka, S.; Nagashima, M.; Takahashi, M.; Kamimura, H.; Onishi, K.; Nishijima, M. Analytical method of 21 coal-tar dyes in protein-rich foods by solid-phase extraction and HPLC. *J. Food Hyg. Soc. Jpn.* **2000**, *41*, 194−199.

(20) Gliszczynska-Swiglo, A.; Koziolowa, A. Chromatographic determination of riboflavin and its derivatives in food. *J. Chromatogr. A* **2000**, *881*, 285−297.

(21) Fuh, M. R.; Chia, K. J. Determination of sulphonated azo dyes in food by ion-pair liquid chromatography with photodiode array and electrospray mass spectrometry detection. *Talanta* **2002**, *56*, 663−671.

(22) González, M.; Gallego, M.; Valcárcel, M. Determination of natural and synthetic colorants in prescreened dairy samples using liquid chromatography-diode array detection. *Anal. Chem.* **2003**, *75*, 685−693.

(23) Ratsimba, V.; García Fernández, J. M.; Defaye, J.; Nigay, H.; Voilley, A. Qualitative and quantitative evaluation of mono- and disaccharides in D-fructose, D-glucose and sucrose caramels by gas−liquid chromatography−mass spectrometry di-D-fructose dianhydrides as tracers of caramel authenticity. *J. Chromatogr. A* **1999**, *844*, 283−293.

(24) Hofer, K.; Jenewein, D. Quick spectrophotometric identification of synthetic food colorants by linear regression analysis. *Z. Lebensm. Unters. Forsch. A* **1997**, *204*, 32−38.

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