Identification and Quantitation of Water-Soluble Synthetic Colors in Foods by Liquid Chromatography/Ultraviolet–Visible Method Development and Validation

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ABSTRACT: A simple and sensitive liquid chromatography method of analysis has been developed and validated for the simultaneous quantitative determination of food colors in a broad range of foods. The method of analysis applies specific extraction solutions to obtain optimal color extraction. The extraction solutions are composed of different proportions of methanol and ammonium acetate, as the ion-pairing agent. Analysis was performed on reverse-phase C18 Poroshell column with ammonium acetate, methanol, acetonitrile, and acetone gradient elution as the mobile phases. Multiple specific wavelengths were used to monitor color additives in the visible range to provide higher sensitivity and expanded scope needed for a large number of analytes. All 27 color compounds showed good linearity with regression coefficients predominantly above 0.990. The limit of detection and limit of quantitation values ranged from 0.10 to 0.43 and from 0.34 to 1.45 μg/g, respectively. The precision of the method ranged from 1.4 to 15.9%, while recoveries averaged 72% across all food commodities tested. The method of analysis offers convenience and adequate sensitivity for the analysis of a wide variety of food matrices containing a broad range of colors.

1. INTRODUCTION

Organic aromatic synthetic dyes are additives that are extensively incorporated into foodstuffs for many purposes. They are often used to enhance sensory perception, to compensate for color loss during manufacturing or storage, as well as to preserve the uniform appearance of food.1 For several years, concern has arisen over the extensive use of synthetic coloring additives in foods. To protect public health and to prevent the indiscriminate use, food colors are comprehensively screened for safety. Many governing bodies have set tight regulations limiting the allowable kinds, concentrations, and purity of dyes.2–4 As a result, the need to monitor permitted and prohibited food colors has increased, particularly in international food trade. Therefore, it is necessary to rely on sensitive and accurate methods to screen, identify, and quantitate color additives in foodstuffs. Analysis of food dyes in foodstuffs by liquid chromatography (LC) with ultraviolet−visible (UV−vis) or MS/MS detection using traditional C18 chromatographic columns has been reported in several publications.26−38 However, few reported analytical methods applied for a wide range of colors, including those permitted in foodstuffs as well as banned dyes for food use.2–4

A broad range of methods have been reported for the determination of dyes in foods. Chromatographic methods of analysis such as paper chromatography,5,6 thin-layer chromatography,7,8 gas chromatography (GC), as well as capillary electrophoresis (CE)9−11 were widely used but are now considered outdated methodologies. GC methods have restricted applications to synthetic colors, typically because of the lack of volatility and their limited stability at high temperature.12 CE methods have also been used for synthetic dyes but have limited use in routine analyses.13−15 Over the past years, LC has been shown to provide much more potential for the analysis of synthetic food colors in terms of qualitative and quantitative determinations, as well as speed of analysis.16 Most of the LC analyses used ion exchange with gradient elution and were conducted to determine the purity of dyes, thus clearly illustrating the power of LC in the field of color analyses.17−30 LC analysis with a UV−vis or diode-array detector (DAD) is the most commonly used detection because food colors absorb strongly at the UV and/or vis wavelengths. Moreover, LC using reverse phase with ion pairing is currently the most-preferred technique because it offers among others, the best overall resolution and sensitivity.17−20 Ion-pairing chromatography consists of salt modifiers added to the mobile phase. Suitable salts are selected as to create large ions of opposite charges to the compounds to be analyzed, thus generating reversible ion-pair complexes.18,22−26

Among all LC methods available for food colors, very few of them offer simultaneous quantitation and resolution of several permitted food colors and nonpermitted dyes in a variety of food commodities. Therefore, an optimized method was...
Table 1. List of Colors and Corresponding Suppliers

