Color Additive Analysis in Foods and Cosmetics using UPLC with Extended Photo-Diode Array Detection

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COLORS MANUAL 2016 describes new approaches for extraction, cleanup, and identification of permitted and non-permitted color additives in a variety of matrices, including difficult ones such as soaps, creams, oil-based cosmetics and various food products. The extraction/purification technique involves three solid-phase extraction (SPE) column types: cation-exchange (CBX), silica gel (SiOH), and hydrophilic-lipophilic balanced (HLB). Colors from the product of interest are extracted with aqueous or organic solvent then concentrated on an SPE column. Once eluted from the column, the colors are re-dissolved in methanol, water, or other solvent and analyzed by Ultra-Performance Liquid Chromatography (UPLC) with extended photo-diode array (PDA) detection. This method can be used for identification of colors by comparison to a spectral library compiled by the FDA Denver Laboratory, and is applicable to quantitative analysis.

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1. Introduction and Scope

Color is a powerful factor in the food and cosmetic industries that can make a product more or less appealing to consumers (1). Product color often produces an expectation on the part of the consumer about taste, quality, or ingredients. While the FDA is responsible for regulating the safe use of color additives in foods, drugs, and cosmetics, many of the current techniques used to extract and identify these dyes and pigments responsible for imparting color to products are inadequate when faced with difficult matrices such as oils, lotions, and soaps. In addition, there are few methods available that can adequately characterize natural pigments.

Dyes and pigments have been used as color additives for hundreds of years to enhance the visual appeal of marketable products. Ancient Egyptian and Roman color additives were derived from either plants (*e.g.*, saffron and turmeric for yellow dyes) or animals (*e.g.,* squid ink for black dye and mucous secretion from a species of predatory sea snails for royal purple and blue dyes) (2). The use of sometimes toxic pigments to improve the palatability of foods was documented in 1820 in England (1,3) and examples included chalk to make bread appear whiter, copper salts to make pickles look greener, and mercury-containing vermilion to make candies look colorful. In 1856, Sir William Henry Perkin characterized the first coal-tar dye, and his discovery was the start of the synthetic dye industry (1). The first application of synthetic dyes was to cloth but they were soon used as food colors.

In the United States, the Pure Food and Drug Act of 1906 was enacted, in part, to prohibit the use of poisonous or deleterious substances (including dyes) in food products. The United States Department of Agriculture (USDA, precursor to the Food and Drug Administration (FDA)) began investigating food colors used throughout the world. In 1907, seven coal-tar dyes were pronounced safe for use as food colors and by 1931, 15 coal-tar dyes had been approved for that use. In 1933, the use of a permanent mascara product called Lash Lure, containing p-phenylenediamine (PPD) as the dyeing agent, resulted in permanent blindness and death in several women (4). As a result, the Food, Drug, and Cosmetic Act of 1938 was enacted with new oversight of cosmetics. A safety standard of "harmless and suitable" was established for coal-tar colors, as well as a requirement for batch certification by FDA before they could be used in foods, drugs, or cosmetics. Following certification, the colors were given "FD&C", "D&C", or "External D&C" designations depending on whether they were allowed in foods, drugs and cosmetics, drugs and cosmetics generally, or drugs and cosmetics for external use only.[1] Additional legislation in the 1960s defined "color additive", provided for the use of certification-exempt color additives, and listed permitted color additives with a safety standard of "reasonable certainty of no harm". Oversight of color additives in medical devices such as sutures and contact lenses was added in 1976. Today, color additives must be pre-approved by the FDA, listed on FDA's approved lists, and declared in product ingredient statements by their FD&C designations, listed names (for those without such designations), or appropriate abbreviations.

[1]*Note that throughout this document, certified color names will either be given as their formal designations according to the FD&C Act (such as FD&C Yellow No. 5, D&C Red No. 22 or External D&C Violet No. 2), or their permitted abbreviations (Yellow 5, Red 22, or Ext. Violet 2). Colour Index designations are given as "C.I. (name)", for instance "C.I. Natural Orange 4". Common names used are selected as one of the most utilized names for a given color, other than its FD&C or Colour Index official designation. However, each dye tends to have many synonyms. Colour Index numbers (i.e. C.I. 42090) are also referred to in some sections. References to "natural" colors in the text refer to colors that are derived from natural sources (such as carotenoids produced by vegetables, anthocyanins produced by fruits, etc.). Note that these are still considered "color additives" when applied to a product to impart color, and those that are permitted in foods are designated as color additives that are "Exempt from certification".*

The majority of available analytical methods address water-soluble dyes. The official AOAC method (5) for the analysis of the dyes permitted for use as food, drug, and cosmetic (FD&C) color additives (FD&C Red Nos. 3 and 40, FD&C Blue Nos. 1 and 2, FD&C Yellow Nos. 5 and 6, and FD&C Green No. 3) in various foods uses a C_{18} cartridge (Waters Sep-Pak® or equivalent), requiring manual application and elution of each aqueous sample extract. Although the AOAC method specifies a multi-step elution scheme involving increasing percentages of isopropanol and water (to collect groups of colors separately), typical FDA Denver Laboratory practice when screening colors has been to apply the water-extracted sample onto a C_{18} cartridge and elute all of the retained colors at once with 10% ammonium hydroxide in methanol. The eluate is collected and reduced to dryness on a steam bath or hot plate prior to re-dissolution in aqueous methanol. The solution is then applied to a thin-layer chromatographic (TLC) plate for comparison to known standards or analyzed spectrophotometrically (6).

Newburger's Manual (2nd edition) includes what is essentially a step-wise guide for the analysis of different types of cosmetic products (14). It has a common base method that is used for most of the relevant products, as well as product-specific preparation steps that lead you back to the common method. It then uses TLC and/or UV-Vis for the final color additive identification.

Yoshioka and Ichihashi (7) developed a high-performance liquid chromatography (HPLC) method with photodiode array detection (PDA) for 40 dyes in drinks, syrups, candies, gelatin-based candies, and marshmallows. In this method, the products are heated and dissolved in water acidified with acetic acid. The acidic (approximately pH 3) extract is applied to a prepared polyamide column and washed with acidified water, then the dyes are eluted with an ammoniated solution containing ethanol. After evaporation and re-dissolution in methanol/water, the dyes are separated on an Agilent XDB-C_{18} rapid Resolution HT (50 mm x 4.6 mm) column in a reverse-phase HPLC mode, monitoring at 450, 490, 520, and 620 nm wavelengths.

Harp *et al.* (8) describe a more recent HPLC-PDA method designed for quantitative dye extraction, though it is primarily used qualitatively, that detects 17 permitted and non-permitted color additives in a variety of food products. This method utilizes a gradient from aqueous to methanolic ammonium acetate solutions on a Waters Xterra RP18 (reverse-phase) column, with an approximate run time of 52 minutes per sample. The same group details its identification of 29 permitted and non-permitted color additives in cosmetics and additional color additives in food products using HPLC-PDA detection in further publications (9-11). Cosmetics, such as lip, eye, and nail products, are dissolved in a methylene chloride/methanol mix. The dyes are then back-extracted into water prior to HPLC analysis. The method also utilizes the RP18 column with a linear gradient advancing from aqueous ammonium acetate to pure acetonitrile, with a run time of approximately 60 minutes.

Existing methods used for color analysis in various matrices by FDA laboratories, including TLC, spectrophotometry, and current HPLC-based methods have several drawbacks. They are often not very sensitive, are time-consuming, work well for a limited selection of matrices, and are applicable mainly to known, commonly occurring dyes. They are quite limited in their abilities to identify unknown colors or in analyzing samples that contain multiple, related dyes.

Colors Manual 2016 was designed to take advantage of the best aspects of the existing methods, as well as incorporating new technologies to make an *evolved*, modern, efficient suite of techniques to analyze a broad spectrum of natural and synthetic color additives in a wide variety of product matrices. Its overall layout is similar to Newburger's Manual, divided into product-specific sections but having a central 'primary' technique that is used for nearly all products. It is meant to be as

universal as possible, to work with the tremendous variety of foods, cosmetics, drugs, herbal supplements, and other products that are on the market. Exceptions and/or expanded methods are published periodically as follow-up LIBs (such as for olives and hair conditioners).

Colors Manual 2016 is also intended to make use of the technological advantages of UPLC analysis (see below), including higher sensitivity (allowing for smaller sample sizes), short run times (improving throughput), and the use of a screening library of spectra for quick identification of undeclared color additives. It employs a new SPE clean-up strategy, coupling a selective SPE with a non-selective SPE (for most products), ensuring that no dyes are lost, and allowing better characterization of a variety of natural colors.

A number of specific improvements have been incorporated into Colors Manual 2016, including: A) Faster and more sensitive LC technology; B) Peak identification and optimization software; C) A custom-built reference library of spectra; and D) Matrix-customized extraction and clean-up protocols. These four improvements are described in greater detail below (with examples to follow within the text):

A. Ultra Performance Liquid Chromatography (UPLC) is used instead of TLC or HPLC. The UPLC system uses a high efficiency, sub-two-micron particle size analytical LC column to achieve superior chromatographic peak resolution. A generic "catch-all" mobile phase gradient allows many synthetic and natural-source dyes to be separated using a rapid (17-minute) run. An extended photodiode array detection (PDA) system from 270-790 nm provides maximum spectral flexibility and identification for a multitude of colors.

B. A new UPLC software algorithm (Waters Corporation's "Maxplot") identifies and displays the maximal absorbance wavelength (lambda max) for a given peak. Prior to this innovation, several discrete wavelengths (associated with known compounds) were typically monitored in a chromatographic run. This algorithm greatly improves the ability to identify undeclared dyes and to apply to quantitative dye analysis.

C. A custom-built PDA spectral reference library containing the profiles of over 150 different dyes and natural pigments, compiled by the FDA Denver Laboratory. This aids greatly in the identification of known and unknown colors in a sample extract, if they are included in the reference library. As part of the UPLC data processing, spectra from unknown peaks are compared to the spectral library to identify the best potential matches, which can also set to require similarity in retention times. A rapid initial screening can be accomplished without running a costly and time-consuming multi-standard dye mixture.

D. A fast, simple, widely-applicable extraction and cleanup procedure scheme is used (summarized in the Flowchart and Table in Section 10). In this new scheme, the sample is initially classified by phase: liquid or solid. Then the sample is further classified as being an aqueous (water-based) or a non-aqueous (oil-based) sample. Liquid aqueous samples are diluted with methanol before Solid Phase Extraction (SPE) cleanup. Solid aqueous-based products are extracted with an acidified methanol/acetonitrile/water solvent mixture before SPE cleanup. Oil-based food samples (both solid and liquid) are extracted with hexane (or dichloromethane). Non-liquid cosmetics are typically extracted with acidified methanol and/or hexane, depending on their degree of lipophilicity. Depending upon the dye, three different SPE column types -- CBX, SiOH, and HLB -- are used for sample cleanup prior to UPLC analysis. Note that tests of various CBX SPE columns have demonstrated that the Avantor/J.T. Baker CBX® SPE was found to be the most effective for this method in

retaining/cleaning up the synthetic dyes of interest, and no other brand of CBX or other SPE column has yet been found that performs as well. These CBX SPEs are also unique in their ability to bind the vast majority of the synthetic dyes while dissolved in methanol. Dyes not retained on the CBX SPE are then applied in a more aqueous phase to the HLB SPE. Using these two columns in tandem, with the specificity of the CBX and the comparatively non-specific binding of the HLB, ensures that no water/methanol-soluble dyes are likely to be missed during the clean-up process. The SiOH SPE columns are used primarily to clean up oil-soluble dyes.

The Denver Laboratory has applied Colors Manual 2016 to a large variety of product types including oil-based liquids, grease-based solids, liquid and bar soaps, creams, lotions, fish, and other liquid and solid food and cosmetic products. It has been successful for rapid screening as well as confirmatory analyses and identification of nearly all dyes (declared and undeclared, permitted and non-permitted) that have been encountered in the various matrices. Although some products may show multiple peaks in their chromatograms from their active and inert (non-colored) ingredients, the use of an extended PDA detector and a color standard library allows quick differentiation and identification of the colored vs. uncolored compounds. It is important to note that colored compounds absorb light specifically within in the visible range (approximately 400-700 nm wavelengths). Other compounds typically need not be considered when looking specifically for color additives.

The Colors Manual 2016 methods have been applied to over 300 regulatory and investigational samples received in the Denver Laboratory to date. Validation of the Colors Manual methods was achieved using negative control products (those devoid of colors) which were fortified at two levels with various combinations of commonly found colors that include both water-soluble and oil-soluble dyes. The fortification levels varied with the products tested. For most products, the added dye concentrations were approximately 5 and 11 mg/kg (ppm). For fish and soda samples, recoveries were evaluated at 1 to 8 mg/kg. Although the intention is for these methods to be used qualitatively (i.e. color additives are present or they are not), quantitative recovery data is a valuable tool to evaluate a method's extraction and cleanup efficiency. However, dry food products such as crackers, cakes, cookies, etc., tend to absorb and hold colors, including those used for fortification, affecting extraction efficiency and quantitative recovery. Limited recovery data is presented within this text, with more extensive data and examples in the method validation packet. Some notable exceptions to the general methods are also given. For instance, some dyes did not retain on the CBX SPE, some peaks co-eluted in the chromatography, and one dye, D&C Red No. 30, was insoluble in most extraction solvents, although tetrahydrofuran (THF) was somewhat effective, as was xylene. Consideration of these exceptions may be helpful when presented with a matrix that exhibits the need for novel extraction or cleanup processes. By far the most challenging aspect of food and cosmetic analysis is the tremendous diversity of matrix types and how they interact with dyes. Several examples of each type of extraction, cleanup, and analysis are described in the sections that follow.

2. Use of UPLC as a separation and identification tool

Currently, the most accepted and utilized detection methods for dyes are thin-layer chromatography (TLC) and ultraviolet-visible (UV-Vis) spectrophotometry. For TLC analysis, small aliquots of both sample and standard solutions are applied about two centimeters from the bottom edge of a silica-coated TLC plate. After the spotting solvents have evaporated, the plate is placed in a tank containing a specific mobile phase. The solvent moves up the plate by capillary action, meets the sample and standard spots, and migrates the dyes at varying rates based on the differences in their attraction to the stationary phase as well as their solubility in the mobile phase. Spot color and

migration distance of the sample, when compared to the same properties of applied standard solutions, define each dye's identity. Migration overlaps can be addressed by altering the mobile phase (development solution). In UV-Vis spectrophotometry, sample and standard extracts are scanned and compared by measuring a light beam's intensity as a function of the dye molecule's absorption. If the spectra of an individual dye extracted from a sample and a reference standard match over the range of 350-750 nm in acid, neutral and basic solutions, the dye is considered to be identified. For the most part, both systems work well when the dyes are common, known, and conform to the product's labeling. However, spectrophotometry, without chromatography, does not support identification of multiple dyes in one solution, necessitating further separation. Furthermore, without product label information and for cases where the product contains new or unusual dyes, it can be difficult or impossible to determine what dyes are present using only TLC and/or spectrophotometric data.

UPLC can be used to separate dyes in a liquid phase and can be coupled with a detector to identify compounds as they elute from the LC column, with a reference library to identify the compounds. To date, the FDA Denver Laboratory has characterized and stored over 150 reference dye spectra in such a library (UPLC-PDA Color Library). Although the use of such a simple clean-up method with wide range UV-Vis range scanning can produce many chromatographic peaks, generally only a few peaks (typically one to three) display spectra in the visible range, and the remaining peaks can be disregarded because they do not contribute to the visible product color. Furthermore, the UPLC gradient described (See 2.1 Instrument Parameters) produces minimal chromatographic overlap for a large library of compounds, with only a 17 minute run time.

Appendix 1 contains a reference table summarizing many of the dyes that have been included in the customized Denver Laboratory Colors Reference Library using Gradient System 1 (below).

2.1. Instrument parameters

Instrument: Waters UPLC (Ultra Performance Liquid Chromatography) Acquity system, with photodiode array detector (270 nm-790 nm) with Maxplot detection algorithm
Instrument software: Empower 3 Software Build 3471; Service Pack D DB ID: 2412999898
Column: Waters UPLC BEH 1.7 micron 2.1 x 50 mm Part #186002350 w/40 °C column temp.
Computer: Dell Optiplex 9020 @ 3.60 GHz w/16 GB RAM and MS Windows 7 SP1
Mobile phase A: 100 mM ammonium acetate (pH 6.5), acidified with 5% HCl (aq.)/1% acetonitrile
Mobile phase B: 75% acetonitrile / 25% methanol
Injection volume: 1 to 2 µL (see notes in Section 9.2); Sample temperature: 26 °C
Flow rate: 0.400 mL/min (constant)

Table 1. Parameters for Gradient System 1 used for the analysis of water-soluble and methanol-soluble dyes.

Time (min)	%A	%B	Curve/Rate
Initial	100	0	--
0.50	100	0	6
12.0	0	100	6
15.0	0	100	6
15.1	100	0	11

Note that for universality, corresponding chromatography conditions are being developed for other UPLC systems as well as HPLC instruments for labs that do not have Waters UPLC instruments. Likewise, investigations are ongoing into other LC column types that give similar

separations of the major color standards, and other SPEs that can substitute in the clean-up. It is important to recognize that the extraction and clean-up portions of this method can be applied to a sample, which can then be analyzed via any available detection method (HPLC, TLC, UPLC, UV-Vis (for single colors), etc.) Thus, the extraction and detection portions are designed to be separable, providing greater flexibility. Use of UPLC/HPLC allows comparison to an established library, however, and exportation efforts for the entire spectral library are underway into formats that are importable for instruments from other manufacturers, as well as in the creation of a standalone spectral 'lookup' database.

2.2. Equipment

SPE cartridges:
(CBX) J.T. Baker BAKERBOND® Wide-Pore carboxylic acid disposable SPE columns, 6 mL (Part # JT7217-6 through VWR Intl) [Recommend no substitution];
(SiOH) J.T Baker BAKERBOND® Silica gel SPE disposable extraction columns, 6 mL (Part # 7086-06 through VWR Intl), or equivalent;
(HLB) Waters Oasis® Hydrophilic-Lipophilic Balanced SPE columns, 6 mL, 200 mg (Part # WAT106202 through Waters Inc.) or equivalent.

Laboratory equipment: Nitrogen Evaporator with temperature-controlled water bath (Organomation Associates, Inc. or equivalent); Refrigerated centrifuge, capable of accelerating 15 and 50 mL tubes to 10,000 rpm (Thermo Fisher RC6+ or equivalent); Robot Coupe food processor (Model# 2Y-1, RSI, Jackson, MS); Stein Mill (Model# M2, the Steinlite Corporation, Atchison, KS); Temperature-controlled Sonicator (Model# 8892, Cole-Parmer or equivalent); Tissumizer (Teledyne Tekmar, Mason, OH); (Multi-tube) Vortex mixer; SPE vacuum manifold; 5-5,000 µL manual pipettors.

Laboratory consumables: Syringe filters: Pall 13mm disposable PTFE, 0.2 µm pore size (Part # 4542, VWR Intl); Whatman 13mm disposable PVDF, 0.2 µm pore size (Part # 6872-1302, VWR Intl); 15 and 50 mL disposable, conical-bottom, graduated, polypropylene tubes with cap (#352097 and #352098, respectively, Becton-Dickinson, Franklin Lakes, NJ); 12 x 75 mm disposable glass tubes (#47729-570, VWR Intl).

Solvents/Reagents: Deionized water (~18.2 MΩ·cm resistivity) (Millipore Corp.); High-purity chromatographic and spectrophotometric grade water (UPLC-MS grade), methanol, acetonitrile, dimethylsulfoxide (DMSO), hexane, dichloromethane (DCM, EMD Chemicals, Gibbstown, NJ, or equivalent), tetrahydrofuran (THF, Sigma Aldrich); Concentrated hydrochloric acid (HCl, 12N) (Sigma Aldrich, St. Louis, MO, or equivalent); Ammonium hydroxide (NH_4OH, 28-30% as NH_3) (Part# BDH3014, VWR Intl); Ammonium acetate (EMD or equivalent).

2.3. Solution and sample preparation

Solution preparation: Prepare a 1% HCl solution in methanol by adding 10 mL 12N HCl to 990 mL methanol in a 1,000 mL graduated cylinder. Mix by inversion. Prepare a 10% ammoniated methanol solution by adding 100 mL NH_4OH to 900 mL methanol in a 1,000 mL graduated cylinder. Invert carefully to mix.

Mobile phase A: 0.1 M ammonium acetate (pH 6.5)/1% acetonitrile is prepared as follows: Accurately weigh 7.7 g ammonium acetate into a 500 mL flask containing 200 mL HPLC-grade water, adjusting pH to 6.5 with 5% HCl in water. Transfer solution to a 1,000 mL mixing cylinder,

add 10 mL acetonitrile, and bring to volume with additional water. Mix thoroughly with inversion, and filter through a 0.2 µm nylon filter prior to instrument use.
Mobile phase B: 75% acetonitrile/25% methanol: Add 250 mL methanol to 750 mL acetonitrile in a 1,000 mL graduated cylinder. Mix by inversion.