| Color                          | Other Name                  | Supplier/Source                               | Purity (%) |
|-------------------------------|-----------------------------|-----------------------------------------------|------------|
| **Group I**                   |                             |                                               |            |
| tartrazine                    | FD&C yellow no. 5, E102     | Sigma-Aldrich/Oakville (ON), Canada           | 99.5       |
| amaranth                      | FD&C red no. 2, E123        | Sigma-Aldrich/Oakville (ON), Canada           | 98.8       |
| indigo carmine                | FD&C blue no. 2, E132       | Sigma-Aldrich/Oakville (ON), Canada           | 87.4       |
| sunset yellow FCF             | FD&C yellow no. 6, E110     | Sigma-Aldrich/Oakville (ON), Canada           | 94.5       |
| allura red AC                 | FD&C red no. 40, E129       | Sigma-Aldrich/Oakville (ON), Canada           | 98.3       |
| ponceau SX                    | E125                        | MP Biomedicals/Solon, OH, USA                 | 94.2       |
| fast green FCF                | FD&C green no. 3, E143      | Sigma-Aldrich/Oakville (ON), Canada           | 83.7       |
| brilliant blue FCF            | FD&C blue no. 1, E133       | Sigma-Aldrich/Oakville (ON), Canada           | 89.4       |
| erythrosine                   | FD&C red no. 3, E127        | Sigma-Aldrich/Oakville (ON), Canada           | 98.0       |
| chlorophyllin (sodium coppered) | E141                  | Alfa Aesar/Ward Hill, MA, USA                 | 36.2       |
| **Group II**                  |                             |                                               |            |
| ponceau 4R                    | cochineal red A, E124       | Sigma-Aldrich/Oakville (ON), Canada           | 96.9       |
| fastred E                     | acid red 88                 | Sigma-Aldrich/Oakville (ON), Canada           | 96.7       |
| bordeaux R                    | acid red 17                 | Sigma-Aldrich/Oakville (ON), Canada           | 86.8       |
| crocein orange G              | acid orange 12              | International Laboratories/San Francisco, CA, USA | 98.0     |
| orange II                     | acid orange 7               | Sigma-Aldrich/Oakville (ON), Canada           | 98.6       |
| 2,4,5-triiodofluorescein      | erythrosin yellowish        | Alfa Aesar/Ward Hill, MA, USA                 | 32.6       |
| 2,4,7-triiodofluorescein      |                             | U.S. FDA, Washington, DC, USA                 | N/A        |
| 4,5-diiodofluorescein         |                             | U.S. FDA, Washington, DC, USA                 | N/A        |
| **Group III**                 |                             |                                               |            |
| carmoisine                    | E122                        | Sigma-Aldrich/Oakville (ON), Canada           | 93.9       |
| green S                       | E142                        | Sigma-Aldrich/Oakville (ON), Canada           | 79.4       |
| quinoline yellow WS           | E104                        | Sigma-Aldrich/Oakville (ON), Canada           | 41.0       |
| patent blue V                 | E131                        | Sigma-Aldrich/Oakville (ON), Canada           | 81.4       |
| orange GGN                    | E111                        | Sigma-Aldrich/Oakville (ON), Canada           | 98.1       |
| eosin Y                       | acid red 87                 | Sigma-Aldrich/Oakville (ON), Canada           | 84.5       |
| patent blue VF                | acid blue 1                 | Sigma-Aldrich/Oakville (ON), Canada           | 91.2       |
| chrysoideine G                | basic orange 2              | Sigma-Aldrich/Oakville (ON), Canada           | 97.0       |
| rhodamine B                   | basic violet 10             | Sigma-Aldrich/Solon, OH, USA                  | 92.9       |

devolved and validated to analyze various foodstuffs where authorized food colors in Canada, in United States of America (USA), and across the European Union (EU), as well as some nonpermitted colors, are resolved and quantitated. In this paper, we present the development, optimization, and validation of a quantitative method of analysis for the determination of colors in foodstuffs. The objectives were to (1) develop a reliable and robust analytical method for the determination of 27 color compounds by keeping detection and quantitation limit levels as low or below those already reported in the literature for specific food dyes, (2) to optimize the extraction step, (3) to optimize the chromatographic conditions to reduce the cost and time of analysis, and (4) to expand the scope of food color analysis to the applicability of a wide range of food commodities in terms of the triangle of composition designed by the Association of Official Analytical Communities (AOAC).

2. MATERIALS AND METHODS

2.1. Chemicals and Materials. The chemicals and reagents used were all of analytical grade and LC grade. Methanol, acetonitrile, and acetone were supplied from EMD (Billerica, MA, USA) and glacial acetic acid by Fisher Scientific (Toronto, ON, Canada). High-purity ammonium acetate and α-amylose from Aspergillus oryzae were purchased from Sigma-Aldrich (Oakville, ON, Canada). ASTM-I grade ultrapure water was used for all LC analyses. When available, high-purity reference standards were purchased from various suppliers. Table 1 lists the 27 chemical compounds used in this study. Standards purity was confirmed by LC. When applicable, the subsidiary dye content of the respective food colour was not taken into account. Subsidiary dyes of erythrosine 4,5-diiodofluorescein, 2,4,5-triiodofluorescein (erythrosin yellowish), and 2,4,7-triiodofluorescein were obtained from the U.S. FDA.

2.2. Instrumentation. Method development, quantification, and validation studies were performed on an Agilent LC 1200 series (Agilent Technologies, Mississauga, ON, Canada) equipped with a binary pump, a high-performance autosampler, a thermostated autosampler unit, a thermostated column compartment, and a diode-array (DAD) VL+ detector. Optimum separation of the 27 color additives was achieved using a Poroshell SB-C18 column (3.0 × 100 mm, 2.7 μm) from Agilent Technologies (Mississauga, ON) coupled with a C18 guard column (3.0 × 4 mm; Phenomenex, Torrance, CA, USA).

2.3. Sample Preparation. Foodstuffs were homogenized using a Retsch Grindomix commercial laboratory blender (Newtown, PA, USA). Extraction procedures were performed using the IKA RO10 multipositions magnetic stirring plate (Wilmington, NC, USA) and the Branson CPX880 ultrasonic bath (Danbury, CT, USA). The centrifugation step was performed using Thermo Sorvall Legend XIR centrifuge (Osterode, Germany) with polypropylene 15 mL centrifuge tubes.

2.4. Chromatographic Conditions. The chromatographic separation was achieved using a ternary mixture of mobile phases. Mobile phases A and B consisted of a mixture of a 50 mM ammonium acetate solution, methanol, and acetonitrile,
acidified by 0.1% (v/v) of glacial acetic acid, with respective ratios of 97:3:0 (v/v) and 10:3:7 (v/v/v), while mobile phase C consisted of a 1 M ammonium acetate solution, methanol, and acetone in a ratio of 1:5:4 (v/v/v). The mobile phases were delivered to the analytical column according to the gradient program in Table 2. Analyses were performed by injecting 25 μL at a flow rate of 0.75 mL/min and a column temperature of 60 °C. The LC column was allowed to re-equilibrate for 1 min (post-time) prior to the next injection. Agilent Technologies ChemStation software was used to monitor and control all analytical conditions and to reprocess chromatographic data, including library search. Seven different wavelengths were used for identifying the color additives; 408, 428, 450, 506, 526, 540, and 610 nm.