Other solutions: 50:20:29:1 methanol:acetonitrile:water:concentrated HCl solution. To prepare, pour 500 mL HPLC-grade methanol into a 1000 mL graduated cylinder. Add 200 mL acetonitrile, 290 mL HPLC-grade water and 10 mL concentrated HCl into cylinder. Invert slowly to mix.
40% Methanol solution: 40% methanol:60% water. To prepare, add 400 mL HPLC-grade methanol into a 1,000 mL graduated cylinder. Add 600 mL HPLC-grade water and invert slowly to mix.

Standards: Reference standards were received from the Center for Food Safety and Nutrition (CFSAN), Division of Color Certification and Technology, as well as purchased from Sigma-Aldrich, Spectrum Chemicals, and other vendors. Portions are accurately weighed individually to obtain an approx. 5 mg/10 mL (500 µg/mL) concentration. Water-soluble colors are dissolved in water or methanol; water-insoluble colors are dissolved in methanol or acetonitrile. To prepare the 50 µg/mL fortification standards, pipette 0.500 mL of each stock standard in each set into a 15-mL polypropylene centrifuge tube and dilute to the 5 mL mark with distilled water. Cap and invert to mix. (Include dye purity in concentration calculations.) Fortification levels are arbitrary, but 10 mg/kg is useful for food analysis. For 1 g sample portions, add 0.200 mL of the 50 µg/mL mixed standard; for 3 g portions of product, add 0.600 mL mixed standard. For higher spike levels, use the stock standards directly. Standards such as the carotenoids (Astaxanthin, Canthaxanthin, Beta-Carotene), D&C Yellow No. 11, D&C Green No. 6, Butter Yellow, Solvent Blue 59, Oil Red N, Sudan III and Sudan IV are typically dissolved in dichloromethane. (Sudans III and IV were found to be soluble in acetonitrile as well.) Aliquots are evaporated to dryness, then re-dissolved in methanol for UPLC analysis. Some oil-soluble dyes are instead dissolved in 5:2 DMSO:methanol for UPLC analysis based on the solubility of concurrently analyzed samples. (For example, an oil-based hair product was analyzed and the final residue was only soluble in the DMSO:methanol solution. Therefore, all standards and controls were dissolved similarly.) For UPLC standards, add 15 µL of each individual standard (those in water, methanol or acetonitrile) to 975 µL methanol, then mix and filter. For standards in methylene chloride, evaporate a ~15 µL aliquot in a test tube, then resuspend in 1 mL methanol (or other injectable solvent) and filter prior to injection. Note that dyes soluble only in hexane or dichloromethane should be analyzed with an alternate method, such as with normal-phase chromatography.

Sample preparation: While liquid samples typically do not require preparation prior to extraction, solid products such as crackers, wafers, and non-homogenous dry materials need to be ground using a Robot Coupe food processor, Stein Mill, or equivalent device. Solid, sticky foods such as fruit and nut pastes and dense candies may require blending using a Tissumizer prior to the extraction process. Seafood and other frozen matrices may need to be blended with dry ice to create a homogenous frozen powder. Each matrix must be considered on a case-by-case basis, trying to ensure a homogeneous and representative mixture is obtained prior to extraction.

2.4. Method selection

When beginning analysis on a new sample, one must first classify the sample as to its character. (Refer to Flowchart in Section 10.) If the sample is primarily a liquid (free-flowing or viscous, aqueous (at least 50% water) or alcohol-based, but not oil-based), apply **Method 3.1**, which is the 'primary' method. This includes foods and cosmetics – such items as drinks, sauces, liquid soaps, shampoos, as well as soft lotions, etc. If the sample is a solid food (low in moisture and oil content), apply **Method 4.1**, which substitutes an acidic solvent mix rather than methanol for the

extractant. This would include such matrices as candies, breads and baked products, dried fruits, etc. If the product is a solid food, particularly high in one component type (such fat, protein, carbohydrate), or is a dry mix intended to be dissolved in water (such as powdered gelatin, gravy or drink mixes, etc.), apply **Method 5.1**, which utilizes methanol as the primary extraction solvent. Otherwise it follows the same steps as the primary Method 3.1. To look for natural carotenoids such as Astaxanthin or Canthaxanthin, in seafood or similar products, apply **Method 5.2**, which uses hexane with the CBX column.

For cosmetics products that are dry, such as eye shadows, blush powders, lipsticks, etc., apply **Method 6.1**, which uses a low volume of acidic methanol as the extractant (due to the small sample size). For oil-based (lipophilic) products such as hair products and lip glosses, or oil-based foods such as vegetable oils, apply **Method 6.2**, which uses hexane as an extraction solvent and a Silica Gel (SiOH) SPE column for clean-up. For extremely viscous or solid oil-based cosmetics, such as hair pomades and waxes, apply **Method 6.3**, which is similar to Method 6.2, but includes steps for keeping the material warm (typically approx. 40 °C) and well-dispersed in solution.

Various exceptions and special product types (such as bar soaps, hair conditioners, etc.) are presented in **Section 7**, with recommended tweaks and alterations to improve performance. Each method section also includes individual notes and tips after the method description, provided based on experience gained using these methods on regulatory products for over a year and noting their successes and difficulties. **Section 8** provides specific examples analyzed with these methods, and **Section 9** has general notes related to the methods and instrument-specificity. **Section 10** provides a 'cheat sheet' guide with a comparison of the different methods and a method selection flowchart.

Note that the analysis scheme presented here is intended to be used as a *starting point*. One may quickly find that the recommended extraction solvent does not appear to be effective, at which point an alternate strategy (such as substituting with acidified methanol or another solvent) should be considered.

3. Primary Extraction Method -- Liquid (aqueous-based) products

3.1. Liquid matrices: Juices, liquid soaps/shampoos, creams, gels, lotions (aqueous-based), etc. (Method 3.1)

This approach to extraction divides products into two main categories: liquids and solids. Liquids include any product which displays colors that are already in a homogeneous fluid state and that typically list water as a primary ingredient. For liquids that are primarily lipophilic in nature (i.e. oils), refer to Section 6.2. Liquids with chunky consistencies, such as tomato-based products (i.e. salsas) may require blending with a homogenizer or may be centrifuged to analyze only the juice in the product. (Superscripts indicate additional notes apply – see Section 3.1.1)

Sample/Control preparation: Weigh approximately 1 g (up to 5 g, based on coloring intensity)[1] liquid product into a 15 mL disposable polypropylene tube. Add 3 mL methanol per gram of sample and vortex vigorously for approximately 0.5 min.[2] If necessary to extract product color, incubate tube in an approximately 50 °C water bath for 5-10 min[3], then vortex for approx. 0.5 min. Centrifuge tube to separate and clarify supernatant as much as possible, typically at 6,000-10,000 rpm at 5 °C for 5 min.[4,5] The supernatant will typically have a colored appearance similar to that of the product, if the extraction has been successful.[6] If not, consider the use of an alternative extraction solvent (see exceptions and other sections/method alternatives that follow).

CBX column prep/loading: Prepare a CBX SPE column on a vacuum manifold by passing 3 mL methanol through column, followed by 1 mL 1% HCl in methanol. Apply 1-5 mL extract (based on color intensity) to the SPE and allow it to pass through column at approximately 5 drops/sec. If colors are observed to pass through the column without forming a band, one may use the HLB protocol described below to attempt to capture these colors.

CBX column wash/elution: Wash CBX column with 6 mL deionized water, then 3 mL methanol. Remove column from vacuum manifold and elute color by passing 3-4 mL 10% ammonium hydroxide (in methanol)[7] through column into a 12x75 mm disposable glass tube using slight air pressure applied to the top of the SPE column. Evaporate eluate under nitrogen in an approximately 50 °C water bath to dryness, then resuspend residue in 0.5 mL or 1.0 mL methanol (or 1:1 methanol:water or water), based on color intensity[8] and instrument type[13]. Pass solution (as well as all control extracts, standards, etc.) through a 0.2 micron PTFE syringe filter prior to injection on the UPLC.

HLB column protocol for unretained colors: If a color does not visibly retain on the CBX column (many natural colors and some synthetic dyes)[9], remove the CBX SPE column from the vacuum manifold and apply slight air pressure to top of column. Collect the color as it elutes into a second 15 mL disposable tube. Apply and collect the 6 mL distilled water wash to the column to elute the remainder of the unretained color. You may also add 1-2 mL of methanol to ensure that the unretained color is fully eluted. Dilute this portion to 10 mL with distilled water, vortex briefly, centrifuge if necessary[4], and apply to conditioned HLB SPE column (see below). Continue cleanup and elution of CBX SPE for retained colors.

HLB column prep/loading: Prepare an HLB SPE column on a vacuum manifold by passing 3 mL methanol through column, followed by 3 mL distilled water. Allow entire diluted CBX eluate (mostly aqueous) to pass through column at approximately 5 drops/sec. Typically, you will see the colors form a band on the SPE. If colors are observed to pass through the column, either the SPE is saturated or an alternate SPE type may be needed to retain them.

HLB column wash/elution: Wash HLB SPE column with 6 mL distilled water, followed by 3 mL 40% methanol/60% water solution.[10] Use vacuum to remove remaining solvent from column prior to elution. Remove column from vacuum manifold and elute color by passing 3-4 mL methanol[11] through column into a 12x75 mm disposable glass tube using slight air pressure applied to top of SPE. Evaporate eluate under nitrogen in an approximately 50 °C water bath to dryness[12], then resuspend residue in 0.5 mL or 1.0 mL methanol (or 1:1 methanol:water or water), based on color intensity[8] and instrument type[13]. Pass solution through a 0.2 micron PTFE syringe filter as above, prior to injection on the UPLC.

Short summary of Primary Method 3.1 (See text for details and additional notes)

Sample Prep:

- Weigh 1-5 g liquid into a 15 mL tube
- Add 3 mL methanol for each gram of sample
- Vortex ~30 sec
- Incubate tube at ~50 °C for 5-10 min
- Centrifuge at ~6-10k rpm at 5 °C for 5 min

CBX Column:

- Wet CBX column with 3 mL methanol

- Prime column with 1 mL 1% hydrochloric acid in methanol
- Apply 1-5 mL of sample supernatant
  - Watch for unretained colors (if so, apply to HLB as below)
- Rinse column with 6 mL distilled water
- Rinse column with 3 mL methanol
- Elute with 3-4 mL 10% ammonium hydroxide in methanol into glass test tube

HLB Column (Only for unretained colors):

- Collect unretained color portions in 15 mL tube
- Dilute up to 10 mL with distilled water
- Wet HLB column with 3 mL methanol
- Prime column with 3 mL distilled water
- Apply all 10 mL of diluted unretained color solution
- Rinse column with 6 mL distilled water
- Rinse column with 3 mL 40% methanol
- Elute with 3-4 mL methanol into glass test tube

Prep for UPLC injection:

- Take eluate(s) to dryness under nitrogen at ~50 °C
- Resuspend with 0.5 to 1 mL methanol (or water); Vortex.
- Syringe filter with 0.2 um PTFE into UPLC vial

3.1.1. Notes on Method 3.1

[1](Sample size) The amount of material to extract may vary outside the 1-5 g recommendation in certain cases. Very lightly colored liquids may require the use of more product, necessitating the use of 50 mL tubes for extraction. Similarly, adjust the amount of extraction supernatant that is loaded on the CBX column based on how intense the color is. Faint colors will require more concentration on the SPE while intense colors may overload it if too much is applied. Overloaded colors may be observed to run off the CBX column, and may be analyzed on an HLB SPE if necessary for confirmation.

[2](Detergents) For liquid soaps, shampoos, or products containing a significant amount of detergents, avoid vortexing and instead invert tube several times to mix, reducing formation of bubbles/emulsions.

[3](Incubation time) Some products may require additional incubation time in the heat bath to sufficiently extract the colors. Keep in mind, however, that some colors may be heat labile and could undergo partial degradation during incubation. Use of a positive control containing the suspected color can help identify such degradation products, if they occur.

[4](Centrifugation) Centrifugation of samples prior to loading on the SPE may be omitted if the sample is completely liquid in nature with no visible insolubles or precipitates. In contrast, additional centrifugation time or steps may be needed for some samples when precipitates are observed to form. For instance, when collecting the portion for the HLB column with a mixture of water and methanol, some insoluble material may form and will require centrifugation to avoid clogging the HLB SPE.

[5](Cloudiness) Soaps and creams with high oil content may appear cloudy after centrifugation at low temperature. Warm these tubes to approx. 40 °C prior to application onto the SPE column.

[6](Fat Layer) For some products, particularly if there are lipophilic components, you may see a layer of fat-soluble material at the top of the tube, with the translucent supernatant sandwiched between this and the precipitate. In this case, pull this translucent layer for application onto the SPE.

[7](Elution) Some colors may bind more aggressively to the column and require a slightly higher concentration of sodium hydroxide, a solvent other than methanol, or even an acidic solution to elute.

[8](Dilution) In some cases, the color intensity of the solution may be so strong that even further dilutions are necessary to avoid saturating the instrument detector. One must be careful, however, to avoid missing minor colors that may also be present, if performing a significant dilution.

[9](Dye retention) Some synthetic dyes such as Rhodamine B, HC Blue No. 2, HC Yellow No. 4, and Methyl Violet 2B do not typically retain on the CBX SPE. Similarly, many natural pigments such as Annatto, Lutein, Astaxanthin, Canthaxanthin, and other xanthophylls, will not retain on the CBX column using Method 3.1. Other dyes such as Carminic Acid, Red 3, and Red 27/Red 28 will form a band on the CBX column, but will slowly migrate down the SPE during the washes, and most will elute off the column. However, a detectable amount will usually remain on the CBX, that will be visible in the UPLC analysis. Alternatively, you can use the HLB column to retain these run-off dyes, or you can reduce or eliminate the rinse volumes, but this may increase the background peaks.

[10](Rinse reduction) In some cases, the 40% methanol wash will cause the retained color(s) to elute. If that occurs, reduce or eliminate this wash.

[11](Elution solvents) Some colors (such as anthocyanins and Carmine) may not elute with neutral methanol. In this case, try 10% ammonium hydroxide in methanol, 1% or 10% hydrochloric acid in methanol, or other solvents. Methylene chloride is generally effective at removing Sudans that retain on the SPE.

[12](Neutralization) Some colors, such as betacyanins, are sensitive to alkaline conditions under heat. To characterize them, one may neutralize the eluate with a small amount of hydrochloric acid prior to the concentration step.

[13](Instrument type) Early eluters such as Yellow 5 can split into two peaks with Waters® Flow-Through-Needle (FTN) Sample Manager Modules. See Section 9.2.2 for details.

4. Solid Products

4.1. Solid matrices: Candies, pastries, crackers, other dry food products (Method 4.1)

Solid foods often require grinding and/or compositing prior to extraction to achieve a sample with as much homogeneity as possible. In general, a representative portion of all the product colors should be included in the sample portion used for analysis.

Sample/Control preparation: Weigh approximately 3-5 g (based on coloring intensity)[1] of product into a 50 mL disposable polypropylene tube. Add 20 mL solid extraction solution (methanol:acetonitrile:water:concentrated HCl (50:20:29:1))[2,3] and vigorously shake or vortex tube for 10-30 sec to incorporate the product into solution, then proceed as in Method 3.1.

Short summary of Method 4.1 (See text for details and additional notes)

Sample Prep:

- Weigh 3-5 g product into a 50 mL tube
- Add 20 mL solid extraction solution
- Vortex ~30 sec
- Incubate tube at ~50 °C for 5-10 min
- Centrifuge at ~6-10k rpm at 5 °C for 5 min

(Proceed as in Method 3.1)

4.1.1. Notes on Method 4.1

[1](Sample size) Some dry products, such as those that are light and bulky, will require a smaller sample weight while others will require a larger amount to ensure all product colors are sampled and are in sufficient quantity for detection.

[2](Extraction solution) Dry products, particularly those with high carbohydrate content such as breads and cookies, tend to absorb and/or emulsify dyes, causing many traditional color extraction methods to fail. Acidic extraction solvents help mitigate these effects. A solid extraction solution consisting of acidified methanol with smaller portions of water and acetonitrile proved to be most effective in extracting colors from these matrices. However, some matrices, particularly those with high fat contents, may not partition well during centrifugation. See Section 5.1 below for these matrix types.

[3](Rawhides) Rawhide-type dog chews should be extracted with neutral water first, not acidified water or methanol. The acidic solution extracts some component from the matrix, making the solution gelatinous, which can easily clog the SPE. Acidic extraction solution also appeared to drive spiked colors into the matrix, and reduced the extraction efficiency overall, for the sample and spikes.

5. Crossover Products

5.1. High protein, fat, salt, or carbohydrate matrices (Method 5.1)

Some products such as powdered drink mixes (which contain many carbohydrates), dried milk (with high fat content) and soy products, creamy icings, as well as fish and shrimp-based products, would be expected to fall under Method 4.1, but are found to extract with far less excipients in 100% methanol. For this reason they are referred to as "crossover products". They typically are dominated in composition by one component type (protein, fat, carbohydrate, etc), or have a high salt content, and often do not centrifuge into discrete layers when using Method 4.1 with the solid extraction solution.

Sample/Control preparation: Follow Method 4.1, substituting 20 mL methanol (preferred) or 20 mL 1% HCl in methanol (if neutral methanol is not effective at color extraction) instead of the solid extraction solution containing water and acetonitrile. Occasionally, such samples still extract better with the solid extraction solution, so Method 4.1 can still be attempted.

Short summary of Method 5.1 (See text for details and additional notes)

Sample Prep:

- Weigh 3-5 g product into a 50 mL tube
- Add 20 mL methanol or 1% HCl in methanol

- Vortex ~30 sec
- Incubate tube at ~50 °C for 5-10 min
- Centrifuge at ~6-10k rpm at 5 °C for 5 min
  (Proceed as in Method 3.1)

5.2. Carotenoids in seafood and other products (Method 5.2)

Astaxanthin and Canthaxanthin are xanthophylls that are part of the carotenoid family of natural pigments. For instance, the flesh of wild salmon is red, pink or orange in color based on its natural diet of carotenoid-rich crustaceans or other fish which ingest these crustaceans. To improve consumer appeal, the color of farm-raised salmonids (with flesh that is typically gray) is enhanced to look more like wild salmon flesh through feed that contains synthetic Astaxanthin and/or Canthaxanthin dyes. Although both Astaxanthin and Canthaxanthin are approved for use in the U.S. in salmonid fish through feed, it must be declared on the label in accordance with federal codes (CFR Title 21 Part 73). These additives have also been commonly seen in shrimp, artificial crab, and other farm-raised seafood products, though it is not currently permitted by regulation.

A common method for the analysis of both carotenoids is LIB 3155 "Determination of Astaxanthin and Canthaxanthin Carotenoids in Salmon Muscle by HPLC" (13). The extraction requires manually grinding salmon tissue with celite resin and chloroform, preparation of a chromatographic column with the celite/salmon/solvent mix, and finally, the elution of the dyes with a large volume of hexane. The HPLC is run in normal-phase mode with an acetone:hexane mobile phase. The CBX-based method described here is less time-intensive and requires less solvent.

Similarly, other food matrices, including other seafood such as farm-raised shrimp, have been found to contain such carotenoids. The CBX column can also be utilized with lipophilic solvents and is effective at retaining many carotenoids and other natural pigments.

Sample/Control preparation: Weigh 3-5 g product into a 50 mL disposable polypropylene tube. Add 10 mL hexane and vortex vigorously for approximately 10 sec. Sample may be incubated in an approximately 50 °C water bath for 5-10 min to enhance color extraction. Tubes may be sonicated at room temperature for 5-10 min, followed by 5-10 min on a vortex mixer, as needed to enhance color extraction. Centrifuge the tubes, typically at 6,000-10,000 rpm at 5 °C for 5 min.

CBX column prep/loading: Prepare a CBX SPE column on a vacuum manifold by passing 5 mL hexane through column. Do not allow the column to become dry. Apply 5 mL of sample extract to column bed, allowing it to pass through column at approximately 5 drops/sec.

CBX column wash/elution: Wash CBX SPE column with 5 mL hexane, using vacuum to remove completely from column prior to elution. Remove column from vacuum manifold and elute color by passing 4-5 mL dichloromethane (or methanol) through column into a 12x75 mm disposable glass tube using slight air pressure applied to top of SPE. Evaporate eluate under nitrogen in an approximately 50 °C water bath to dryness, then resuspend residue in 0.5-1 mL methanol, 1:1 methanol:water, water, or other solvent as appropriate for the colors and instrument in question. Vortex eluate for 30 sec and sonicate for 5 sec, if necessary to completely dissolve dried material. Pass 0.5 mL through a 0.2 micron syringe filter (PVDF for dichloromethane, PTFE for methanol) prior to injection on UPLC.

Short summary of Method 5.2 (See text for details and additional notes)

Sample Prep:

- Weigh 3-5 g product into a 50 mL tube
- Add 10 mL hexane
- Vortex ~10 sec
- Incubate tube at ~50 °C for 5-10 min
- Sonicate tube at room temp for 5-10 min
- Vortex tube for 5-10 min
- Centrifuge at ~6-10k rpm at 5 °C for 5 min

CBX Column:

- Wet CBX column with 5 mL hexane (keep column wet)
- Apply 5 mL of sample supernatant
- Rinse column with 5 mL hexane
- Elute with 4-5 mL dichloromethane into glass test tube

Prep for UPLC injection:

- Take eluate(s) to dryness under nitrogen at ~50 °C
- Resuspend with 0.5 to 1 mL methanol or other solvent; Vortex.
- Syringe filter with 0.2 um PTFE (or PVDF, depending on solvent) into UPLC vial

6. Cosmetics

Cosmetic products, having a wide variety of allowed color additives, can be particularly challenging matrices for analysis. Current laboratory practice utilizes a complicated scheme of analysis based on product type and material, usually involving multiple rounds of TLC and other cleanup procedures (14). Given the diverse nature of cosmetics currently on the market and their complex manufacturing processes, these methods often are partially or wholly unsuccessful. The methods that follow are more efficient, simpler alternatives for cosmetic analysis.

6.1. Powder-based products, lipsticks (including lip crayons), and non-lipophilic cosmetics (Method 6.1)

Cosmetics often have multiple color additives, and since many have hydrophilic and lipophilic ingredients, they can contain both water/methanol-soluble and oil-soluble color additives. Method 6.1 is intended to extract methanol-soluble color additives from eye shadows, lipsticks, highlighters, mascaras, and many other cosmetic matrices that contain some hydrophilic ingredients (for strictly oil-soluble matrices, see Sections 6.2 and 6.3).

Sample/Control preparation: Weigh approximately 0.2 g product (0.1-0.5 g depending on color intensity) into a 15 mL disposable polypropylene tube. For samples that contain several similar products such as eye shadows, combine approximately 0.1 g of each color. Add 4-5 ml 1% HCl in methanol as the extraction solution. Vortex or vigorously shake tube for 0.5-1 min, followed by 10 min of sonication (if necessary). Sample may be incubated in an approximately 50 °C water bath for 5-10 min to enhance color extraction. Centrifuge the tubes, typically at 6,000-10,000 rpm at 5 °C for 5 min.

CBX column wash/elution: After loading sample extract, wash SPE column with 3 mL methanol, followed by 3 mL distilled water, then another 3 mL methanol. Otherwise, follow CBX column wash method as described in Method 3.1.

Short summary of Method 6.1 (See text for details and additional notes)

Sample Prep:

- Weigh 0.1-0.5 g product into a 15 mL tube

- Add 4-5 mL 1% HCl in methanol
- Vortex 0.5-1 min
- Sonicate for 10 min (if necessary)
- Incubate tube at ~50 °C for 5-10 min, as needed
- Centrifuge at ~6-10k rpm at 5 °C for 5 min
  (Proceed as in Method 3.1)

6.2. Oil-based (lipophilic) products: Cosmetics such as hair products and lip glosses; Foods such as vegetable oil, etc. (Method 6.2)

Dyes used in oil-based hair and some lip gloss products are generally not soluble in water, methanol or acetonitrile. Instead, they dissolve most effectively in non-water-miscible hydrocarbons. For this reason, these products can often be extracted in hexane and applied to a Silica Gel (SiOH) SPE column. After drying, if the residue is not soluble in methanol, the extract can be re-dissolved in dimethyl sulfoxide (DMSO) and methanol.

Sample/Control preparation: Weigh approximately 0.5 to 3 g product[1] (depending on color intensity) into a 15 mL disposable polypropylene tube. Add 5-10 mL hexane and mix by inverting the tube multiple times. If necessary to extract product color, incubate tube in an approximately 50 °C water bath for 5-10 min, then vortex for 0.5 min. If there is a visible precipitate, centrifuge tube at approx. 6,000-10,000 rpm at 5-10 °C for 5 min.

SiOH column prep/loading: Prepare a SiOH SPE column on a vacuum manifold by passing 5 mL hexane through column. Do not allow the column to become dry. Apply 2-5 mL sample extract to column and allow to pass through column at approximately 1 mL/min.

SiOH column wash/elution: Wash SiOH SPE column with 4 mL hexane, followed by another 4 mL hexane. Use vacuum to remove remaining solvent from column prior to elution. Remove column from vacuum manifold and elute color by passing 4-5 mL acetonitrile[2] through column into a 12x75 mm disposable glass tube using slight air pressure applied to top of SPE. Evaporate eluate under nitrogen in an approximately 50 °C water bath to dryness.

Attempt to resuspend the product residue in 0.5 mL methanol.[3] If sample becomes a viscous residue, re-dry the sample and suspend the resulting residue in 0.5 mL DMSO:methanol (5:2)[4]. Pass solution through a 0.2 micron PVDF syringe filter prior to injection on UPLC. If sample is dissolved in DMSO:methanol, keep instrument auto sampler temperature above 18.5 °C (the freezing point for DMSO).

Short summary of Method 6.2 (See text for details and additional notes)

Sample Prep:

- Weigh 0.5-3 g product into a 15 mL tube
- Add 5-10 mL hexane; Mix by inversion
- Incubate tube at ~50 °C for 5-10 min
- Vortex tube for ~0.5 min
- Centrifuge at ~6-10k rpm at 5-10 °C for 5 min (if necessary)

SiOH Column:

- Wet SiOH column with 5 mL hexane (keep column wet)
- Apply 2-5 mL of sample supernatant
- Rinse column with 4 mL hexane; Repeat

- Elute with 4-5 mL acetonitrile into glass test tube

Prep for UPLC injection:

- Take eluate(s) to dryness under nitrogen at ~50 °C
- Resuspend with 0.5 to 1 mL methanol or other solvent
- Syringe filter with 0.2 um PTFE (or PVDF, depending on solvent) into UPLC vial
- Raise injection temperature to 20 °C if DMSO:methanol is used

6.2.1. Notes on Method 6.2

[1](Sample size) Oils and other liquids will typically require 1-3 g (though this can be increased for faintly colored oils), while lip glosses may require only 0.5 g.

[2](Elution) If acetonitrile is not effective at eluting the retained colors from the SPE, may need to substitute methylene chloride or another solvent.

[3](Resuspension solvent) As described in Section 9.2.2, early eluters such as Yellow 5 can show a split peak when injected in methanol on some injectors. For water-soluble colors, this problem can be avoided by using water or 50% methanol as the resuspension solvent. However, many oil-soluble dyes are not soluble in water, though they behave in methanol. Thus, water may not be an option, and split peaks will need to be noted (compare with concurrently run standard) or run in a second injection in water to confirm their identities.

[4](Dye solubility) If extracted colors are not soluble in the DMSO-methanol mix, they may represent oil-soluble colors that will only be dissolved by hexane, methylene chloride, THF, DMF, etc. These color additives can be analyzed on a UV-Vis spectrophotometer to determine their spectral profile, or possibly on an HPLC/UPLC set up for normal phase chromatography, but this is outside the scope of the method(s) described here.

6.3. Solid oil-based (lipophilic) products: Pomades, waxes, etc. (Method 6.3)

A pomade is a greasy or waxy substance that is used to style hair, making it look slick and shiny. Pomades of the eighteenth and nineteenth centuries consisted mainly of bear fat or lard (15). Lanolin, beeswax, and petroleum jelly are typically used in the manufacture of modern pomades (16). Because of the dense, grease-based nature of the product, initial chemical extraction includes heat as a means of dispersing the product into solvent.

Sample/Control preparation: Weigh approximately 1-3 g product (depending on coloring intensity) product into a 15 mL disposable polypropylene tube. Centrifuge tube briefly (~1 min at 1,000-5,000 rpm at room temperature) to draw product to base of tube. Place tube in water bath at approximately 50 °C for 1 min. Add 5-10 mL hexane and mix by inverting the tube multiple times and/or vortex for 1 min to incorporate product into the hexane. Return tube to heated water bath for 2 min, then repeat inversion/vortex step. Repeat heating and mixing steps until product is well dispersed in the hexane. Centrifuge sample at approx. 6,000-10,000 rpm at room temperature for 5 min, then return tube to the heated water bath until ready to load onto SPE column.

SiOH column prep/loading: Prepare a SiOH SPE column on a vacuum manifold by passing 5 mL hexane (one column volume) through column. Do not allow the column to become dry. Apply 2-4 mL sample extract (based on color intensity) to column and allow it to pass through the SiOH column at approximately 1 mL/min. Perform column wash/elution as in Method 6.2.

Short summary of Method 6.3 (See text for details and additional notes)

Sample Prep:

- Weigh 1-3 g product into a 15 mL tube
- Centrifuge tube briefly (~1 min) at 1-5 krpm at room temp
- Incubate tube at ~50 °C for 1 min
- Add 5-10 mL hexane; Invert or vortex to mix
- Incubate tube at ~50 °C for 2 min; Invert or vortex to mix
- Repeat heating steps until product well-dispersed into hexane
- Centrifuge at ~6-10k rpm at room temp for 5 min
- Return tube to water bath until ready to load onto SPE

SiOH Column:

- Wet SiOH column with 5 mL hexane (keep column wet)
- Apply 2-4 mL of sample supernatant
- Rinse column with 4 mL hexane; Repeat
- Elute with 4-5 mL acetonitrile into glass test tube
  (Proceed as in Method 6.2)

7. Exceptions and special considerations

The procedures described above have performed well for the vast majority of matrices tested. However, due to the nearly infinite number of possible product matrices, slight variations in these protocols may be necessary to optimize color additive extraction in certain cases. These variations and notes on specific dyes and product types are described in the sections that follow.

7.1. Products containing tightly-bound carmine

Imitation crab meat is a seafood product that is frequently analyzed for carmine. Better known as surimi, it is composed of fish proteins (from fish such as walleye pollock), starch, sugar, sorbitol, egg whites and flavorings to improve and stabilize the products gel-like texture. Once formed into various shapes resembling crab legs, crab cakes, etc., the product is often externally dusted with color additives such as Carmine, Paprika, and Annatto. Due to the proteinaceous nature of this product, methanol alone does not effectively extract the carmine. Other food products such as taco mixes may also contain carmine that does not readily extract into solution. For these products, follow Method 5.1, but extract with 15-20 mL 10% HCl in methanol. Use of a Tekmar Tissumizer (or similar homogenizer) for 30 sec may also help disperse the product into the solution and allow exposure of the colors to the solvent. Incubate the mixture in an approx. 50 °C water bath for 10 min, followed by another 1-5 min of vigorous shaking using a Vortex mixer. If effective, the supernatant should be visibly pink. Centrifuge the mixture to avoid clogging the SPE columns, and proceed as in Method 5.1. The majority of the Carmine (which will likely convert to Carminic acid) and the Annatto pigments will retain on the HLB SPE column, and can be more efficiently eluted with 10% ammoniated methanol than straight methanol alone.

7.2. Bar soaps

Making soap from fat with lye involves the process of saponification, which often produces a product that is basic in pH due to remaining alkali. While soap pH values range widely depending on brand and ingredients, the majority have a pH of 9-10 (19). Though soaps are solids, the extraction solvent must not contain water, to avoid the formation of excessive bubbles. Thus, Method 3.1 can be applied (with no vortexing), treating it similarly to a liquid soap. However, if the extracted bar soap's (or liquid soap's) supernatant is alkaline, the majority of water-based dyes will not retain on the CBX SPE.

To compensate for the pH variations in (primarily bar) soaps (Dove® is pH 6, whereas Dial® is 10), dilute a 2 mL portion of the methanol extract with 4 mL 1% HCl in methanol prior to application onto the CBX column. Note, the supernatant may have to be warmed briefly in an approx. 50 °C water bath prior to dilution). For liquid soaps or shampoos with dyes that do not appear to retain on the CBX SPE, try extracting with 1% HCl (or higher % acid, depending on how alkaline the extract is) in methanol and reapplying to the CBX column.

7.3. Hair conditioners

As opposed to typical hair shampoos, which usually contain non-ionic or anionic detergents, conditioners (which are usually acidic in pH overall), often contain cationic surfactants. These can interfere with the binding of Red 40, Blue 1, and other common dyes to the CBX column, possibly by filling the active sites so that the colors pass right through. Conditioners can also form thick emulsions in the presence of methanol, which can typically be broken up using 2 g of product with 3-4 mL of THF (Tetrahydrofuran) as extractant. Once the emulsion is no longer visible (becomes translucent), apply the colored portion to the acidified CBX column and continue as in Method 3.1.

7.4. Alkaline foods

Similar to the issues with bar soaps, some food products, such as juices and bean products, can be alkaline in nature. Extraction with neutral methanol can lead to the dyes not retaining at all on the CBX SPE. In these cases, switch to 1% HCl in methanol as the extraction solvent and proceed as in Method 3.1.

7.5. Foods and cosmetics with waxy coatings

Several types of cosmetic products (i.e. lip balms), and some foods (i.e. candies), have a waxy coating covering water-soluble colors. Use of Methods 4.1, 5.1, or 6.1, with neutral or acidic methanol as the extraction solvent, may not extract the dyes effectively until the coating is removed. A pre-treatment of the sample portion with 5 mL hexane or dichloromethane (DCM), followed by centrifugation and removal of the solvent, will then allow those methods to work as expected. This is also a way to screen for oil-soluble dyes in the products. Keep in mind, however, that some permitted water-soluble dyes are slightly soluble in hexane and/or DCM, even though their appearance may change slightly in hexane. For instance, D&C Red No. 27 is somewhat soluble in DCM, but it will appear orange rather than pink. (In contrast, D&C Red No. 28 is not soluble in DCM.)

7.6. Dye modification and degradation

Some color additives are known to undergo chemical modification and/or degradation under certain conditions, such as heat, high/low pH conditions, etc. For instance, a product that contains carmine may only appear to have carminic acid, as likely it has been converted to the acidic form during extraction and processing. Similarly, D&C Red Nos. 6 and 7 can undergo acid-catalyzed degradation into a product with a different absorbance maximum (~505 nm vs. 493 nm for the standard in methanol). Thus, products (usually cosmetics) that declare these color additives can be extracted using neutral methanol instead of (or in addition to) acidified methanol as described in Method 6.1.

Yellow 5 has also been observed to produce a degradant under acidic conditions or in the presence of some (undetermined) matrix components. The degradant was observed to have an absorbance maximum of approx. 418 nm versus the 427 nm peak normally seen for this standard (in methanol).

Similarly, some dyes (such as Blue 1) can undergo reversible chemical changes when heated in the presence of 10% or greater hydrochloric acid. The standard methods described above, involving the drying of eluates in 10% ammonium hydroxide, have not been observed to chemically change any of the typically found synthetic dyes (except for Red 6 and Red 7). If an acidic elution is necessary, consider neutralizing or alkalizing the eluate prior to drying down on the Nitrogen-evaporator to prevent such effects.

Non-color additives in foods and cosmetics may also cause chemical modifications to color additives in certain conditions. For instance, products with high levels of sulfites as preservatives may, in the presence of water and heat, cause sulfonation of certain dyes such as FD&C Red No. 40 and FD&C Yellow No. 6. When possible, the use of matrix-matched controls and color additives/spikes that are suspected to be in the product should be used to ensure that any such effects can be noted.

7.7. Natural colors

Often natural colors are used to alter the appearance of products in lieu of or in addition to synthetic dyes. A number of natural alternatives are approved for use in food and cosmetic products. The most commonly used natural colors are Caramel color, carotenoids (carotenes and xanthophylls), flavonoids (including anthocyanins and anthoxanthins), chlorins (chlorophylls and pheophytins), and quinones (including anthroquinones and naphthoquinones). Natural blue colors such as Spirulina (Phycocyanin) are also seeing expanded use in industry.

Many natural pigments are sensitive to heat and light, and they often do not bind to the CBX SPE column at all. In these cases, where only natural colors are anticipated, one can use Method 3.1, substituting acidified water (1% HCl in water instead of methanol) as the extraction solution, and load the extract directly onto an HLB SPE, bypassing the CBX column. This will likely result in a notable rise in the background peaks, but these should be easily identified as not having significant levels of absorbance in the visible region (400-700 nm).

Some natural colors, such as Caramel color, anthocyanins, and phycocyanin can be extracted and purified using Methods 3.1, and 4.1 on the HLB SPE, but will not form a well-behaved chromatographic peak with the LC method described here. In these cases, a simple UV-Visible spectrum can sometimes be obtained with a spectrophotometer. An alternate mobile phase and/or chromatographic column will be necessary, however, to further characterize these pigments. Note that the anthocyanins and phycocyanins will typically form tight bands on the CBX or HLB columns, but eluting them usually requires stronger eluents than those described in the method. Anthocyanins are often easy to identify, as they will usually appear red in color while in an acidic solution and will turn blue or purple in the presence of base.

Many other natural colors, including Astaxanthin and Canthaxanthin (xanthophylls), Carmine and Carminic acid, Curcumin (from turmeric), Crocetin and Crocin (from saffron), betalains (from beets), Lawsone (from henna) and others have been successfully extracted, purified, and analyzed by UPLC as described in the methods above. Most of these pigments also have commercial reference standards available for comparison. Often, however, naturally-obtained pigments will be observed as ‘families’ of closely related compounds in several chromatographic peaks.

The characterization of chlorophylls, chlorophyllins, and pheophytins, such as those that give color to many green fruits and vegetable such as olives, often require more nonpolar solvents for extraction (such as THF or DMF), and are outside the scope of this method. Similarly, other porphyrin-based dyes such as hemes in meats, require similar approaches. Alternate analyses have

been developed for the characterization of such complex pigment classes, and can be found in separate LIB publications.

7.8. Further analysis

In some cases, such as for co-eluting colors or those with closely-related spectra, additional information is needed to identify and/or discriminate the dyes in question. In these cases, a Waters UPLC-PDA coupled with a mass spectrometer (MS), such as the Waters QDa® UPLC-MS system or similar, is a relatively straightforward method of obtaining mass data to complement the retention time and UV-Vis spectral data that is obtained on the UPLC-PDA. The same mobile phases, gradient, and column can be used on these UPLC-MS systems, adding 0.1% Formic Acid to both mobile phases, resulting in reproducible data for the majority of commonly used dyes. A separate LIB is in preparation which describes the use of MS and MS/MS to characterize and confirm the identities of unidentified dyes that are observed in the analysis methods described here.

8. Examples and recovery information

The sections below present specific examples of products analyzed using these methods, and illustrate the advantages of the methods over traditional techniques such as TLC or standalone UV-Vis Spectrophotometry. These sections also provide some specific recovery information obtained during the validation process (see also Figure 45). Note also that the analytical process cannot determine whether a certified batch of color additive was used in a product. Thus, even when a color such as "Yellow 5" is described in the text as "detected", it is actually Tartrazine that is analytically confirmed, since the method is not able to tell if a certified batch of FD&C Yellow No. 5 was used.

8.1. Examples: Liquid matrices (foods & cosmetics)

Traditional methods of cosmetic analysis, including those used for liquid matrices such as shampoos and primarily water-based creams, are often frustrating and prone to failure due to matrix complexity and emulsion formation. Tables 2 and 3 below show example dye recoveries for creams and shampoo-type products using Method 3.1 described above. The dyes selected for fortification were some of those most commonly found in these types of products. Recoveries ranged from 88-121% for fortified levels at 5 and 11 mg/kg. For liquids spikes, added dyes remain in solution (versus dry products which tend to absorb the dyes used for fortification) which accounts for their generally higher recoveries.

Table 2. % Recovery (and %RSD) at 5 mg/kg level for each dye from matrix based on solvent standard curve for creams (N=6) and shampoos (N=7)

Color	Cream	Shampoo
FD&C Yellow No. 5	101 (7)	94 (5)
FD&C Red No. 4	107 (3)	98 (2)
FD&C Yellow No. 6	106 (2)	97 (3)
D&C Red No. 6	121 (3)	96 (4)
FD&C Red No. 40	109 (2)	98 (3)
D&C Red No. 28	102 (3)	99 (3)
FD&C Blue No. 1	104 (3)	99 (3)
Ponceau 4R	103 (3)	96 (3)
Brilliant Black BN	111 (2)	92 (7)
Acid Red 73	103 (2)	101 (3)

Table 3. % Recovery (and %RSD) at 11 mg/kg level for each dye from matrix based on solvent standard curve for creams (N=6) and shampoos (N=7)

Color	Cream	Shampoo
FD&C Yellow No. 5	86 (13)	83 (11)
FD&C Red No. 4	100 (2)	99 (10)
FD&C Yellow No. 6	103 (2)	98 (10)
D&C Red No. 6	103 (1)	105 (10)
FD&C Red No. 40	99 (3)	98 (11)
D&C Red No. 28	101 (1)	101 (10)
FD&C Blue No. 1	102 (1)	100 (11)
Ponceau 4R	91 (4)	91 (12)
Brilliant Black BN	88 (11)	87 (10)
Acid Red 73	98 (2)	98 (11)

8.1.1. Liquid body soap (negative control)

As an example, Figure 1 shows a chromatogram of a control liquid soap that had been extracted with Method 3.1, cleaned up on a CBX SPE column, and injected on the UPLC-PDA. No significant peaks were detected, and this material was used as a negative control. This same product was wet-spiked with nine dyes at 5-8 mg/kg and extracted/analyzed similarly. All of the fortified dyes retained and eluted as expected using the CBX SPE column. A chromatogram for the fortified soap sample is shown in Figure 2, showing the dye peaks and their retention times.