### 2.5. Standard Preparation.
Individual standard stock solutions of 200 μg/mL of each dye category (see Table 1) were prepared by weighing 10 mg of each compound and dissolving into a 50 mL volumetric flask using the corresponding diluent. On the basis of their physicochemical properties, the analytes were divided in three groups: group I, II, and III. For group I, ultrapure water was used as the diluent, whereas groups II and III used mixtures of methanol and ultrapure water at ratios of (1:25 v/v) and (1:10 v/v), respectively. Volumetric flasks were sonicated for 1 min and wrapped with aluminum foil to protect them from light. Stock solutions were stable for about 70 days when kept refrigerated. Working standard solutions were prepared fresh on a daily basis by pipetting aliquots of stock solutions and serial dilutions with ultrapure water were made at concentrations to cover the range of 0.025–50 μg/mL.

### Table 2. Gradient Timetable

| time (min) | mobile phase A (%) | mobile phase B (%) | mobile phase C (%) |
|------------|--------------------|--------------------|--------------------|
| 0          | 100                | 0                  | 0                  |
| 1          | 100                | 0                  | 0                  |
| 1.01       | 97                 | 3                  | 0                  |
| 2          | 97                 | 3                  | 0                  |
| 4          | 67                 | 33                 | 0                  |
| 5          | 50                 | 50                 | 0                  |
| 6          | 25                 | 75                 | 0                  |
| 7          | 0                  | 75                 | 25                |
| 8          | 0                  | 75                 | 25                |
| 8.01       | 0                  | 50                 | 50                |
| 9          | 0                  | 25                 | 75                |
| 11         | 0                  | 0                  | 100               |
| 11.01      | 100                | 0                  | 0                  |
| 15         | 100                | 0                  | 0                  |

### Table 3. Calibration Parameters for Individual Color Additives from Three Different Calibration Curves

| peak            | color additive       | calibration range (μg/mL) | slope (±SD) | Y-intercept (±SD) | r²      | LOD† (μg/g) | LOQ‡ (μg/g) |
|-----------------|----------------------|---------------------------|-------------|-------------------|--------|-------------|-------------|
| Group I         |                      |                           |             |                   |        |             |             |
| 1               | chlorophyllin        | 0.025–50                  | 31.56 ± 2.08 | −0.87 ± 0.19      | 0.97314 ± 0.00424 | 0.43   | 1.45        |
| 2               | tartrazine           | 84.50 ± 0.28              | −0.03 ± 0.06 | 0.99993 ± 0.00007 | 0.23   | 0.78        |
| 3               | sunset yellow FCF    | 80.46 ± 0.42              | −0.09 ± 0.11 | 0.99996 ± 0.00002 | 0.07   | 0.23        |
| 4               | alizarin red AC      | 88.07 ± 0.22              | −0.0003 ± 0.0366 | 0.99994 ± 0.00003 | 0.14   | 0.45        |
| 5               | ponceau SX           | 81.19 ± 0.96              | −0.05 ± 0.01 | 0.99990 ± 0.00007 | 0.14   | 0.45        |
| 6               | amaranth             | 66.17 ± 1.30              | −0.53 ± 0.18 | 0.99987 ± 0.00062 | 0.08   | 0.28        |
| 7               | erythrosine          | 118.48 ± 2.01             | −0.05 ± 0.42 | 0.99814 ± 0.00148 | 0.11   | 0.37        |
| 8               | indigo carmine       | 78.89 ± 1.08              | −0.19 ± 0.16 | 0.99973 ± 0.00018 | 0.07   | 0.23        |
| 9               | fast green FCF       | 231.14 ± 3.27             | 0.31 ± 0.32 | 0.99758 ± 0.00020 | 0.08   | 0.28        |
| 10              | brilliant blue FCF   | 166.67 ± 1.40             | −0.03 ± 0.09 | 0.99939 ± 0.00020 | 0.08   | 0.28        |
| Group II        |                      |                           |             |                   |        |             |             |
| 11              | orange II            | 0.025–20                  | 65.98 ± 0.75 | −0.13 ± 0.23      | 0.99997 ± 0.00002 | 0.38   | 1.28        |
| 12              | ponceau 4R           | 63.97 ± 0.55              | −0.02 ± 0.04 | 0.99999 ± 0.00001 | 0.15   | 0.49        |
| 13              | fast red E           | 33.82 ± 0.36              | −0.02 ± 0.04 | 0.99990 ± 0.00007 | 0.15   | 0.49        |
| 14              | 4,5-diodofluorescein | 60.82 ± 1.86              | −0.12 ± 0.05 | 0.99971 ± 0.00020 | 0.16   | 0.52        |
| 15              | bordeaux R           | 51.59 ± 0.28              | −0.04 ± 0.09 | 0.99884 ± 0.00012 | 0.13   | 0.44        |
| 16              | 2,4,5-triodofluorescein | 66.11 ± 2.02         | −0.13 ± 0.06 | 0.99971 ± 0.00020 | 0.14   | 0.46        |
| 17              | 2,4,7-triodofluorescein | 76.03 ± 2.32           | −0.15 ± 0.06 | 0.99971 ± 0.00020 | 0.16   | 0.53        |
| Group III       |                      |                           |             |                   |        |             |             |
| 18              | quinoline yellow WS  | 0.025–20                  | 162.79 ± 2.72 | −0.08 ± 0.22      | 0.99991 ± 0.00007 | 0.40   | 1.33        |
| 19              | chrysoidine G        | 90.99 ± 0.90              | −0.70 ± 0.48 | 0.99853 ± 0.00097 | 0.41   | 1.37        |
| 20              | orange GGN§          | 42.65                     | −0.16        | 0.99888            | 0.16   | 0.52        |
| 21              | carmine              | 65.91 ± 1.09              | −0.07 ± 0.03 | 0.99988 ± 0.00011 | 0.14   | 0.46        |
| 22              | eosin Y              | 185.00 ± 0.73             | 0.36 ± 0.63 | 0.99854 ± 0.00017 | 0.14   | 0.46        |
| 23              | rhodamine B          | 159.50 ± 0.77             | −2.38 ± 0.58 | 0.99161 ± 0.00500 | 0.14   | 0.46        |
| 24              | green S              | 192.27 ± 1.14             | −0.002 ± 0.044 | 0.99971 ± 0.00011 | 0.10   | 0.34        |
| 25              | patent blue VF       | 160.17 ± 3.56             | −0.08 ± 0.12 | 0.99983 ± 0.00008 | 0.10   | 0.34        |
| 26              | patent blue V        | 187.15 ± 4.93             | −0.18 ± 0.03 | 0.99975 ± 0.00011 | 0.10   | 0.34        |

Where r² = determination coefficient (calibration from different injection days; n = 3), LOD and LOQ = limits of detection and quantitation, respectively (instrumental). Calibration data from one single injection day (±1). Calculated with a dilution factor of 10 (e.g., 10 g sample weighed into 100 mL v.f.). Calculated with a dilution factor of 10 (e.g., 10 g sample weighed into 100 mL v.f.).
2.6. Extraction Solutions. 2.6.1. Ammonium Acetate Extraction Solutions. Two different solutions were prepared to conduct separate extractions. A solvent mixture of methanol and 100 mM ammonium acetate solution in proportions of 3:10 (v/v) was used as extraction solution 1, and a mixture of the same solvents in proportions of 1:20 (v/v) was used as extraction solution 2.

2.6.2. α-Amylase Solution. A solution of about 900 units per mL (100 U/mg solids) was used for the enzymatic treatment of starch-containing products during the extraction step.

2.7. Extraction Procedure. 2.7.1. Starch-Containing Commodities. A 10 g portion of each food commodity was weighed in duplicate and transferred to 125 mL Erlenmeyer containing a magnetic stirring bar. To each replicate, 5 mL of the α-amylase solution and 30 mL of the 100 mM ammonium acetate solution were added. Samples were stirred on a magnetic stirrer plate for 15 min. Then, 3 mL of methanol and 25 mL of 100 mM ammonium acetate solution (extraction solution 1) were added to the first duplicate, and 30 mL of methanol and 25 mL of 100 mM ammonium acetate (extraction solution 2) were added to the second duplicate. Agitation was maintained for 15 min. Samples were transferred into an ultrasonic bath filled with water at a temperature of about 60 °C for 15 min. Extracts were quantitatively transferred into 100 mL volumetric flasks, then cooled down to room temperature prior to filling to mark with 100 mM ammonium acetate solution. Volumetric flasks were shaken, and about 12 mL was transferred into polypropylene centrifuge tubes. Extracts were centrifuged for 5 min at 14 000 rpm at room temperature. The supernatant was transferred to LC vial, and 25 μL was injected onto the LC system.

2.7.2. Other Commodities. Food colors were extracted using the same procedure as described previously without the enzymatic treatment step. The initial addition of the extraction solution was performed with 35 mL of 100 mM ammonium acetate solution.

2.8. Method Validation. The LC-DAD method developed was fully validated for selectivity, limit of detection (LOD), limit of quantitation (LOQ), linearity, precision, accuracy, recovery, and stability according to IUPAC and AOAC guidelines for single-lab validation.32,33 Measurement of uncertainty was estimated according to IUPAC and ISO guidelines.32,34,35

2.9. Selectivity. Selectivity is the degree to which a method can quantify the analyte accurately in the presence of interferences.35 Matrices without any food colors (blank) were tested for interferences, and selectivity was evaluated with the chromatograms obtained with those of the same blank matrices spiked with food colors, by comparing retention times and diode-array detection at the corresponding wavelength.

2.10. LOD, LOQ, and Linearity. In broad terms, the LOD is the smallest amount or concentration of the analyte in the test sample that can be reliably distinguished from the blank or background noise, which is equal to signal-to-noise ratios of 3 and 10, respectively.36 Linearity of the detector response was verified with standard preparations for each individual color additives included in the study over the range of 0.025–50 μg/mL. Standard stock solutions were appropriately diluted on each experiment day, and the corresponding fresh calibration curve solutions were used to calculate the concentration of the color additives in the samples. Calibration curves for groups I to III were generated by linear regression based on the peak area using quadratic (1/x² amount) weight fit to determine the linearity of the responses. The parameters of the regression equations obtained for the calibration curves are presented in Table 3.

2.10.1. Precision. The repeatability precision ($s_p$, relative standard deviation (RSD)) of the LC method was evaluated by analyzing six replicates of samples spiked with standards. The repeatability precision was expressed as the mean RSD for the repeated determinations across all food commodities. An intermediate precision was also tested by analyzing six replicates of one single food commodity (Peppermint candies) spiked with standards and was expressed as the mean RSD for the analysis of the candies commodity (Jelly beans and Peppermint candies).

2.10.2. Accuracy. Accuracy (trueness) was calculated as the relative difference between the determined concentrations by LC from spiked recovery and the nominal concentrations (100%) of all the color additives in sample preparations. The accuracy of the method was expressed as the mean of recoveries of the color additives for all twelve food matrices tested.