As discussed in section 7.2, if the extracted dyes do not retain on the CBX SPE, it is possible that the soap's extract is alkaline, and therefore acts similarly to the ammoniated methanol eluent, preventing the dyes from binding to the SPE. In this case, extract the liquid soap with 1% to 5% HCl in methanol and continue with Method 3.1.

In this example, the nine dyes used for fortification and their observed retention times were: FD&C Yellow No. 5 (1.578 min), Ponceau 4R (2.533 min), FD&C Yellow No. 6 (2.587 min), FD&C Red No. 40 (2.993 min), former External D&C Red No. 10 (3.959 min), FD&C Red No. 4 (4.214 min), FD&C Blue No. 1 (4.498 min), D&C Red No. 6 (4.778 min), and D&C Red No. 28 (5.911 min). Even though the mobile phase solutions are prepared identically each day, there can be slight differences (on the order of 0.01 min) in observed RT values from run to run. Furthermore, with over 100 dyes profiled in the library, retention time overlaps are possible and could potentially result in a misidentification. For this reason, relevant standards should be run concurrently with the samples after an initial 'screening' process is performed.

Figure 1. Control soap chromatogram (no significant peaks detected)

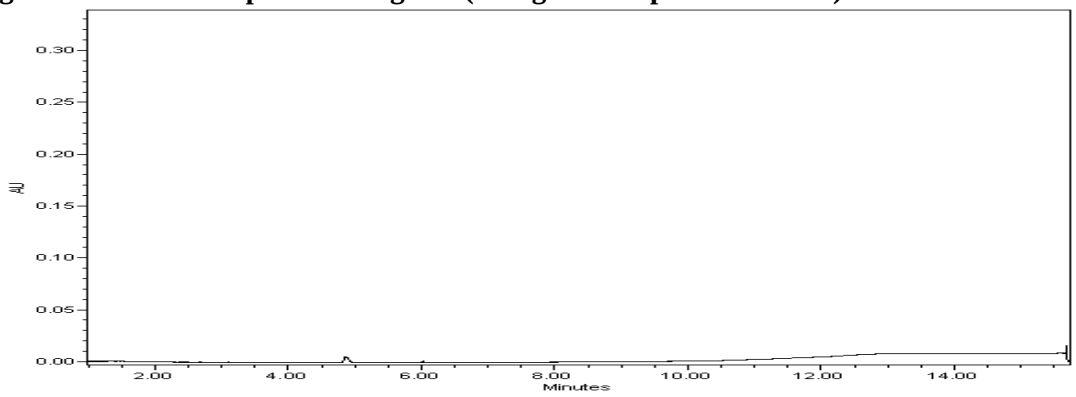
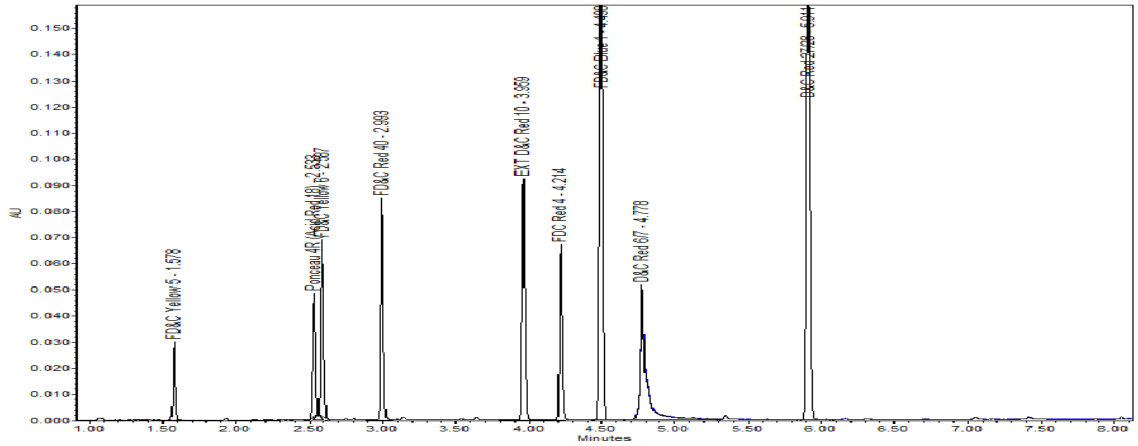


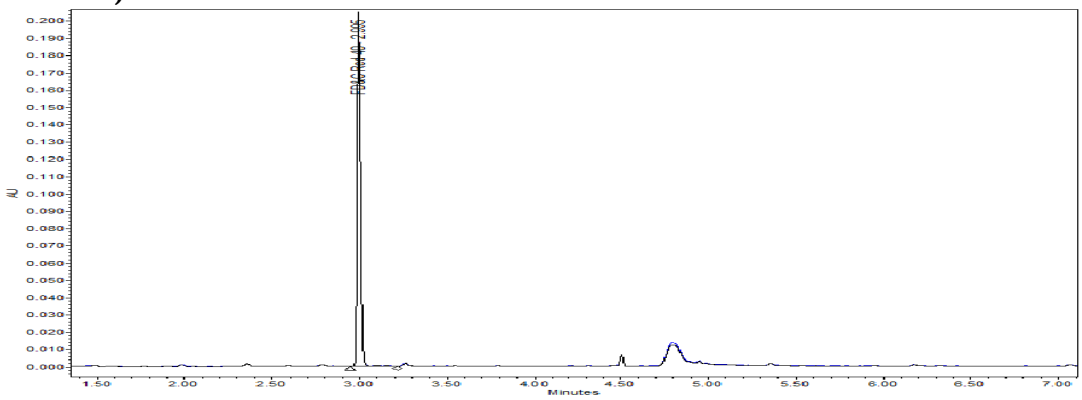
Figure 2. Soap spiked with dyes at 5-8 mg/kg. Elution order: Yellow 5 (1.578 min), Ponceau 4R (2.533 min), Yellow 6 (2.587 min), Red 40 (2.993 min), former External Red 10 (3.959 min), Red 4 (4.214 min), Blue 1 (4.498 min), Red 6 (4.778 min), and Red 27 (5.911 min).[1]



8.1.2. Pink body cream

Figure 3 displays an example chromatogram for a pink body cream product that did not declare the presence of any dyes. Analysis using Method 3.1 and CBX SPE column cleanup, followed by UPLC-PDA injection, indicated the presence of FD&C Red No. 40, based on a match with the RT and UV-Visible spectrum with the spectral library and concurrently run reference standards.

Figure 3. UPLC chromatogram for a pink body cream with no declared color additives. Labeled peak is Red 40 at 2.995 min RT. Unlabeled peaks did not absorb in the visible region (400-700 nm).



8.1.3. Shampoo with henna and walnut

Although most of the commonly used dyes retain well on the CBX column, some colors in a product may pass right through the SPE. For this reason, the method makes use of the C_{18}-based reverse-phase (hydrophilic-lipophilic balanced) HLB SPE column, to capture and purify dyes that are not retained on the CBX column. While the less selective HLB column will have a greater number of peaks, filtering out the non-colored components is fairly straightforward.

[1]*Note: Due to software limitations, the chromatograms and spectra presented here are limited in visual quality. Peak identities and retention times are given in the figure captions when relevant. Unlabeled peaks represent uncolored co-extractants.*

For example, a shampoo "with Henna and Walnut" was received for analysis which declared HC Yellow 4, HC Blue 2, Disperse Black 9, Disperse Violet 1, FD&C Yellow No. 5, FD&C Blue No. 1, and FD&C Red No. 40 on its product label. Therefore, both the CBX SPE (to capture the FD&C dyes) and the HLB SPE (to capture any unretained dyes, such as HC Blue 2 and Yellow 4) were required. The resulting chromatograms are shown in Figures 4 and 5, respectively. In Figure 4, only two dyes were collected on the CBX SPE: Red 40 and External Violet 2, whose spectra matched their respective reference standards. Figure 5 displays the HLB SPE extract which displayed a number of large peaks. Only two of the peaks in the chromatogram had UV-Vis spectra that absorbed in the visible range (approx. 400-700 nm), greatly simplifying the analysis. The spectra obtained for these two peaks overlaid with the reference standard spectra for HC Blue 2 and HC Yellow 4, respectively. In this case, External Violet 2 was not declared on the label. The Disperse dyes that were declared, as well as Yellow 5 and Blue 1, were not detected in the product.

Note: HC Blue No. 2 is one of the dyes that is sensitive to pH. In acid, it changes to yellow and reverts back to blue in base. For products with dyes such as this, extract in 1% HCl in methanol and apply a portion of the extract onto the CBX SPE. While the dye will not retain on the CBX, it will retain on the HLB SPE (in this case, the CBX SPE is just a cleanup step). Elute the HLB SPE with 10% ammoniated methanol (to convert HC Blue 2 back to the blue form) rather than neutral methanol, and continue with the UPLC analysis. As previously mentioned, extracts must be acidic (or neutral) prior to application onto the CBX SPE for color additive retention.

Figure 4. CBX extract of "Shampoo with Henna and Walnut" product displayed two peaks (at 3.030 min and 6.406 min) with spectra in the visible range. The peaks were identified as Red 40 and External Violet 2, respectively.

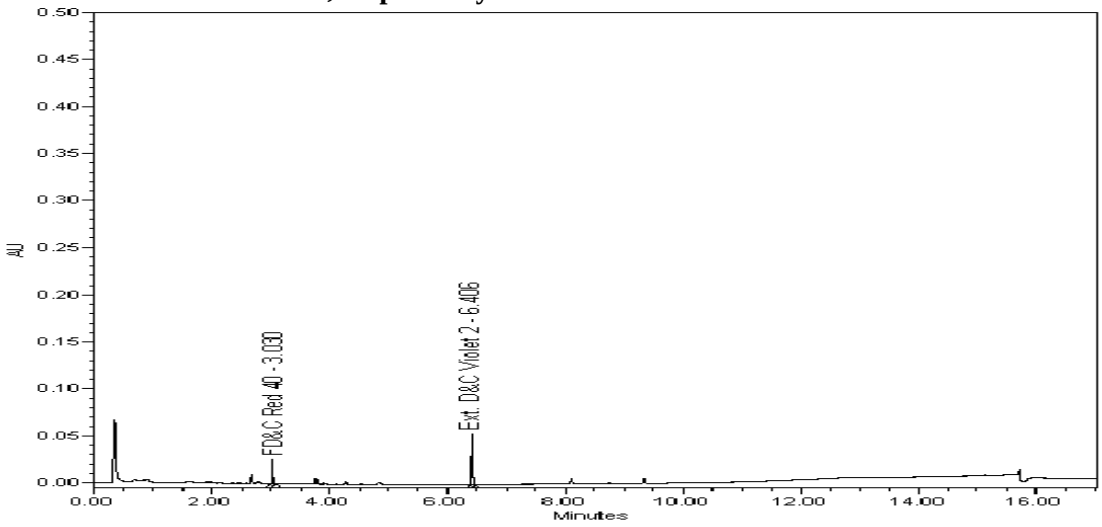
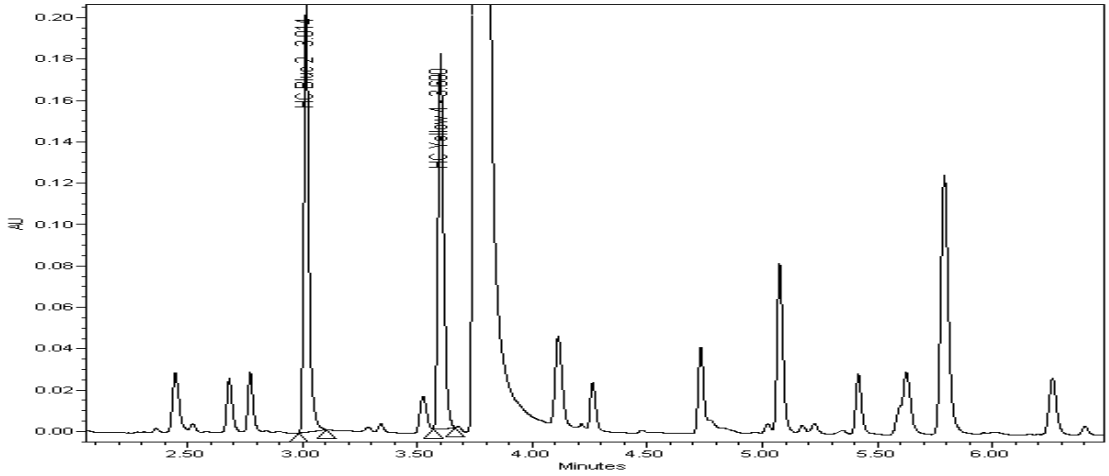


Figure 5. HLB extract of "Shampoo with Henna and Walnut" product displayed two peaks (at 3.014 min and 3.600 min) with spectra in the visible range (400-700 nm). The peaks were identified as HC Blue 2 and HC Yellow 4, respectively.



8.1.4. Purple-colored cosmetic

Another example product analyzed with this method was a purple-colored, alcohol-based cosmetic product that was analyzed by LIB 3420 but produced non-definitive results by TLC and UV-Vis spectrophotometry. Using Colors Manual 2016 - Method 3.1, a portion of the sample was diluted in methanol and applied to a CBX column. The purple dye was not retained on the CBX SPE. The 'pass-through' eluate was collected, diluted and applied to an HLB SPE column, which retained the dye. The HLB SPE column was washed as described in the method and the dye was eluted from the SPE with methanol. The eluate was dried and the residue was suspended in methanol, filtered, and injected onto the UPLC-PDA.

Three major peaks were observed in the visible range, at retention times (RT): 6.984, 7.509, and 7.997 minutes (Figure 6), and these produced the UV-Vis spectra shown in Figure 7. Comparison to the UPLC-PDA Color Library indicated a potential match of Methyl Violet 2B, which is often observed to be a mixture of polymethylated pararosaniline chloride dyes (12) that produces three observed peaks. The library reference spectra for Methyl Violet 2B is shown in Figure 8. The sample was then analyzed concurrently with the Methyl Violet 2B reference material and relevant controls to confirm its identity.

Because Methyl Violet 2B in solution is a mixture of three distinct dye molecules, neither TLC nor UV-Vis spectrophotometric-based methods were capable of correctly identifying this dye unless the exact reference dye was known and used for comparison. Additionally, traditional analysis had suggested similarity in color and TLC migration properties with D&C Violet 2 and External Violet 2, but could not produce a definitive identification. Without a declaration of the specific dye used for a starting point, TLC-based methods would be inconclusive or could potentially misidentify the violative dye.

Figure 6. Diagnostic analysis by UPLC found Methyl Violet 2B in the product based on spectral comparison of the three peaks at 6.984 min (a), 7.509 min (b), and 7.997 min (c) to the three peaks of the Methyl Violet 2B reference standard.

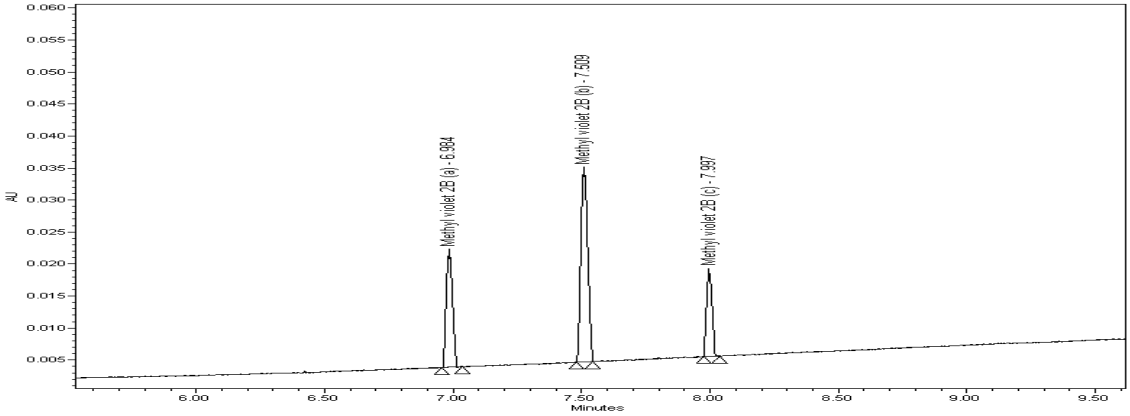


Figure 7. Sample peak spectra for three peaks (a, b, and c) with maxima wavelengths of 575.8 nm, 583.2 nm, 591.7 nm, respectively.

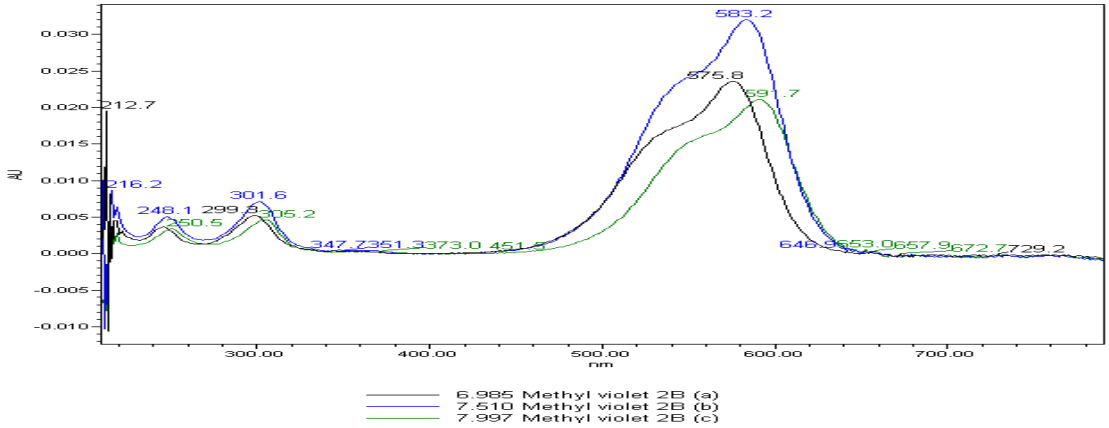
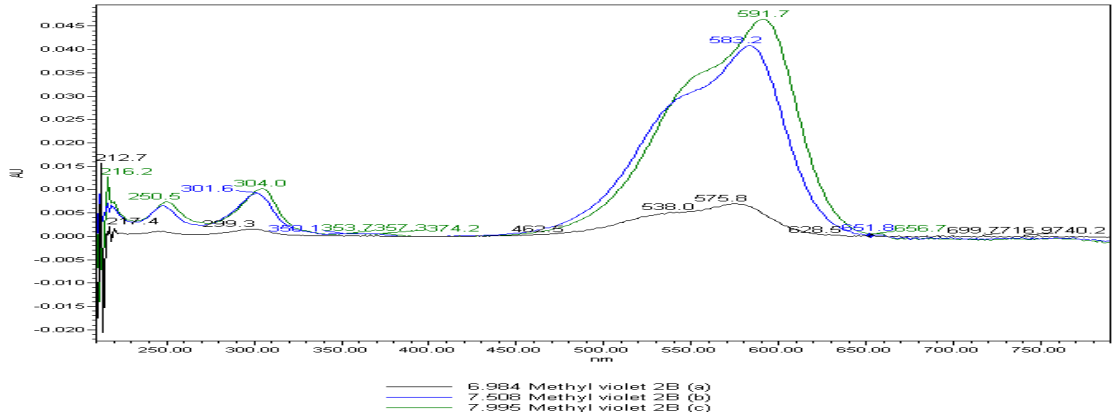


Figure 8. Three peaks for methyl violet 2B standard (a, b, and c) from the spectral library; Absorbance maxima for the peaks are 575.8 nm, 583.2 nm, 591.7 nm, respectively.



8.1.5. Liquid food matrices

Liquid food matrices analyzed with Method 3.1 produced similar recovery results to the liquid cosmetic products tested. Table 4 presents the recovery data for seven commonly found food dyes that were spiked into a control carbonated soda: Yellow 5, Yellow 6, Red 3, Red 40, Blue 1, Ponceau 4R (a.k.a. Cochineal Red A or C.I. Acid Red 18), and former External D&C Red No. 10 (Carmoisine/Azorubine). Recovery values ranged from 90-107% for fortification levels of 1 and 3 mg/kg. Chromatograms for control and fortified soda are shown in Figures 9 and 10, respectively.