2.10.3. Recovery. Food colors were spiked with standard solutions at three concentration levels. Twelve replicates were prepared and analyzed for all twelve foodstuffs included in the validation study. Triplicate fortified samples were prepared for the low and high levels, respectively, at 5 and 20 μg/g, while six replicates were analyzed for the mid-level at 10 μg/g. The dilution factor used to express final results in μg/g was 100 (final volume = 100 mL). The recovery (R) was calculated by the method described in Appendix D of AOAC guidelines37 as $R_{\text{marginal}} = \frac{|C_{\text{sp}} - C_{\text{re}|}}{C_{\text{ref}}} \times 100$, where $C_{\text{sp}}$ is the concentration found in the spiked sample, $C_{\text{ref}}$ is the concentration in the unfortified sample, whereas $C_{\text{sp}}$ is the added concentration in the spiked sample. Typically, $C_{\text{sp}}$ is calculated by injecting standard preparations with the same concentrations as in spiked samples.

2.10.4. Stability. The stability of the standard preparations was conducted over a period of time. The stock standard solutions containing all color additives were respectively diluted to a 0.5 μg/mL concentration. The standard preparations were injected, and the corresponding concentrations were calculated against the calibration curves injected the same day. The same standard stocks were re-diluted, and this was repeated over a period of time; 1, 7, 14, 30, 60, and 90 days. The concentrations were calculated according to the calibrations curves originally injected (day 1). The three standard solutions were injected after 60 days using the calibration curves generated on day 1. The % difference was calculated as the absolute value of the concentration calculated with the corresponding calibration curve over the mean value of both concentrations.

2.10.5. Measurement of Uncertainty. Uncertainty of a measurement is defined as “a parameter associated with the result of a measurement that characterizes the dispersion of the values that could reasonably be attributed to the measurand”.35 The sources of measurement uncertainty (i.e., weighing, standard stock solutions, sample preparations, calibration curves, operator’s bias, as well as overall precision from analyzed matrices) associated with the analysis of color additives in food commodities were evaluated. Error components were estimated and calculated as an expanded uncertainty (U) using a coverage factor (k) of 2 at the confidence level of 95%. RSD values from the precision study were used to evaluate the measurement of uncertainty. The pool of data for color additives recoveries obtained on all food products tested are expressed as the RSDpool values.
Recoveries from the accuracy (trueness or bias) study were used to calculate the standard uncertainty ($\mu R_m$). Therefore, the mean and standard deviation of the recoveries obtained are included in the measurement of uncertainty.

3. RESULTS AND DISCUSSION

3.1. Analytical Characteristics. 3.1.1. Optimization of Chromatographic Analysis. To develop a method of analysis applicable to all color additives cited in this study, a combination of several LC columns and mobile phases were tested. Short and long LC columns as well as sub-2 $\mu m$ and core–shell packings were tested and evaluated to achieve efficient resolution, satisfactory peak shape, and retention factor ($k'$). Using the traditional LC system, high back pressure was experienced with sub-2 $\mu m$ columns, and the flow rate had to be reduced considerably, thus affecting the total duration of the chromatographic analysis. Several mobile phases composed of sodium phosphate buffers, ion-pairing agents such as tetrabutyl...
ammonium bisulphate (TBAB), tetrabutyl ammonium dihydrogen phosphate (TBAP), ammonium formate, and ammonium acetate, mixed with different ratios of methanol and acetonitrile, were used to achieve optimal separation for all color additives. The results demonstrated that the 50 mM ammonium acetate solution was the most suitable ion-pairing agent in providing optimal chromatography. In addition, the use of ammonium acetate solution was highly efficient. Typical chromatograms obtained from groups I, II, and III calibration curves injections are shown in Figure 1, while Table 4 lists the appropriate chromatographic data.

3.1.2. Identification and Quantitation. The retention times and DAD absorption spectra of detected colors were compared with standard preparations recorded in the spectral library, and a corresponding factor for "analyte matching" was generated. Although the wavelengths do not all correspond to respective maximum absorption wavelengths for the individual compounds, they were selected as to fit as the optimal ones for detecting all of the color additives in one single analysis. Positive matching of color additives triggered the quantitation step. Specifically, when a color additive peak corresponding to either crocein orange G or orange II was detected and identified, one single peak assignment was made. The method was highly efficient. Typical chromatograms obtained from groups I, II, and III calibration curves injections are shown in Figure 1, while Table 4 lists the appropriate chromatographic data.

Table 5. Recovery Results for Color Additives in Multicolored Candies Extracted with Ammonium Acetate Solution

| color additive                  | recovery (%) – 20 mM NH₄Ac methanol (v/v %) | recovery (%) – 50 mM NH₄Ac methanol (v/v %) | recovery (%) – 100 mM NH₄Ac methanol (v/v %) |
|---------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|
| Group I                         |                                              |                                              |                                              |
| chlorophyllin                   | 78.7                                        | 158.4                                       | 124.9                                       |
| tartrazine                      | 106.0                                       | 80.4                                        | 92.6                                        |
| sunset yellow FCF               | 102.6                                       | 106.8                                       | 100.3                                       |
| allura red AC                   | 102.6                                       | 106.6                                       | 100.5                                       |
| ponceau SX                      | 104.9                                       | 106.6                                       | 99.9                                        |
| amaranth                        | 100.5                                       | 105.0                                       | 100.5                                       |
| erythrosine                     | 105.1                                       | 107.0                                       | 101.3                                       |
| indigo carmine                  | 94.7                                        | 79.8                                        | 97.0                                        |
| Group II                        |                                              |                                              |                                              |
| orange II                       | 102.0                                       | 101.7                                       | 101.3                                       |
| crocein orange G                |                                              |                                              |                                              |
| ponceau 4R                      | 101.3                                       | 101.7                                       | 99.2                                        |
| fast red E                      | 101.4                                       | 101.6                                       | 99.9                                        |
| bordeaux R                      | 101.4                                       | 102.7                                       | 102.0                                       |
| 4,5-diodofluorescein            | 67.9                                        | 73.0                                        | 73.4                                        |
| 2,4,5-triodofluorescein         | 100.1                                       | 88.6                                        | 101.7                                       |
| 2,4,7-triodofluorescein°F        | N/A                                         | N/A                                         | N/A                                         |
| Group III                       |                                              |                                              |                                              |
| quinoline yellow WS             | 108.1                                       | 107.2                                       | 103.1                                       |
| chrysoidine G                   | 52.2                                        | 39.0                                        | 53.0                                        |
| orange GGN                      | 98.9                                        | 98.8                                        | 99.0                                        |
| carmoisine                      | 100.9                                       | 97.4                                        | 97.4                                        |
| eosin Y                         | 98.5                                        | 98.3                                        | 98.1                                        |
| rhodamine B                     | 97.3                                        | 97.2                                        | 100.3                                       |
| green S                         | 101.7                                       | 99.2                                        | 99.3                                        |
| patent blue VF                  | 100.8                                       | 99.9                                        | 98.6                                        |
| patent blue V                   | 101.3                                       | 99.4                                        | 99.2                                        |

2,4,7-Triiodofluorescein was not included in the solution used to spike color additives of group II. The concentration of the spiked solutions for groups I, II, and III were at about 20 μg/g.
the other two FDA compounds were therefore used to conduct a study for determining correction factors with respect to erythrosin yellowish. When the presence of subsidiary dyes of erythrosin (4,5-diiodofluorescein and 2,4,7-triiodofluorescein) was detected in foodstuffs, the quantitation was performed by applying the corresponding correction factors. The factors used were 0.92 for 4,5-diiodofluorescein ($\lambda = 506$ nm) and 1.15 for 2,4,7-triiodofluorescein ($\lambda = 540$ nm). Color additives quantitation was calculated using the external calibration curves from neat standard solutions.

### 3.2. Extraction Step
#### 3.2.1. Sample Preparation
Laboratory blenders were used to achieve satisfactory homogenization, while efficient magnetic stirring coupled with adequate sonication step helped in the extraction step.

#### 3.2.2. Optimization of Extraction Efficiency
Several extraction solutions were tested during this study, where the concentration of the ammonium acetate solution and the proportion of methanol were varied. Three concentrations of ammonium acetate solution were used, and 4 different proportions of methanol were tried and tested. Table 5 shows the recovery results for color additives at different compositions of extraction solutions for one food commodity (multicolored candies). Recoveries for color additives show fairly similar results in terms of the concentration of ammonium acetate solution at 20 and 50 mM. Specific color additives such as tartrazine, 4,5-diiodofluorescein, and chrysoidine G showed results slightly lower than the average across the four proportions of methanol tested. We observed that the best recoveries for tartrazine were obtained with the lowest methanol proportion (5%), while the opposite was observed for 4,5-diiodofluorescein and chrysoidine G. The same trend was observed in terms of results for the 100 mM ammonium acetate concentration. From those results, the 100 mM ammonium acetate solution was the most efficient and suitable concentration. This ion-pairing agent concentration facilitated the extraction of the color additives from food matrices, while methanol further improved recoveries of some of the color additives, particularly for erythrosine and its subsidiary dyes, rhodamine B as well as chrysoidine G. The use of extraction solution 1 demonstrated the optimal extraction and chromatography of dyes in food commodities, while the extraction solution 2 showed the optimal chromatography specifically for three analytes in group I (tartrazine, amaranth, and indigo carmine). These food colors are the first compounds eluted by LC with respect to their chromatographic affinity and chemical structure. The difference in organic strength between the extracts injected and the mobile phase used at the beginning of the gradient elution led to a modification in the extraction solution for these compounds. When methanol is over 10% (v/v) in the extraction solution, we clearly saw the impact on the chromatographic peak shape. A slight change in the proportion of methanol was made to extract, recover, and improve the

| Colour additive | Group I | Recovery (%) |
|-----------------|---------|--------------|
| Chlorophyllin   | 9.8     | 20.7         |
| Tartrazine      | 77.5    | 98.1         |
| Sunset Yellow FCF | 64.6 | 90.9         |
| Allura Red AC   | 55.1    | 79.0         |
| Ponceau SX      | 47.2    | 65.0         |
| Amaranth        | 52.9    | 74.1         |
| Erythrosine     | 1.1     | 0.8          |
| Indigo Carmine  | 67.9    | 87.1         |
| Fast Green FCF  | 52.6    | 70.8         |
| Brilliant Blue FCF | 62.2 | 77.7         |

#### Group II

| Colour additive | Recovery (%) |
|-----------------|--------------|
| Orange II       | 36.0         |
| Crocein Orange G| 70.6         |
| Ponceau 4R      | 30.5         |
| Fast Red E      | 18.2         |
| Bordeaux R      | 4.2          |
| 4,5-diiodofluorescein | 32.4 | 234.6$^a$ |
| 2,4,7-triiodofluorescein | 7.6   | 14.5        |
| 2,4,7-triiodofluorescein$^b$ | N/A | N/A         |