Table 4. % Recovery (and %RSD) at 1-3 mg/kg level for each dye from matrix based on solvent standard curve for carbonated soda (N=6)

Color	Soda @ 1mg/kg	Soda @ 3mg/kg
FD&C Yellow No. 5	96 (7)	103 (3)
Ponceau 4R	95 (7)	106 (2)
FD&C Yellow No. 6	98 (5)	106 (1)
FD&C Red No. 40	96 (7)	107 (1)
former External Red 10	90 (14)	106 (1)
FD&C Blue No. 1	96 (6)	107 (1)
FD&C Red No. 3	91 (9)	104 (2)

Figure 9. UPLC chromatogram of soda negative control

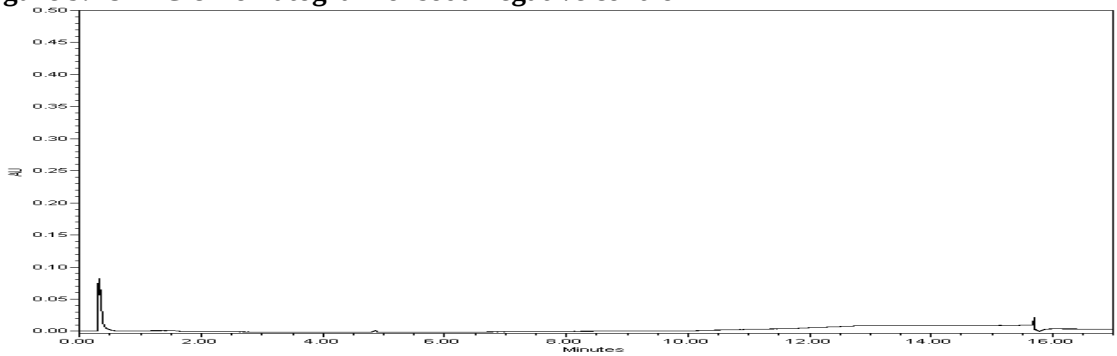
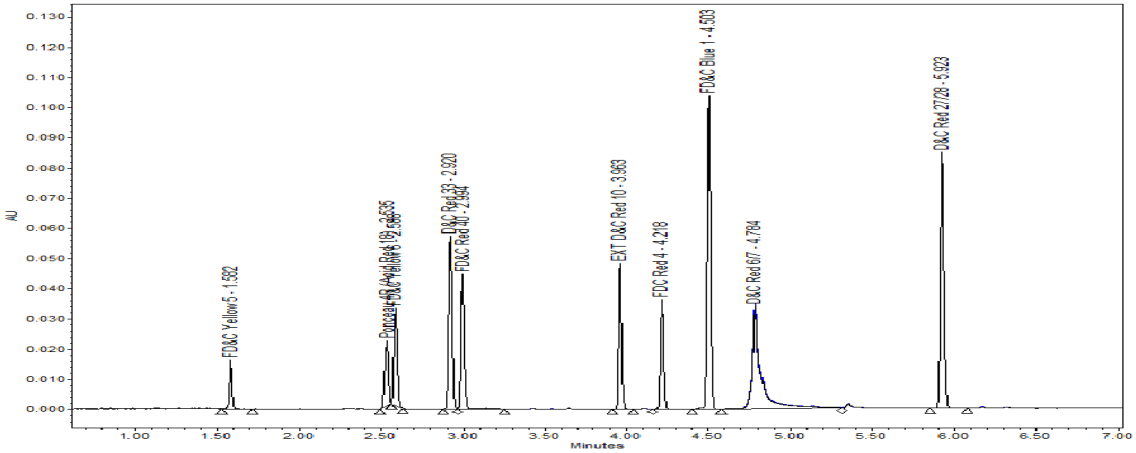


Figure 10. UPLC chromatogram of soda fortified with 10 dyes at approximately 1 mg/kg (7 of the 10 were used for recovery calculations). Color additive order and retention times are: Yellow 5 (1.582 min); Ponceau 4R (2.535 min); Yellow 6 (2.588 min); Red 33 (2.920 min); Red 40 (2.994 min); former External Red 10 (3.963 min); Red 4 (4.218 min); Blue 1 (4.503 min); Red 6 (4.784 min); and Red 27 (5.923 min).



8.2. Examples: Solid matrices (primarily foods)

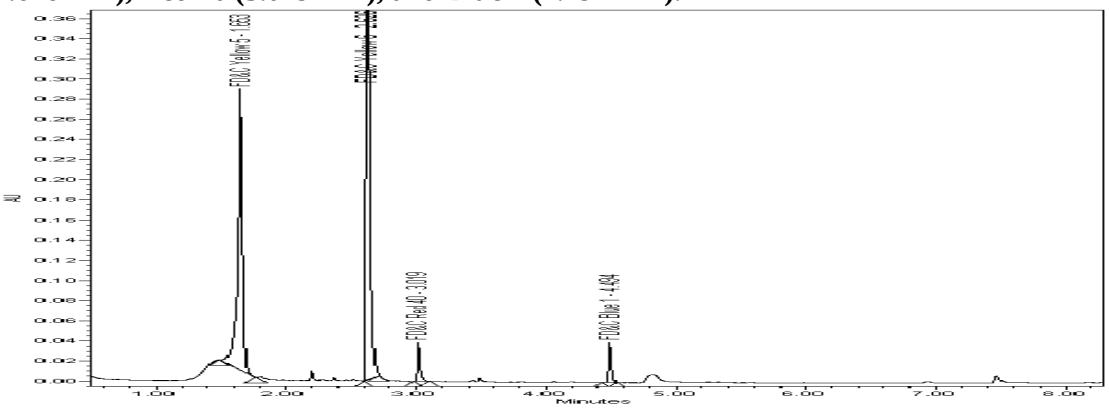
Traditional methods of color analysis on bread-type products such as cakes, cookies, and crackers often fail to extract sufficient amounts of certain dyes such as FD&C Yellow No. 5. Such samples were typically subjected to Nun's wool dying, a time-consuming, outdated color extraction method commonly used at the beginning of the 20th century. The Nun's wool extraction technique could be more effective than the aqueous acid digestion at extracting Yellow 5, but it did not improve the detection of low-level dyes. Dyes at low levels (Red 40, Yellow 6, etc.) in bread-type matrices were often missed completely, since they were not extracted in sufficient quantity to be visually detected on a TLC plate.

8.2.1. Potato chip with declared colors and lakes

Colors Manual 2016 Method 4.1 was used to analyze a potato chip product with high levels of fats. Homogenization using a Robot-Coupe or similar food processor is recommended for most dry, cracker-type products prior to analysis to ensure that small sample portions are representative of the sample as a whole. While most dry products produce a fine, evenly ground material, high-fat snacks produce an homogenate with a clumpy, greasy appearance, resulting in a layer of visible fat at the surface of the extract supernatant. This layer is not generally applied to the CBX column, using the translucent supernatant layer instead (which is usually where the dyes segregate to), as the lipid layer can reduce flow or clog the SPE column.

The label of the potato chip product declared four dyes: Yellow 5, Yellow 6, Red 40, and Blue 1, as well as their lake counterparts. In this case, Red 40 and Blue 1 would likely not have been identified using TLC due to their low concentrations in the product. Because of the higher sensitivity of the UPLC system, Method 4.1 readily detected all four dyes (See Figure 11). The lack of sufficient sensitivity for the TLC method was a persistent problem with the older methods, leading to false-negative conclusions that dyes listed on the labeling were not present in the product, based on TLC results alone.

Figure 11. UPLC Chromatogram of a potato chip sample which declared Yellow 5, Yellow 6, Red 40, and Blue 1 (both lake and straight dyes). Elution order: Yellow 5 (1.653 min), Yellow 6 (2.626 min), Red 40 (3.019 min), and Blue 1 (4.494 min).



8.2.2. Spiked cracker control

As mentioned above, dry products tend to resist complete dye extraction, and this effect tends to be greatest in bread and cracker-type matrices. To demonstrate the ability of Method 4.1 to resist these types of matrix interference, a cracker product was spiked with 10 commonly found dyes at an approximately 10 mg/kg level for each dye, and then it was analyzed using the method. Note that when spiking to make a positive control, the amounts of dye to use should be well above the

typical detection threshold (i.e. use 10 mg/kg or greater) to sufficiently fortify the product given its absorption tendencies, depending on the negative control matrix. Figures 12 and 13 show the resulting chromatograms from the analysis. As previously discussed, the dye Rhodamine B did not retain on the CBX column, but was collected and applied to an HLB SPE column as part of the clean-up procedure. In the HLB chromatogram, one also sees small amounts of some of the dyes that did retain on the CBX SPE. This is not unusual, for if the extract has large amounts of these dyes, one will sometimes see 'bleed through' due to column saturation. The HLB then gives a second peak that can be used for dye identification/confirmation.

The dyes (other than Rhodamine B) retained on the CBX column, and the resulting chromatogram is shown in Figure 12, in which the following colors were observed: Yellow 5 (1.580 min), Ponceau 4R (2.534 min), Yellow 6 (2.588 min), Red 40 (2.993 min), former External D&C Red No. 10 (3.962 min), Red 4 (4.217 min), Blue 1 (4.502 min), Red 6 (4.783 min), and Red 27 (5.923 min).

Figure 1. UPLC chromatogram of a color-fortified cracker sample (approx. 10 mg/kg levels) showing dyes that retained on the CBX SPE. Dye identities and elution order are given in text.

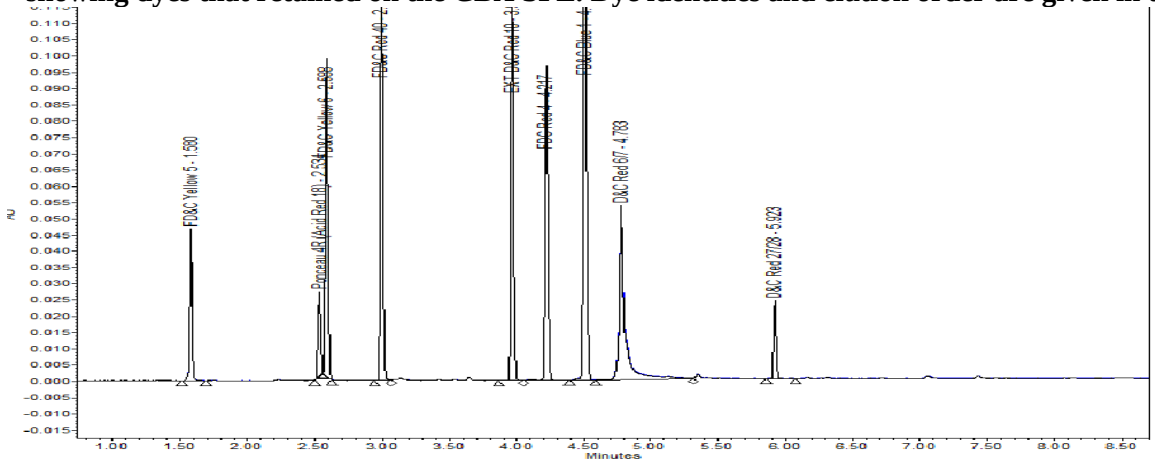
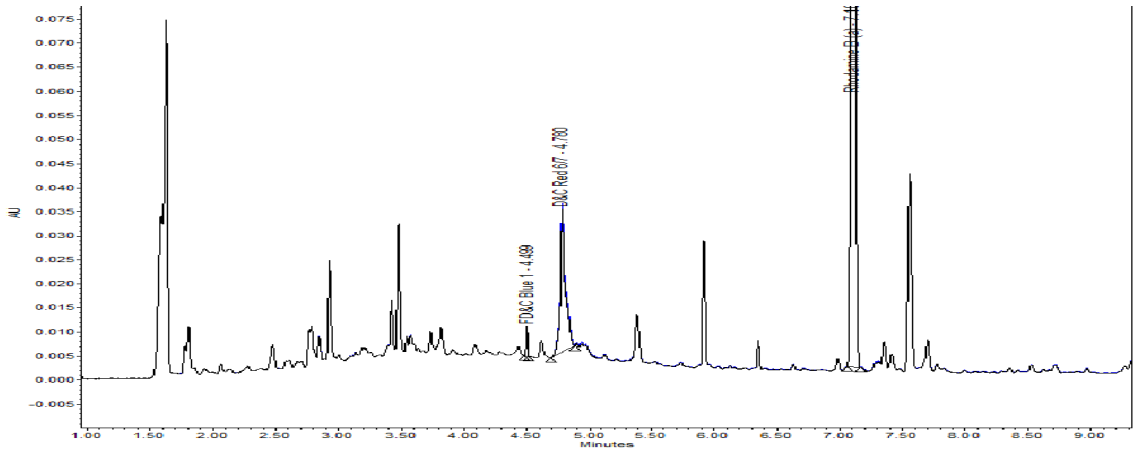


Figure 13. UPLC chromatogram of the HLB portion of a color-fortified cracker control containing a small amount of Blue 1 (4.499 min), Red 6 (4.780 min), and a large peak for Rhodamine B (7.105 min) which does not retain on the CBX SPE. Unlabeled peaks did not absorb in the visible region (400-700 nm).



8.2.3. Wafer-type products

Another example of the analysis of a solid, bread-type matrix was a wafer-type product received for color analysis that declared Yellow 5 and Blue 1, but no other colors. Upon extracting the product using Method 4.1 and applying to a CBX SPE column, several color bands were retained but one red dye was observed to pass through the column. The red eluate was collected and applied to an HLB SPE for cleanup as described in the method. The UPLC chromatogram and PDA spectrum confirmed its identity as the non-permitted color Rhodamine B (former D&C Red No. 19) (Figures 14 and 15).

The CBX-retained colors from the wafer produced a chromatogram with peaks corresponding to the declared Yellow 5 and Blue 1, and the non-permitted colors Ponceau 4R, and former Ext. D&C Red No. 10, as well as former FD&C Violet No. 1 (Figure 16). PDA spectral overlays of the Ponceau 4R and the violet dye peak in the product extract with reference standards are shown in Figure 17.

Figure 14. UPLC chromatogram of colors from a wafer sample extract which did not retain on the CBX SPE, but was further purified using an HLB SPE. The 7.137 min peak was identified as Rhodamine B based on comparison to the spectral reference library. Unlabeled peaks did not absorb in the visible region (400-700 nm).

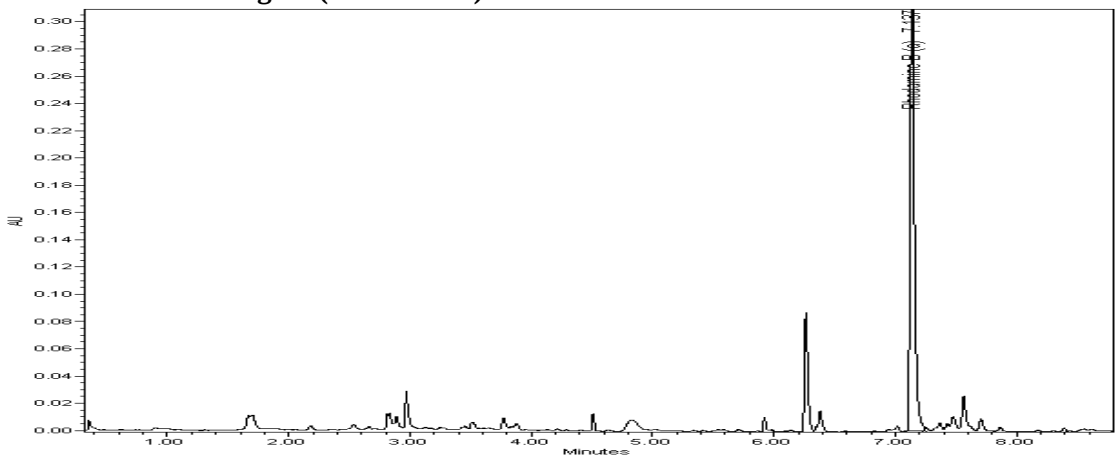


Figure 15. Overlay of UV-Visible spectra for the wafer extract peak at RT=7.137 min and a Rhodamine B reference standard peak

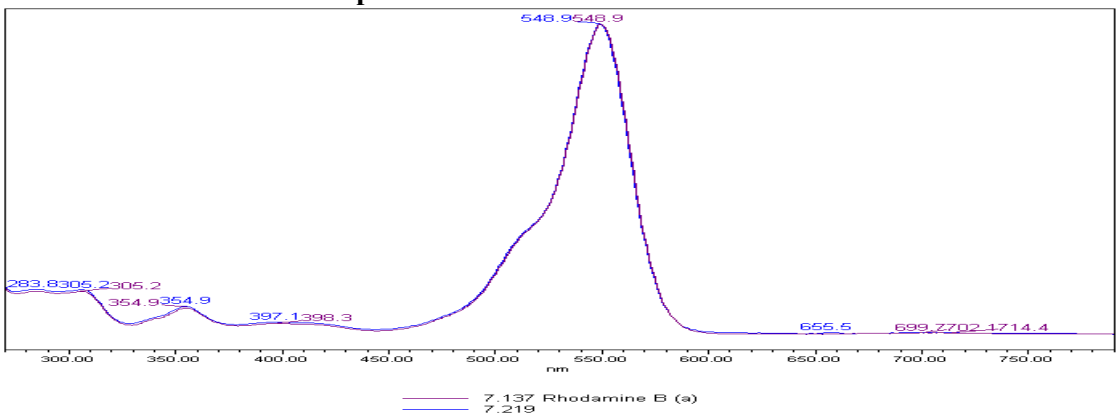


Figure 16. UPLC chromatogram of dyes extracted from wafers and retained on the CBX SPE. The dyes were positively identified by comparison to the visible spectra of reference standards as: Yellow 5 (1.659 min), Ponceau 4R (2.577 min), former External Red 10 (3.956 min), Blue 1 (4.510 min) and former Violet 1 (6.266 min).

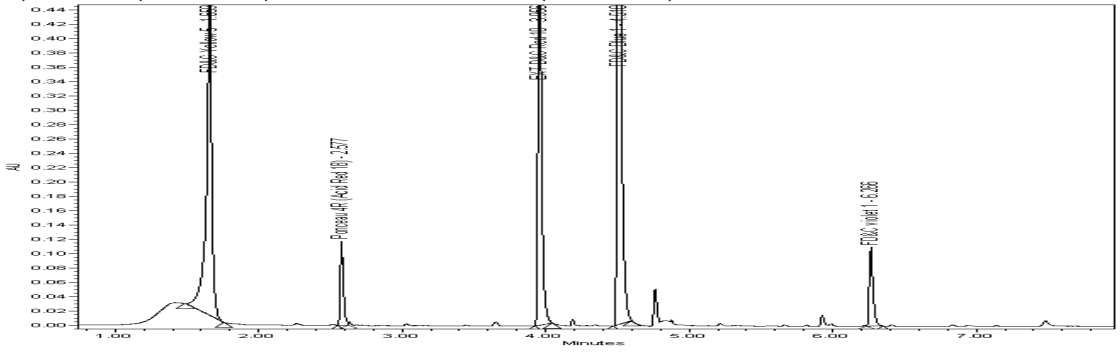
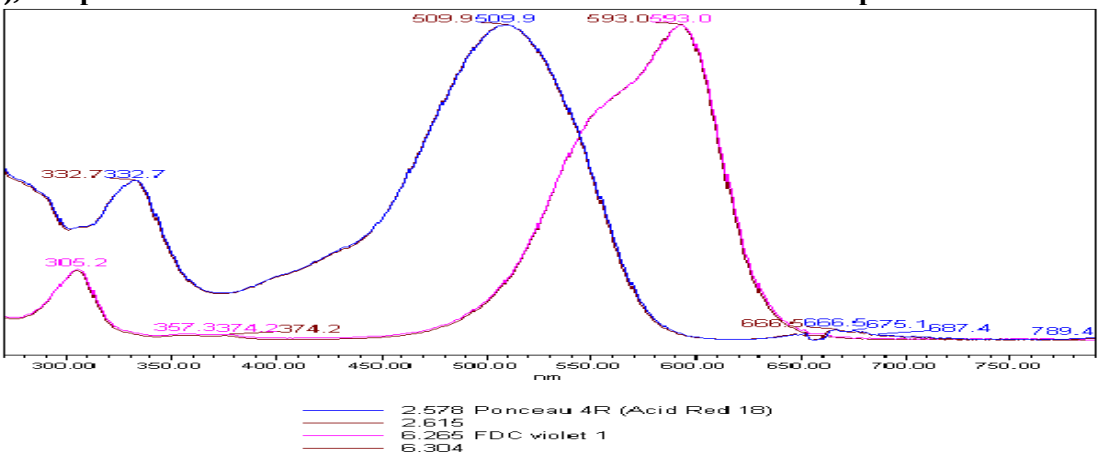


Figure 17. Overlay of UV-Vis spectra for product peaks identified as Ponceau 4R (2.577 min with 509.9 nm absorption max) and former Violet 1 (6.266 min with 593.0 nm absorption max), compared to Ponceau 4R and former Violet 1 reference standard spectra.



8.2.4. Almond paste product

An almond paste dessert was analyzed by TLC for colors. The product could be described as a marzipan "persimmon" in that it resembled a fruit: round and soft in shape, intensely orange in color, with a small greenish spot on one end. The acidified water extraction detailed in LIB 3420 produced one spot by TLC identified as Sunset Yellow FCF (certifiable as Yellow 6).