#### Group III

| Colour additive | Recovery (%) |
|-----------------|--------------|
| Quinoline Yellow WS | 61.1 | 92.2 |
| Chrysoidine G    | 10.4         |
| Orange GGN       | 72.3         |
| Carmoisine       | 31.2         |
| Eosin Y          | 11.5         |
| Rhodamine B      | 44.9         |
| Green S          | 63.9         |
| Patent Blue VF   | 86.7         |
| Patent Blue V    | 82.6         |

Table 6. Recovery Results for Color Additives in Chips with and without $\alpha$-Amylase Treatment

$^a$Outlier value. $^b$2,4,7-Triiodofluorescein was not included in the solution used to spike color additives of group II. The concentration of the spiked solutions for groups I, II, and III were at about 20 $\mu$g/g.
chromatographic elution of these compounds. Overall, two different extractions solutions were necessary for quantitative determination of color additives in food commodities: extraction solutions 1 and 2, respectively with 100 mM ammonium acetate and methanol, at ratios of 70:30 and 95:5 (v/v).

3.2.3. Enzymatic Treatment. An α-amylase solution was used to perform an enzymatic hydrolysis of the polysaccharide links [α(1 → 4)] present in the foodstuffs analyzed into maltose disaccharides fragments. The extraction and recovery of color additives were considerably increased when starch-containing commodities were treated with α-amylase during the extraction step, followed by the sonication step. Recovery results are presented in Table 6.

Hot chilli powder was selected to investigate the effect of filtration and centrifugation on the recoveries. An average of 8% loss was observed after filtration on the polytetrafluoroethylene membrane. In some cases, the color adsorption resulted in an intense staining of the filter. Given the results obtained, the centrifugation step was chosen and applied to the method validation.

3.3. Method Validation. Twelve different foodstuffs including beverages, candies,5 baked goods, chips, spices, fish and fish roe, dairy products, breakfast cereals,5 as well as savory sauces were purchased from retail stores. Food commodities chosen were selected to cover as many sectors of the AOAC food triangle. Figure 2 shows the AOAC food triangle based on the relative levels of fat, protein, and carbohydrate, as well as the triangle sectors covered with commodities analyzed. Although commodities are analyzed among several food categories, a specific trend was studied in starch-containing and other commodities.

3.3.1. Selectivity. First, several matrices were tested to determine if they contained color additives or not. The selected food matrices were chips, sauces, spices, fish roe and fish meat, orange juice, and yogurt and were chosen so as to reflect as closely as possible the different sectors (sectors 2, 5, and 9) of the food triangle covered with all food commodities tested during method validation. None of the tested commodities showed interferences when compared with spiked samples.

3.3.2. LOD, LOQ, and Linearity. Instrumental LOD and LOQ were determined by injecting diluted standard prepara-

Figure 2. AOAC food triangle and food commodities analyzed during the validation study.

the concentration corresponding to the limit of quantitation was recorded as the lowest level of the calibration ranges. The linearity of the detector response was evaluated by injecting seven concentration levels from 0.025 to 50 μg/mL for food colors of group I, and five concentration levels from 0.025 to 20 μg/mL for color additives of groups II and III. The different LODs and LOQs, as well as calculated parameters for linearity are presented in Table 3. Corresponding sample LODs and LOQs were determined by including the dilution factor for samples16 into the calculation. Sample LOQs ranged from 0.2 to 1.5 μg/g with an average of 0.6 μg/g, demonstrating the sharp sensitivity of the method. For instance, LODs and LOQs for amaranth (acid red 27) and for fast red E (acid red 88) are substantially similar and in the same order of magnitude as those cited in the literature for the same food dyes.18

3.3.3. Precision. The repeatability precision (sR, RSDr) was expressed as the mean RSD and was calculated from repeated determinations across the twelve food commodities tested. RSD was calculated with color additive recoveries from replicate sample preparations (n = 6) at one single concentration level (10 μg/g). On the other hand, intermediate precision was calculated with recoveries of color additives from replicate sample preparations on a different day. Data from the candies tested (jelly beans and peppermints), corresponding to sector no. 5 of the AOAC food triangle, were used to calculate the intermediate precision mean RSD. The overall precision was below 10%. The full intraday and interday precision data are shown in Table 7.

3.3.4. Accuracy. An average of about 70% accuracy was recorded for each compound group evaluated (I, II, and III). Lower results (<50%) were encountered for chlorophyllin, erythrosin, bordeaux R, 2,4,5-triodofluorescesin, chrysoidine G, and eosin Y. Accuracy data are summarized in Table 7.

3.3.5. Recovery. The different food commodities tested were spiked with standard solutions of all color additives at three spiking concentrations. Recoveries of color additives16 from spiked samples for all food commodities tested16 averaged about 70% along the three spiking levels run. Similar recovery results were obtained for all food dyes in the same types of spiked food commodities as those cited in the literature.19,27 Lower recovery results were found in specific commodities and for individual food dyes such as erythrosine and chlorophyllin which appeared to be related to the dyes’ binding to the proteins present in the samples analyzed. Results are presented in Table 7.

3.3.6. Stability. Given the difference in concentrations across all color additives, a difference of 10% (overall mean) or less was obtained and corresponded to a lifetime of 60 days. Therefore, 0.5 μg/mL of standard stock solutions was kept to re-prepare diluted standard preparations for not more than 61 days, refrigerated, and kept away from light.