The product was analyzed in parallel using Method 4.1, with a portion of the product extracted using the methanol:acetonitrile:water:HCl mixture, followed by concentration of the dyes on the CBX SPE column prior to UPLC analysis. Using the PDA detector, peaks were detected and measured within the selected wavelength range (270-790 nm). The Waters Maxplot algorithm then displayed the UV-Visible spectrum for each compound at its highest absorbing wavelength. Each compound in the extract is displayed in the chromatogram, including impurities and peaks that do not absorb in the visible (400-700 nm) color range.

Several peaks showed absorbance in the visible range, at retention times (RT): 1.674, 2.654, 4.575, and 5.320 minutes (Figure 18), which matched the retention times of Yellow 5, Yellow 6, Blue 1 and Orange 4 reference standards (Figure 19), respectively, and were confirmed using spectral overlays with the UPLC-PDA Color Library.

Figure 18. UPLC chromatogram of marzipan product which contained multiple colors. Detected: Yellow 5 (1.674 min), Yellow 6 (2.654 min), Blue 1 (4.575 min), and Orange 4 (5.320 min). Unlabeled peaks have no visible absorbance.

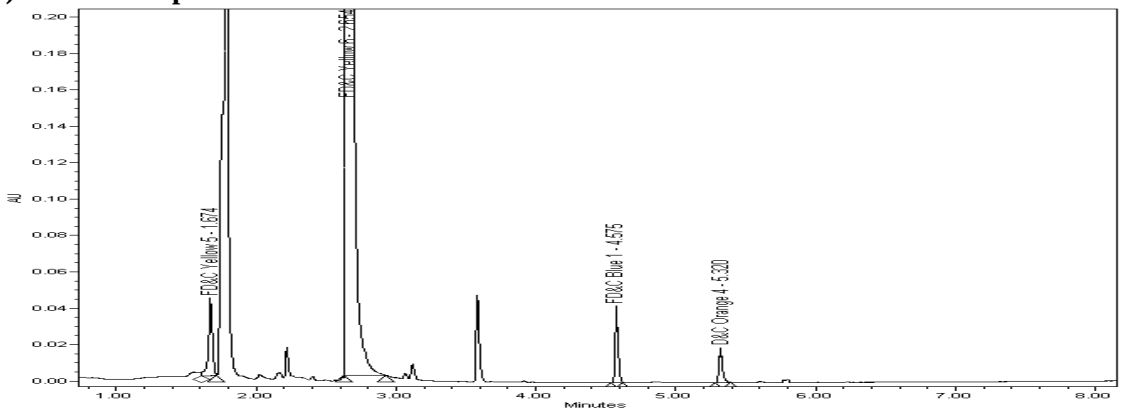
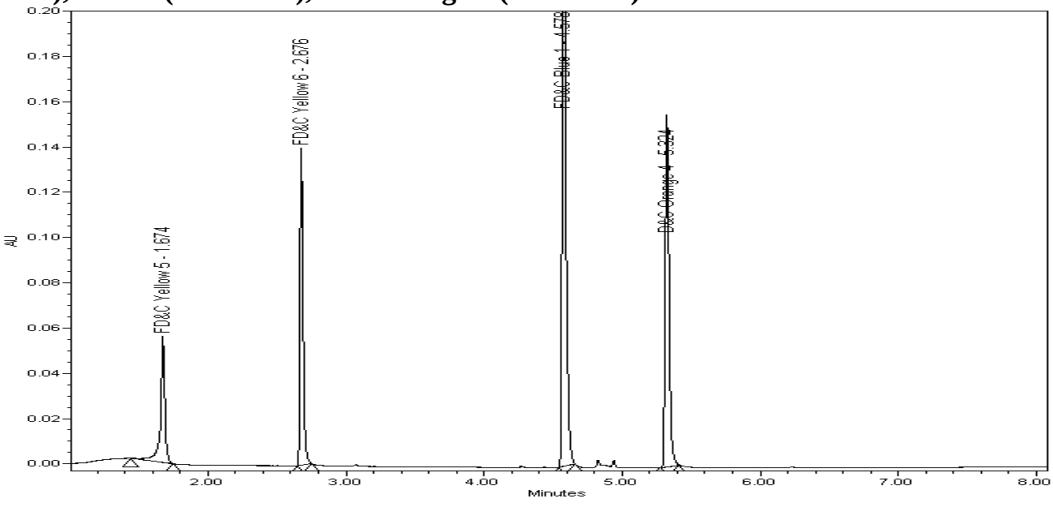


Figure 19. UPLC chromatogram of reference standard mix: Yellow 5 (1.674 min), Yellow 6 (2.676 min), Blue 1 (4.578 min), and Orange 4 (5.324 min).



8.3. Examples: Seafood and added colors

There are few traditional methods capable of detecting Carminic acid or carotenoid (natural or added) pigments in food matrices. Carminic acid is commonly added to seafood products to enhance or embue a pink color, such as coloring whitefish (i.e. pollock) to be sold as artificial crab meat. Xanthophylls (a class of carotenoid) such as Astaxanthin and Canthaxanthin are frequently added to feeds for farmed fish and crustaceans, but may also be added directly to the flesh to enhance coloring. Colors Manual 2016 has approaches for both types of seafood coloration.

8.3.1. Imitation crab with Carminic acid

The UPLC chromatograms for an imitation crab meat product analyzed by Method 5.1 (extracted with 1% hydrochloric acid in methanol), including the CBX and HLB portions, are displayed in Figures 20 and 21. Note: natural colors such as Annatto and Turmeric (Curcumin) respond similarly to Carmine/Carminic acid and will pass through the CBX onto the HLB SPE. However, as seen in Figure 20, a small amount of Carminic acid is often retained on the CBX. This product was found to contain both Carminic acid and Annatto (C.I. Natural Orange 4), as shown in Figure

21. The spectral comparisons of Carminic acid and Annatto in the product to the library reference standards are shown in Figure 22.

Figure 20. UPLC chromatogram of dye extracted (purified on a CBX SPE) from an imitation crab meat product identified by comparison to the visible spectra of reference standards as Carminic acid (1.792 min).

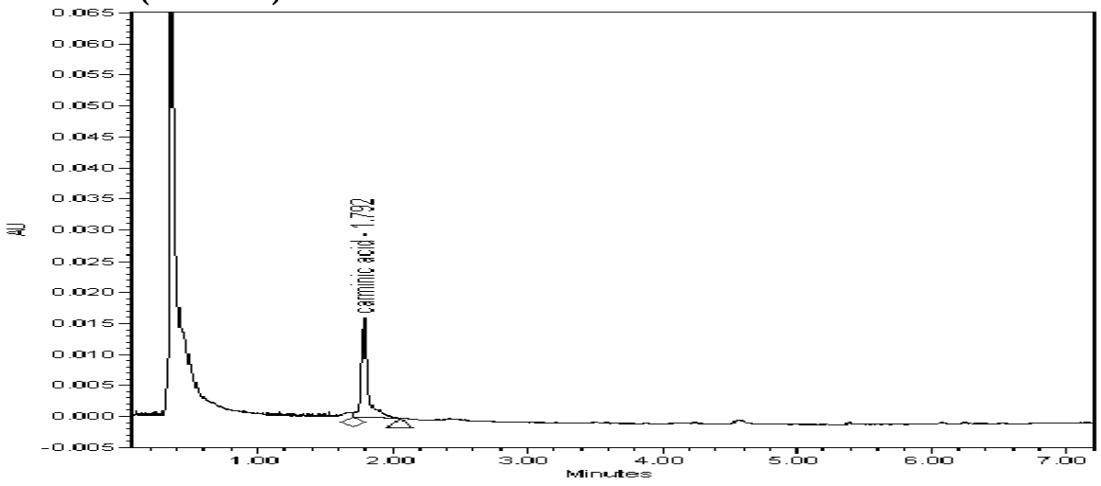


Figure 21. UPLC chromatogram for extract from imitation crab meat product which passed through the CBX SPE and was collected on an HLB SPE. The peak at 1.795 min was identified as Carminic acid and the peak at 6.146 min was identified as Annatto (C.I. Natural Orange 4) by comparison to reference library spectra. Unlabeled peaks did not absorb in the visible region (400-700 nm).

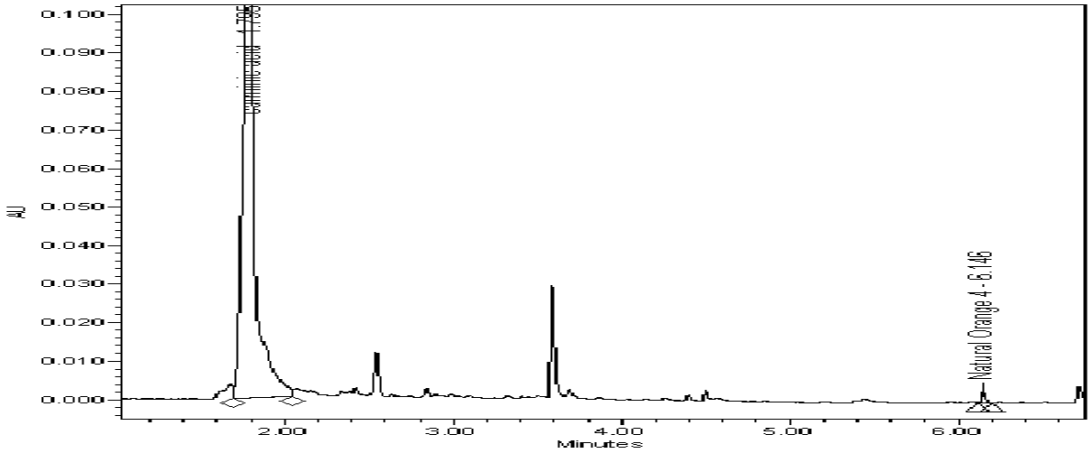
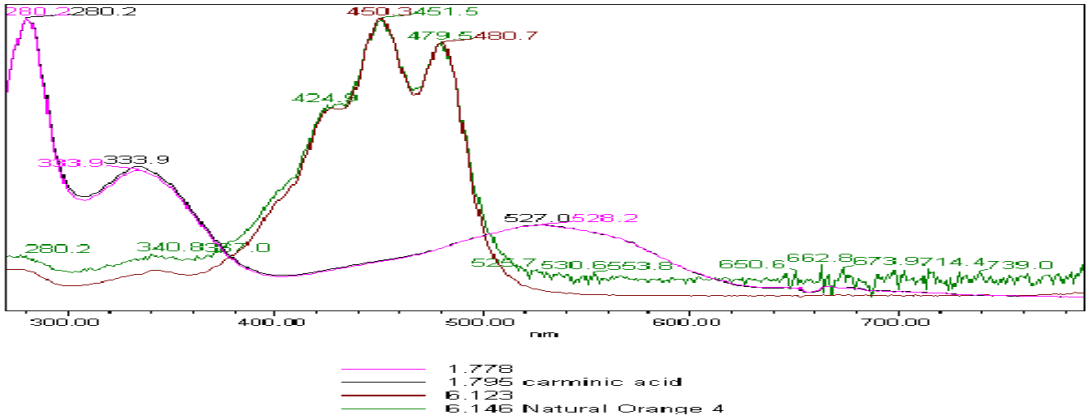


Figure 22. UPLC UV-Vis spectral comparison of reference standards for Carminic acid (visible absorbance max at 528.2 nm) and Annatto (absorbance max at 480.7 and 450.3 nm) and that obtained from the product peaks of interest (527.0, 479.5 and 451.5 nm, respectively).



8.3.2. Seafood and crossover products spiked with common dyes

Shrimp and fish are frequently analyzed for color additives, most often for red dyes. Method 5.1 is typically used for the analysis of red dyes such as Red 40 and Ponceau 4R (C.I. Acid Red 18) in these high-protein products. Although farm-raised salmon are often colored through the addition of carotenoids in their feed, other common food dyes have also been utilized. Shrimp samples have also been found to contain Red 40, and Zhou Qing, in his 2004 exposé on China's lack of safe food practices, reported that C.I. Acid Red 73 is used to dye shrimp during the cooking process (17). C.I. Acid Red 73, a water-soluble azo-type dye, is mainly metabolized in the intestinal lining and liver of humans, producing free aromatic amines that are potentially carcinogenic and mutagenic (18).

Three seafood matrices were spiked with dyes at different fortification levels, and Table 5 presents the recovery data for the control shrimp, tilapia, and salmon analyzed. Recovery values for shrimp and tilapia ranged from 79-109% for fortification levels of 2 and 8 mg/kg for the three dyes tested. Salmon proved more difficult since it tended to absorb the dyes, resulting in lower recoveries of 8-65% at 8 mg/kg. Ponceau 4R was the analyte most affected by the matrix components. Chromatograms for the control and fortified shrimp are shown in Figures 23 and 24, respectively.

Figure 25 shows a UPLC chromatogram of a soy herbal drink (also a 'crossover' product, analyzed by Method 5.1) that was found to contain Ponceau 4R and former External D&C Red No. 10.

Table 5. % Recovery (and %RSD) from aquaculture products based on the solvent standard curve (N=6)

Color	Shrimp @ 8mg/kg	Shrimp @ 2mg/kg	Tilapia @ 8mg/kg	Salmon @ 8mg/kg
Ponceau 4R	94 (3)	87 (3)	79 (2)	8 (2)
Red 40	100 (3)	92 (3)	94 (1)	65 (3)
C.I. Acid Red 73	109 (2)	92 (3)	99 (1)	41 (3)

Figure 23. UPLC chromatogram of shrimp negative control

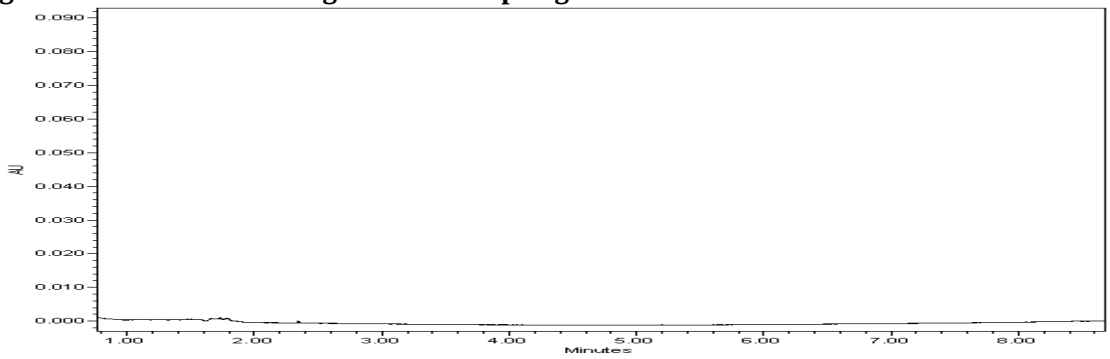


Figure 24. UPLC chromatogram of a shrimp control fortified with Ponceau 4R (2.647 min), Red 40 (3.097 min), and C.I. Acid Red 73 (5.185 min).

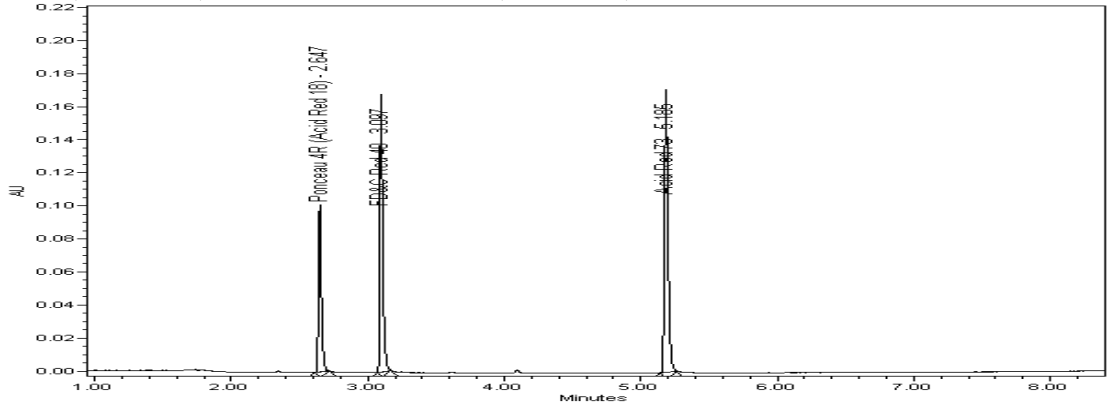
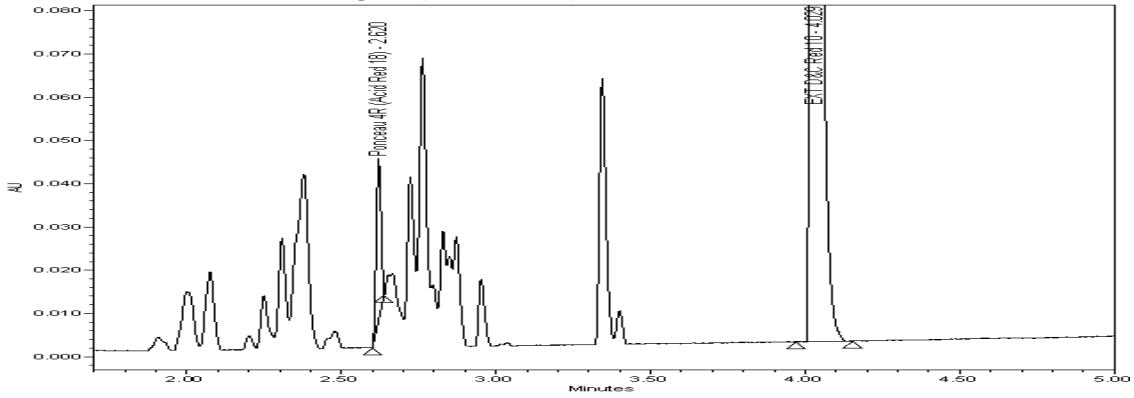


Figure 25. UPLC chromatogram of dyes detected in a soy herbal drink ('crossover' product), identified as Ponceau 4R (2.620 min) and former Ext. Red 10 (4.029 min). Unlabeled peaks did not absorb in the visible region (400-700 nm).



8.3.3. Seafood products spiked with carotenoids

In the case of farm-raised seafood, carotenoids such as Astaxanthin and Canthaxanthin are the most commonly used color additives, typically provided through feed. A control tilapia, shown not to contain any detectable colors, was spiked with these two carotenoids at approx. 1 and 2 mg/kg, then extracted them using Method 5.2. The resulting chromatogram is shown in Figure 26 and the UV-Visible spectra for these closely related carotenoid dyes are shown in Figure 27. A standard curve was created using reference standards for these dyes, and recovery levels were calculated as shown

in Tables 6 and 7. While observed recoveries were 75-78%, indicating some level of matrix interference, sufficient levels of these dyes were recovered using Method 5.2 to perform a positive identification from retention times and UV-Visible spectral matches. However, this analysis method is unable to distinguish naturally- and synthetically-produced Astaxanthin and Canthaxanthin.

Figure 26. UPLC chromatogram of fortified tilapia control at approximately 1 mg/kg for each standard: Astaxanthin peak is at 11.117 min and Canthaxanthin peak is at 12.068 min. Unlabeled peaks did not absorb in the visible region (400-700 nm).

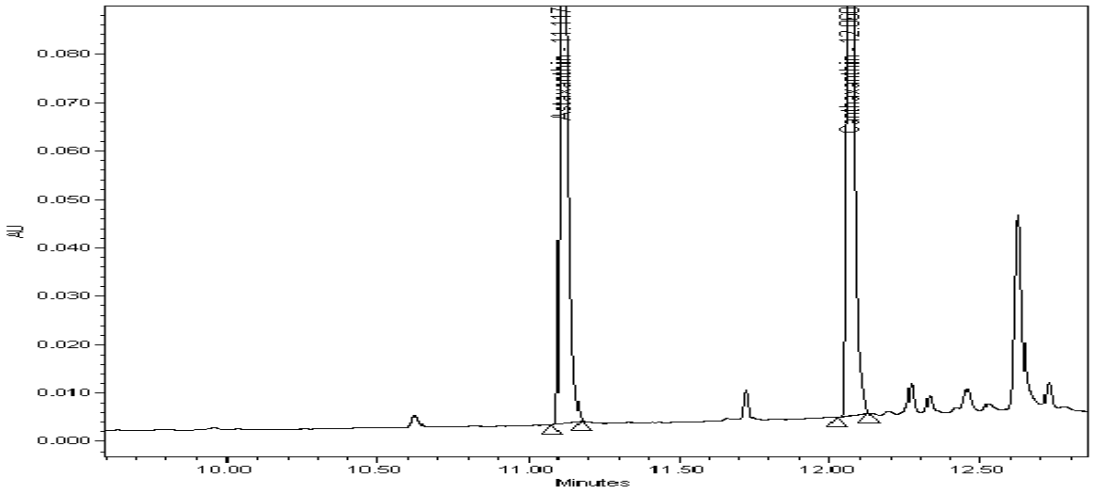


Figure 27. UV-Visible spectral scan for Astaxanthin and Canthaxanthin. Observed absorbance maxima for each dye was 478.3 nm for Astaxanthin and 475.8 nm for Canthaxanthin.

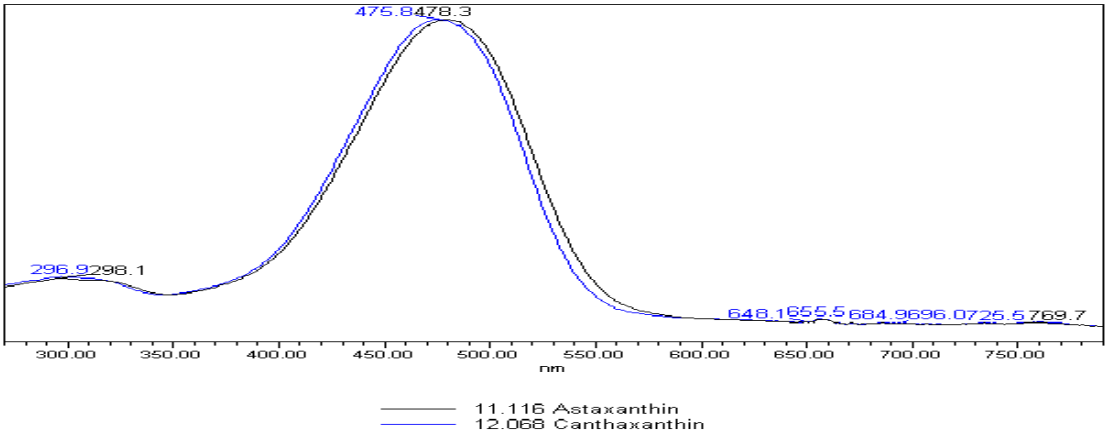


Table 6. Astaxanthin % recovery (and %RSD) with matrix-based standard curve. (N = 6 for each concentration).

Dye	1 mg/kg	2 mg/kg
Astaxanthin	78 (4)	77 (4)

Table 7. Canthaxanthin % recovery (and %RSD) with matrix-based standard curve. (N = 6 for each concentration).

Dye	1 mg/kg	2 mg/kg
Canthaxanthin	75 (2)	75 (6)

8.4. Examples: Cosmetic products

While some cosmetic products can be analyzed using the extraction simple liquids (Method 3.1), many cosmetics are complex mixtures which require additional clean-up steps and involve more unusual color additives. Often they contain mixtures of water and lipid-soluble dyes, and have matrix components that interfere with traditional analytical techniques.

8.4.1. Eye shadow palette

An eye shadow product, which declared Blue 1 and Red 40, was analyzed by Method 6.1. The water-soluble dyes are easily extracted with the 1% HCl/methanol solution. However, most of these cosmetic products contain iron oxide pigments that are not soluble in this solvent mix. This product palette contained one white and eight colored eye shadows. Portions of the eight colored eye shadows were combined into one extraction, in a simultaneous screening of all the product shades. The sensitivity of this method allows for such screenings, whereas older methods required more material from each to ensure that no colors were missed on the TLC plates. The white eye shadow was used as a control (after initially determining that no dyes were present) and was fortified with seven dyes. Figures 28-30 display the chromatograms of the control eye shadow, the fortified control, and the mixed color extract, respectively. The UPLC-PDA analysis identified Blue 1 in the product as well as Red 40 and Red 6 (a color not permitted for use in the eye area), which were confirmed by spectral comparisons to their respective library standards (Figure 31).

Figure 28. White eye shadow control which contained no undeclared dyes.

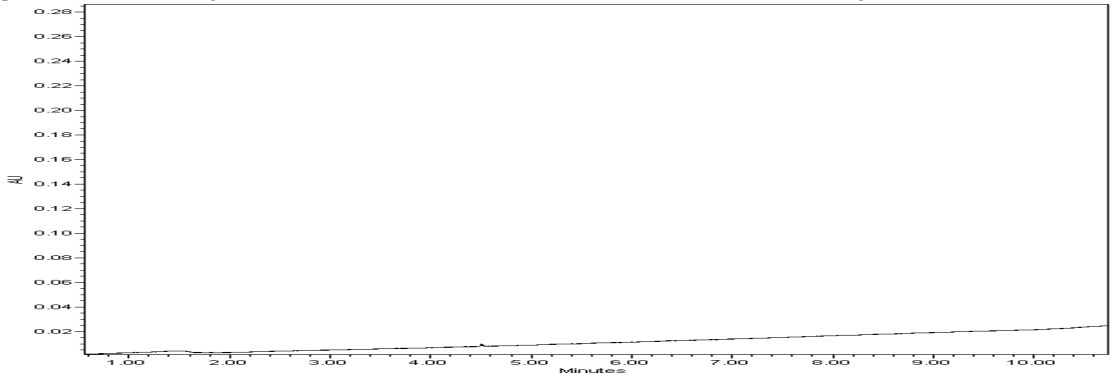


Figure 29. Control eye shadow product fortified with seven dyes: Yellow 5 (1.657 min), Ponceau 4R (2.581 min), Yellow 6 (2.635 min), Red 40 (3.024 min), Blue 1 (4.504 min), Red 6 (4.769 min), and Orange 4 (5.257 min).

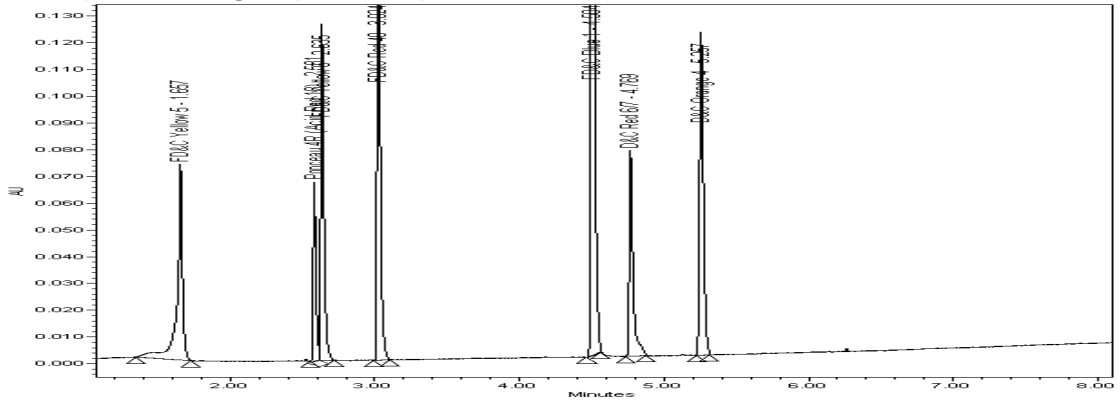


Figure 30. Eye shadow product (mixed colors) which declared Blue 1 and Red 40 aluminum lakes. Red 40 (3.026 min) and Blue 1 (4.510 min) were detected, as was Red 6 (4.767 min) (non-permitted), which was undeclared. Unlabeled peaks did not absorb in the visible region (400-700 nm).

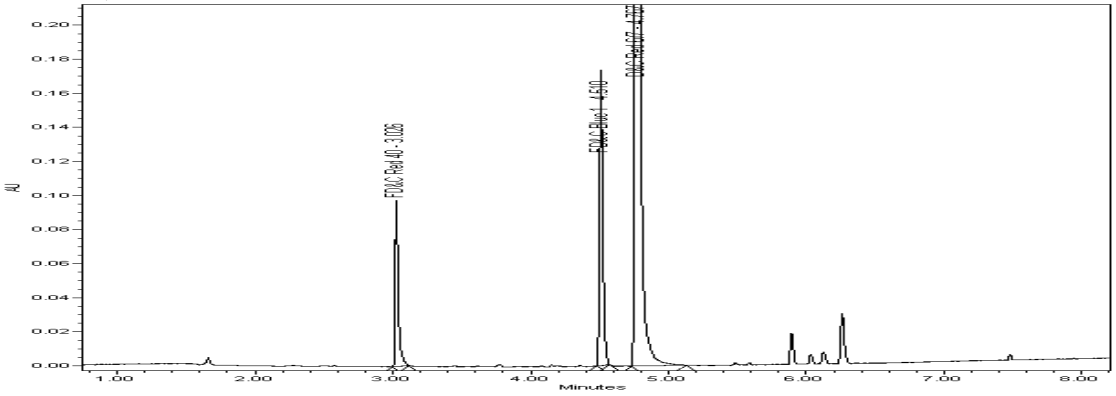
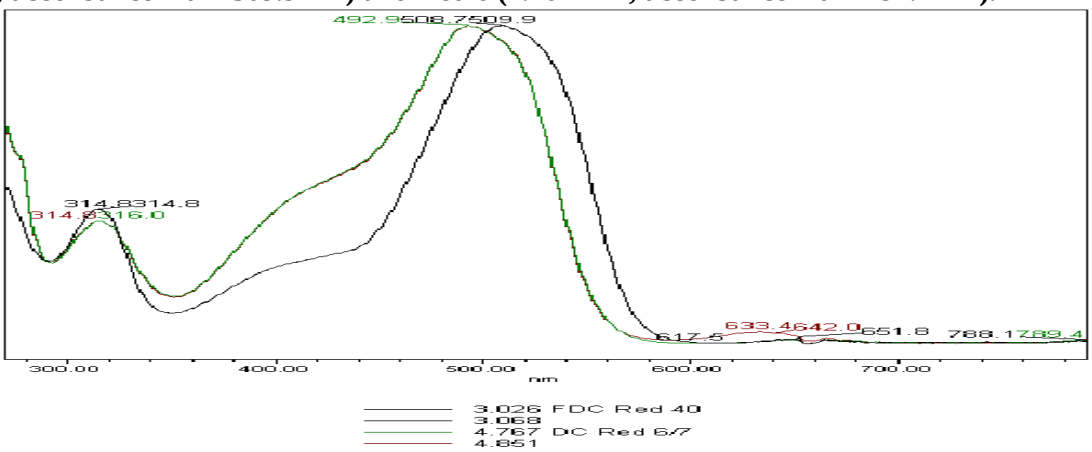


Figure 31. Spectral overlay of eye shadow peaks and reference standards for Red 40 (4.510 min, absorbance max=509.9 nm) and Red 6 (4.767 min, absorbance max=492.2 nm).



8.4.2. Green hair oil and mineral oil-based products

Method 6.2 was applied to a green hair oil product to be analyzed for color additives. The product's composition included 70% mineral oil and 27.5% arachis or peanut oil, with the remainder being perfume and olive oil. Traditional methods, following the Newburger's Manual scheme (14) were attempted but did not produce a suitable extract. The extract from Method 6.2 had a chromatogram that was significantly more complex than mineral oil or olive oil alone, but only two peaks absorbed in the visible region: RT=6.762 and 11.666 min (Figure 32). UV-Visible spectral comparisons identified these peaks as D&C Yellow No. 11 and D&C Green No. 6 (Figure 33). For this sample and the related controls, DMSO:methanol (5:2) was used to dissolve the extract residues prior to UPLC analysis.

Based on these findings, mineral oil was fortified with the two dyes at 0.7 mg/kg and 1.5 mg/kg and analyzed using Method 6.2. Mineral oil produced a far cleaner extract and the final residue was soluble in methanol, unlike the sample which was not methanol-soluble. Table 8 shows recoveries ranging from 92-115% for the two dyes examined.

A mineral oil-based lip gloss product, which did not declare any color additives on its label, was also analyzed using Method 6.2. Figure 34 shows a chromatogram of its extract, which was found to contain undeclared D&C Violet No. 2 and Sudan III, identified using PDA spectral matches with reference standards.

Figure 32. Chromatogram for an oil-based hair product. Two peaks displayed spectra in the visible range: RT=6.762 min and 11.666 min, identified as Yellow 11 and Green 6.

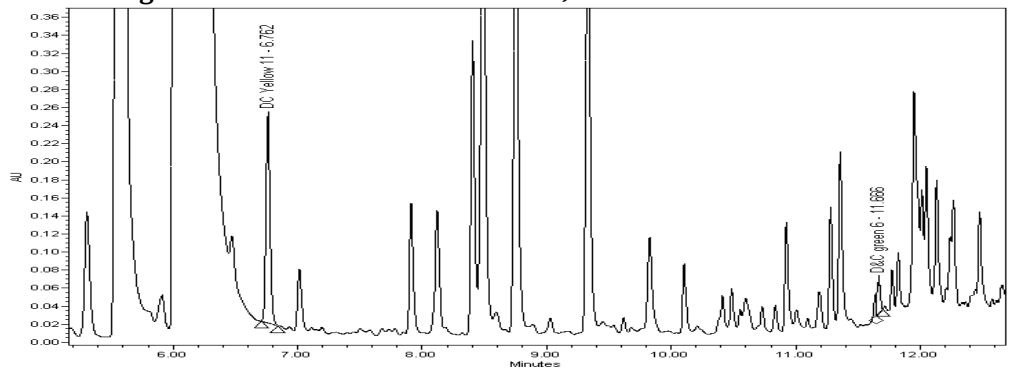


Figure 33. Overlay of product peak spectra and standard spectra. Yellow 11 (absorbance maxima at 410.4 nm and 427.3 nm); and Green 6 (abs. max at 635.9 nm and 404.3 nm).

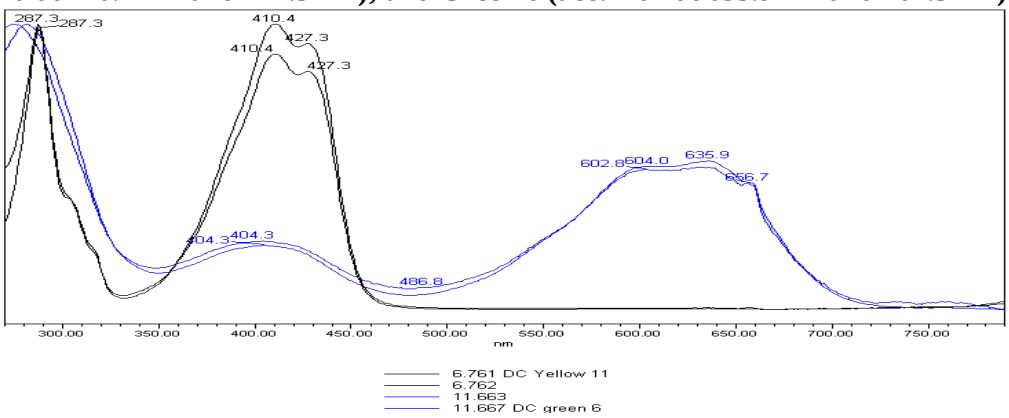


Figure 34. UPLC chromatogram of a mineral oil-based lip gloss product (no color additives declared on label), displays peaks corresponding to Violet 2 (10.313 min) and Sudan III (10.885 min). Spectral matches to reference standards were obtained. Unlabeled peaks did not absorb in the visible region (400-700 nm).

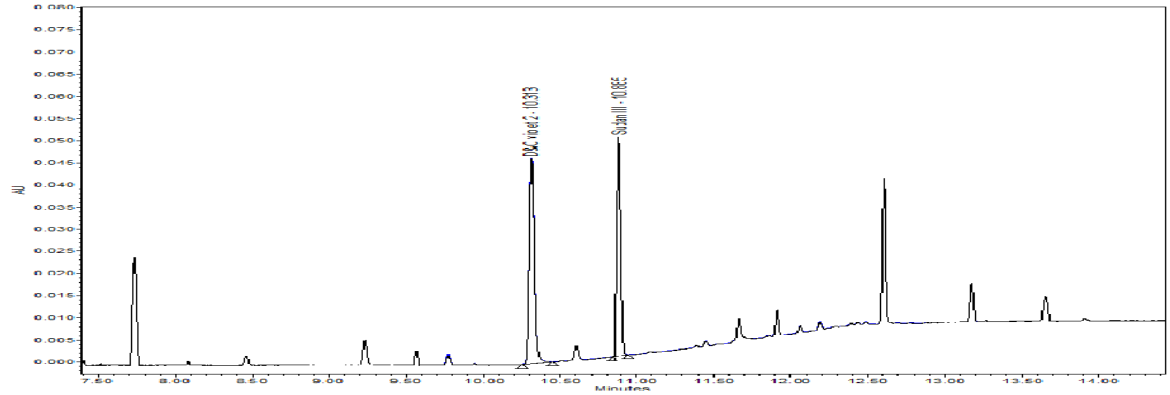


Table 8. % Recovery (and %RSD) calculations for color-fortified mineral oil based on the solvent standard curve (N = 6 for each concentration)

Dye	0.7 mg/kg	1.5 mg/kg
D&C Yellow No. 11	106 (2)	92 (12)
D&C Green No. 6	115 (3)	109 (10)

8.4.3. Pomade hair product

Pomade products, primarily petrolatum-based, are essentially oil-based products, but require heating prior to their extraction with hexane and application onto the SiOH SPE column (see Method 6.3). Figures 35 and 36 display UPLC chromatograms for a control Vaseline product, as-is and fortified with D&C Yellow No. 11, Butter Yellow, Sudan Blue II, Sudan III, and D&C Green No. 6, respectively. (Note: Some oil-soluble dyes, such as Butter Yellow and C.I. Solvent Blue 59 overlap in retention time, as do Sudan IV and D&C Green No. 6. Spectral comparisons are essential in differentiating these dyes.)

Two petrolatum-based hair products with no declared dyes were analyzed using Colors Manual Method 6.3. The first was a yellow-colored pomade and the second was a mango-colored hair gel-like product. The first product, shown in Figure 37, was found to contain Butter Yellow, and the second, displayed in Figure 38, was found to contain Yellow 11 and Sudan III, based on peak retention times and spectral matching to their respective library reference standards (Figure 39).

Figure 35. UPLC chromatogram of control Vaseline product.

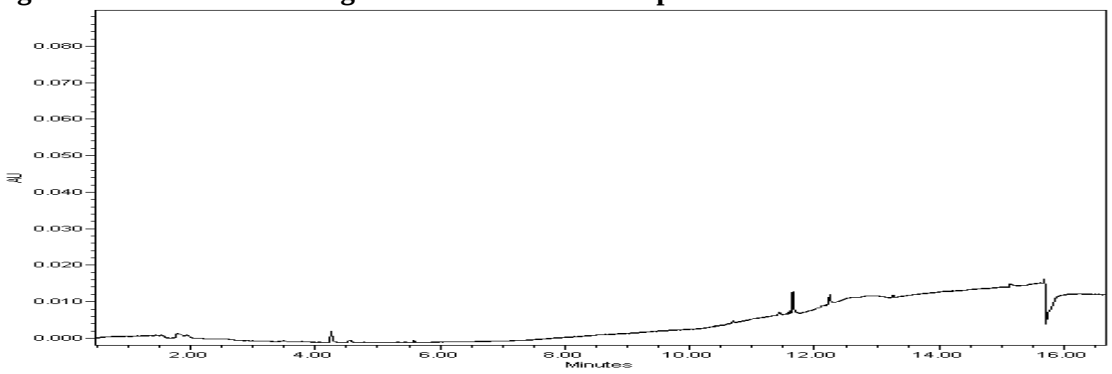


Figure 36. UPLC chromatogram showing fortified Vaseline control extract peaks corresponding to Yellow 11 (6.707 min), Butter Yellow (8.549 min), Sudan Blue II (10.671 min), Sudan III (10.888 min), and Green 6 (11.633 min).

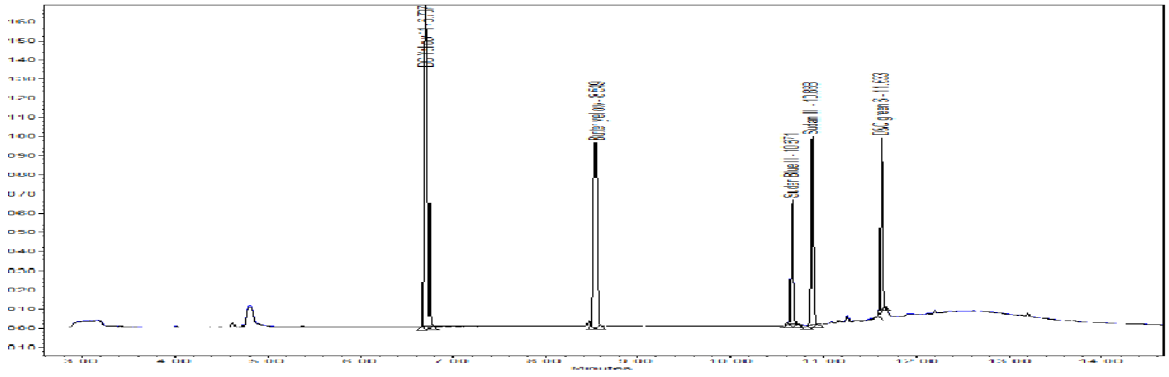


Figure 37. UPLC chromatogram obtained from a yellow pomade sample with no colors declared. Using Method 6.3, one peak was detected and identified, (retention time and spectrum match with the reference standard) as Butter Yellow (8.551 min). Unlabeled peaks did not absorb in the visible region (400-700 nm).