3.4. Measurement of Uncertainty. The combined standard uncertainty (Uc) for each color additive was calculated, and the confidence factor of k = 2, with a confidence level of 95%, was applied to calculate the expanded uncertainty. Overall, the expanded uncertainty (U) for all color additives and foods is averaged to 29%.

4. CONCLUSIONS

The validation results demonstrated the purpose, sensitivity, and fitness for purpose of the method to simultaneously quantitate color additives in food matrices. The use of two
extraction solutions was the best compromise to maximize efficiency, chromatographic resolution, and ensure short analysis time. In terms of color additives extraction, the use of ammonium acetate as the ion-pairing agent offers similar performance as other cationic ion-pairing agents at a fraction of the price. The method uses successful color additives extraction and cleanup procedures to avoid the interference from complex food matrices, thereby offering a combination of selectivity and ease of use at low cost. The LC UV−vis analytical method described herein is a successful approach to separate and quantitate color additives in food commodities; it therefore expands the scope to analyze a broad variety of market samples. Since its implementation, the method has been used to analyze nearly 3000 food samples.

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**Table 7. Precision Data Calculated from Recoveries of Color Additives in Jelly Beans and Peppermints**

| Group | color additive      | RSD (%) | recovery (%) |
|-------|---------------------|---------|--------------|
|       | repeatability       | intermediate | accuracy (average) | low-level (5 μg/g) | mid-Level (10 μg/g) | high-Level (20 μg/g) | standard deviation |
|       | precision<sup>a</sup> | precision<sup>b</sup> |          | (n = 3)            | (n = 6)            | (n = 3)            | (μg/g) (average)    |
| Group I | chlorophyllin       | 5.7      | 33.5         | 39.2               | 36.6               | 39.2               | 36.1               | 24.9               |
|        | tartrazine          | 12.4     | 13.8         | 96.9               | 91.4               | 96.9               | 93.6               | 11.9               |
|        | sunset yellow FCF   | 12.7     | 9.4          | 96.2               | 88.2               | 96.2               | 95.0               | 21.4               |
|        | allura red AC       | 16.1     | 20.9         | 87.4               | 88.8               | 87.4               | 90.6               | 19.6               |
|        | ponceau SX          | 3.1      | 3.0          | 75.3               | 73.4               | 75.3               | 78.8               | 16.7               |
|        | amaranth            | 6.9      | 5.6          | 72.6               | 72.8               | 72.6               | 68.7               | 25.4               |
|        | erythrosine         | 5.5      | 14.2         | 30.7               | 32.6               | 30.7               | 30.4               | 40.2               |
|        | indigo carmine      | 5.9      | 13.7         | 77.3               | 74.0               | 77.3               | 80.8               | 13.9               |
|        | fast green FCF      | 3.1      | 3.0          | 81.3               | 79.4               | 81.3               | 85.0               | 13.9               |
|        | brilliant blue FCF  | 11.0     | 8.5          | 85.2               | 83.4               | 85.2               | 87.9               | 13.4               |
| Group II | orange II          | 0.7      | 7.7          | 72.7               | 75.6               | 72.7               | 68.1               | 15.4               |
|        | crocein orange G    |          |              |                    |                    |                    |                    |                    |
|        | ponceau 4R          | 2.3      | 4.2          | 76.0               | 80.1               | 76.0               | 70.7               | 16.1               |
|        | fast red E          | 3.9      | 4.2          | 65.3               | 63.6               | 65.3               | 62.8               | 16.2               |
|        | bordeaux R          | 5.2      | 7.3          | 50.7               | 53.4               | 50.7               | 49.7               | 20.8               |
|        | 4,5-diiodofluorescein| 7.4      | 31.1         | 97.0               | 86.7               | 97.0               | 94.5               | 28.8               |
|        | 2,4,5-triiodofluorescein| 4.9  | 8.8          | 44.4               | 45.8               | 44.4               | 38.7               | 34.5               |
|        | 2,4,7-triiodofluorescein<sup>b</sup> | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| Group III | quinoline yellow WS | 5.9      | 5.1          | 74.7               | 76.3               | 74.7               | 98.4               | 12.9               |
|        | chrysoidine G       | 4.5      | 14.8         | 52.2               | 52.1               | 52.2               | 50.3               | 19.8               |
|        | orange GGN          | 6.1      | 5.6          | 81.3               | 78.2               | 81.3               | 79.7               | 10.3               |
|        | carmoisine          | 2.4      | 4.3          | 63.5               | 60.5               | 63.5               | 61.3               | 18.7               |
|        | eosin Y             | 2.9      | 13.5         | 43.2               | 51.5               | 43.2               | 39.3               | 29.2               |
|        | rhodamine B         | 2.1      | 2.2          | 84.4               | 79.2               | 84.4               | 77.4               | 15.2               |
|        | green S             | 1.6      | 2.7          | 78.2               | 69.5               | 78.2               | 77.5               | 11.3               |
|        | patent blue VF      | 1.3      | 1.9          | 84.1               | 75.8               | 84.1               | 81.3               | 7.4                |
|        | patent blue V       | 1.5      | 2.2          | 81.2               | 73.8               | 81.2               | 78.7               | 9.6                |
|        | mean (overall)      | 5.4      | 9.7          | 72                 | 70                 | 72                 | 71                 | 19                 |

<sup>a</sup>Precision data was calculated from recoveries of color additives in jelly beans and peppermints.<br><sup>b</sup>2,4,7-Triiodofluorescein was not included in the solution used to spike color additives of group II. The concentration of the spiked solutions for groups I, II, and III were at about 10 μg/g (n = 6).
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