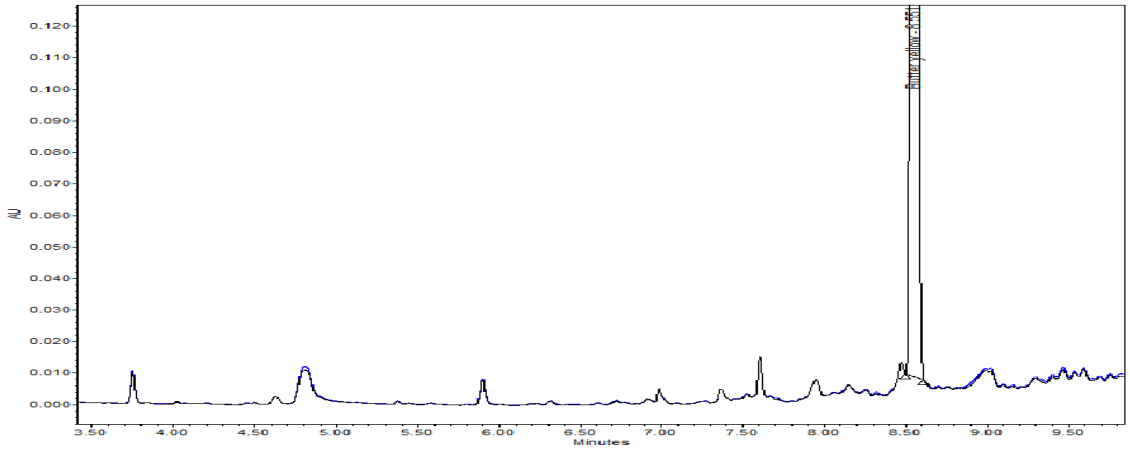


Figure 38. Mango-colored pomade hair product which declared no color additives. UPLC analysis detected both Yellow 11 (6.708 min) and Sudan III (10.888 min). Unlabeled peaks did not absorb in the visible region (400-700 nm).

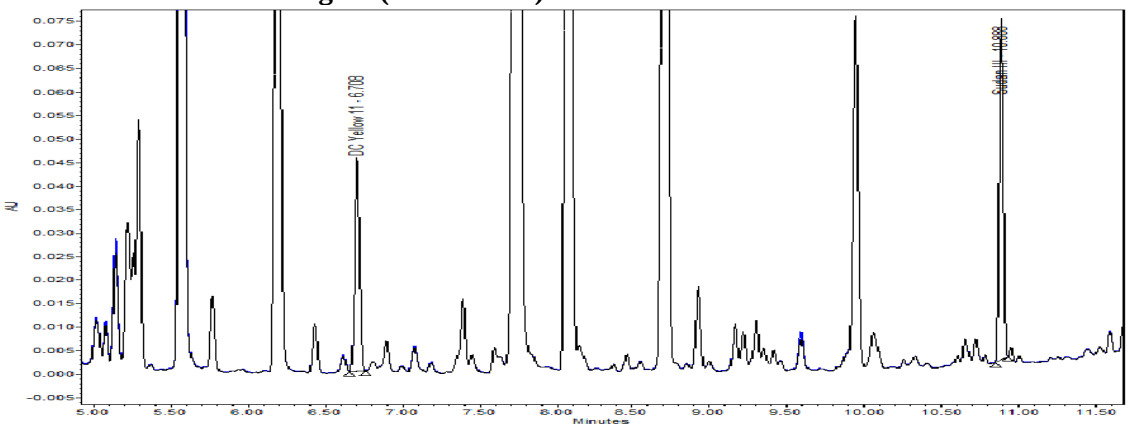
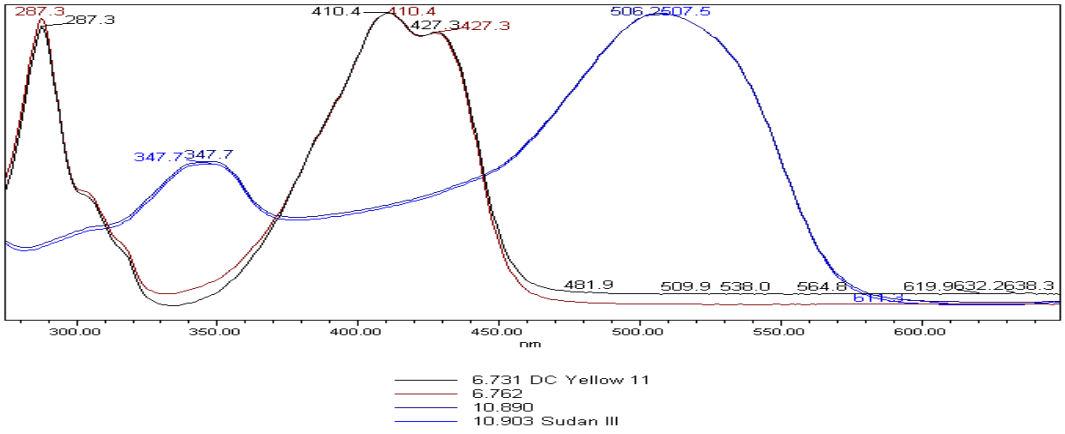


Figure 39. Overlay of product peak spectra and standard spectra. Yellow 11 (abs. max at 410.4 nm and 427.3 nm); Sudan III (abs. max at 507.5 nm and 347.7 nm).



8.5. Insoluble dyes and difficult matrices

During the analysis of various types of sample matrices, some dyes prove to be analytically difficult either because they do not extract sufficiently from the matrix or they are not retained on the CBX SPE column. For example, a toothpaste product declared D&C Red No. 30 and FD&C Blue No. 1, but produced one chromatographic peak when analyzed, corresponding to FD&C Blue No. 1. The Red 30 was not soluble in any of the extraction solvents attempted. Thus, it was nearly impossible to extract it from the product matrix in appreciable quantities for detection. D&C Green No. 8, which is sometimes used in combination with FD&C Green No. 3, was also not soluble in methanol, water, or acidified methanol.

Henna powders and products also prove difficult since the natural henna dye (primarily Lawsone) is derived from a flowering plant, and often contains several closely related peaks. Products labeled as "henna" are also sometimes adulterated with artificial colors. Figure 40 displays the chromatogram of a henna powder extracted using Method 3.1 in which three dyes were identified: Former External D&C Red No. 10, Naphthol Blue Black, and D&C Orange No. 4. Their spectra are overlaid with reference standards in Figure 41.

Figure 40. Henna powder product in which three undeclared dyes were identified: Former Ext. Red 10 (4.011 min), Naphthol Blue Black (5.285 min), and Orange 4 (5.334 min). Unlabeled peaks did not absorb in the visible region (400-700 nm).

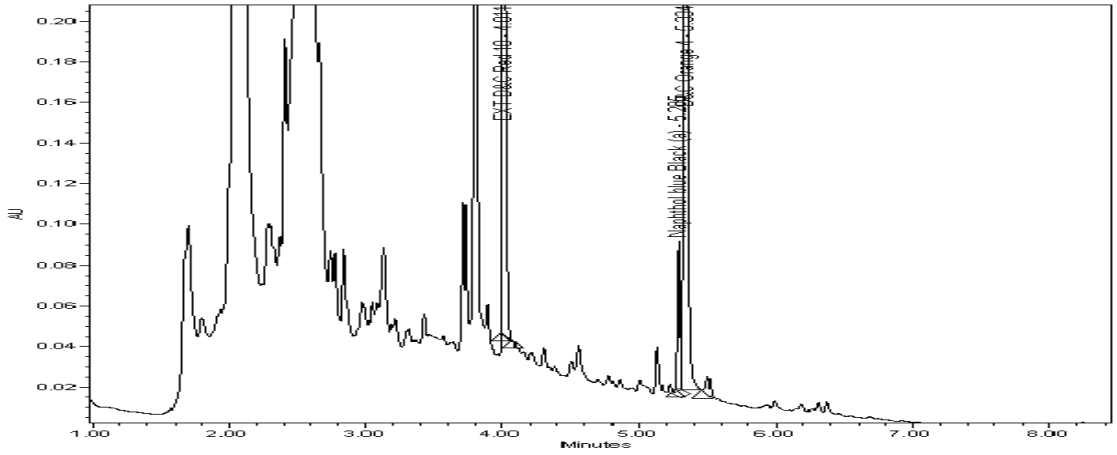
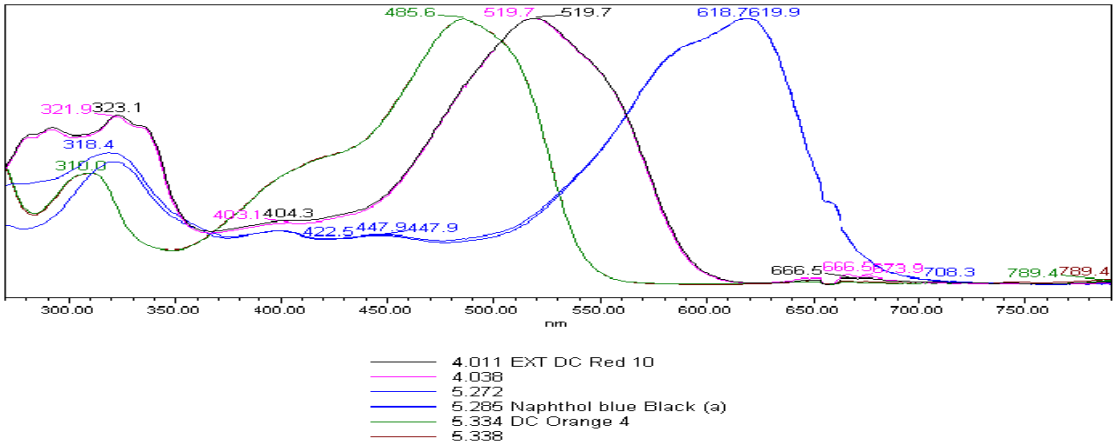


Figure 41. Overlay of Henna product and reference standard spectra of former Ext. Red 10 (absorbance max at 519.7 nm), Naphthol Blue Black (abs. max at 618.7 nm), and Orange 4 (abs. max at 485.6 nm).



9. Method information

9.1. Method development

Several combinations of extraction solutions were studied in the development of Method 4.1 for solid (aqueous-based) food products. Research began with a 50:49:1 methanol:water:concentrated HCl solution only to find that lower density cookie-type products tended to produce too many suspended particulates in the supernatant. Three additional solutions were used and their results are displayed in Figures 42-44. The product used for the method testing was a commercially-processed chip that identified Yellow 6, Yellow 6 Lake, Blue 2, Blue 1 Lake and Red 40 Lake as color additives, similar to other products tested in the past and found to be problematic for color additive analysis using traditional methods. The solvent systems that were compared were 50:20:29:1 methanol:acetonitrile:water:concentrated HCl (Figure 42), 70:29:1 methanol:water: concentrated HCl (Figure 43); and, 90:9:1 methanol:water:concentrated HCl (Figure 44).

Figure 42. Chromatogram of a commercial chip product, displaying peaks for Blue 2 (a, 1.919 min; b, 2.553 min), Yellow 6 (2.658 min), Red 40 (3.075 min), and Blue 1 (4.549 min). Extractant: 50:20:29:1 methanol:acetonitrile:water:HCl

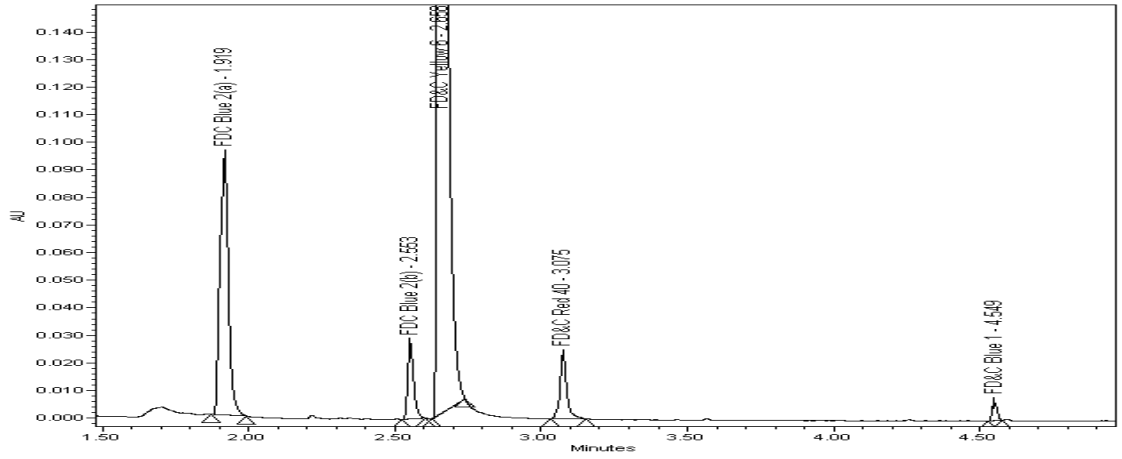


Figure 43. Chromatogram of a commercial chip product displaying peaks for Blue 2 (a, 1.922 min; b, 2.555 min), Yellow 6 (2.662 min), Red 40 (3.078 min), and Blue 1 (4.549 min). Extractant: 70:29:1 methanol:water:HCl

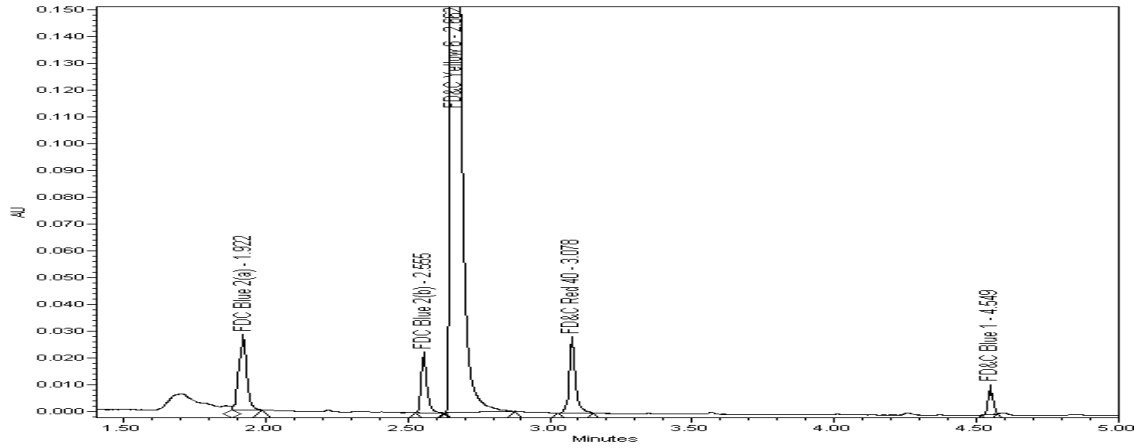
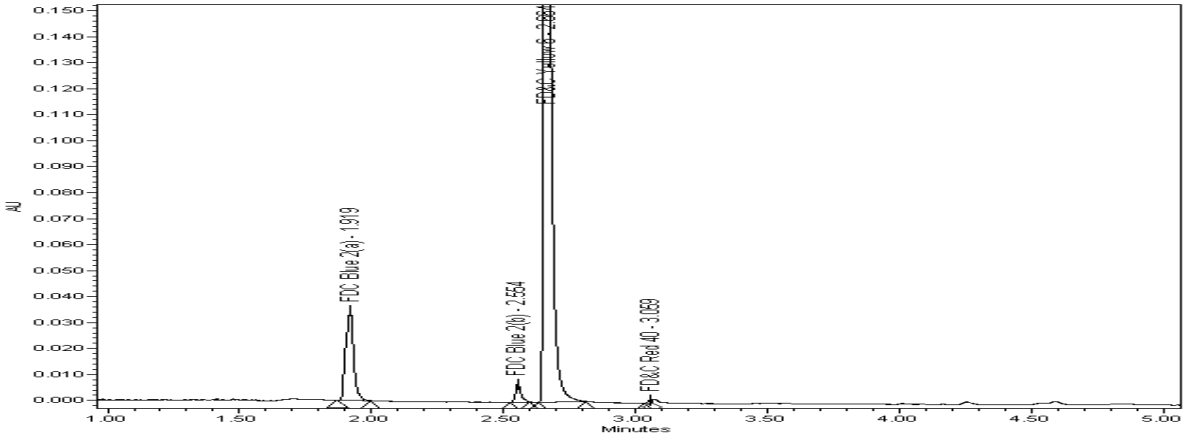


Figure 44. Chromatogram of a commercial chip product displaying peaks for Blue 2 (a, 1.919 min; b, 2.554 min), Yellow 6 (2.661 min), and small or no peak for Red 40 (3.059 min) and Blue 1. Extractant: 90:9:1 methanol:water:HCl



The difference between the first two extractants was the substitution of 20% of methanol for acetonitrile in the second solution. Acetonitrile appeared to improve the extraction of Blue 2(a) (compare Figures 42 and 44). The use of less water (Figure 44) in the extractant affected the overall recoveries for all of the dyes, especially Red 40 and Blue 1, which essentially disappear from the chromatogram (when at low levels) in the third solution. Several other samples, including potato chips, crackers, candies declaring color additives were tested to ensure that all dyes were detected using the optimal solvent mix (which is the solid extraction solution described in Method 4.1).

9.2. Method notes

9.2.1. CBX SPE columns

Avantor, the manufacturer of the CBX SPE columns described in this method, transitioned their production from India to Europe during 2016. During this time the columns were back-ordered, so tests were made of the regenerative properties of the CBX column and to answer the question: How many times can one column be reused for analysis? One column was subjected to over 36 sample applications using Method 3.1 (including standards in methanol, soap and soda samples, and spikes), Method 4.1 (including potato chip samples and spikes), and Method 6.1 (including an eye shadow product). The results indicated that recoveries of standards through a CBX SPE regenerated six times remained consistent (% RSD values ranged from 3.09 to 9.28). Average recoveries ranged from 90% (Yellow 5) to 100% (Red 6). Even after 36 regenerations the CBX column displayed no significant deterioration**.** Product peak spectra matched within 5 nm of each standard's absorbance maximum wavelength and product peak retention times were within 5% of each standard's retention time based on a comparison of peaks over the range of 270-790 nm by UPLC with photodiode array detection. Regeneration consisted of washing the column with two column-volumes (CV) of water, two CVs of 40% methanol/water, two CV's of methanol, followed by two mL of 1% HCl/methanol in preparation for the next sample application.

Columns should be discarded, however, when noticeably discolored with natural dyes (anthocyanins, etc.) from products such as tomato-based salsas, peppers, and chocolates, etc., or if materials begin to collect on top of the column bed and cannot be washed off. Additionally, the columns should be periodically tested as "reagent blanks" to confirm that no colors remain to

contaminate sample runs, if opting to re-use SPE columns. Finally, CBX columns that have been treated with hexane should not then be used with aqueous solvents, and vice-versa.

9.2.2. Instrumentation differences

Analysts should be aware that the Waters UPLC instruments (Acquity, H-Class, and I-Class) are sensitive to overloading of color extracts. The concentrations of solutions to be injected should be selected so that absorbance values for individual colors are less than 1.0 AU when possible, though this may not be possible for samples with large amounts of some color additives and low amounts of others. There are also subtle differences between injectors with flow-through needles (FTN) and those without, which can affect injection behavior. Generally, the final sample diluent should match the initial mobile phase mix as close as possible to avoid peak splitting. The gradient described in this paper is approximately 99% aqueous at the time of injection. However, since this method includes both water-soluble and some oil-soluble dyes, methanol was selected as the most universal solvent. If analyte peaks appear to split with 2 µL injections, try reducing injection volumes to 1 µL and (if possible) dilute samples in ≤50% methanol/water or water prior to injection. Keep in mind some splitting may still occur for early eluters such as FD&C Yellow No. 5 and Carminic acid, if there is any methanol in the injected solution.

There also may be slight differences in the gradients obtained with the use of a Waters® Binary Solvent Module (BSM) versus a Quaternary Solvent Module (QSM), due to differences in the mixing valves and solvent line lengths. Thus, a slight time offset may be required to obtain identical Retention Times between instruments, which can be calculated with the Waters Columns Calculator® software that comes with the Empower® software package.

9.2.3. Quantitative analysis

Color additive analysis in the U.S. to date has primarily been qualitative, except for those colors and products where there is a regulatory tolerance threshold. The extractions described in the methods presented here were designed to be qualitative, sampling all dyes present but not necessarily completely extracting all of the dye present in the product (which can be quite challenging). However, the detection method via UPLC-PDA has demonstrated a linear response for the dyes tested, with high reproducibility and accuracy for repeated injections (see examples in Figure 45). Thus, the instrument method could be applied to quantitative color analysis, as long as appropriate controls are used and steps are taken to ensure complete dye extraction from the matrix.

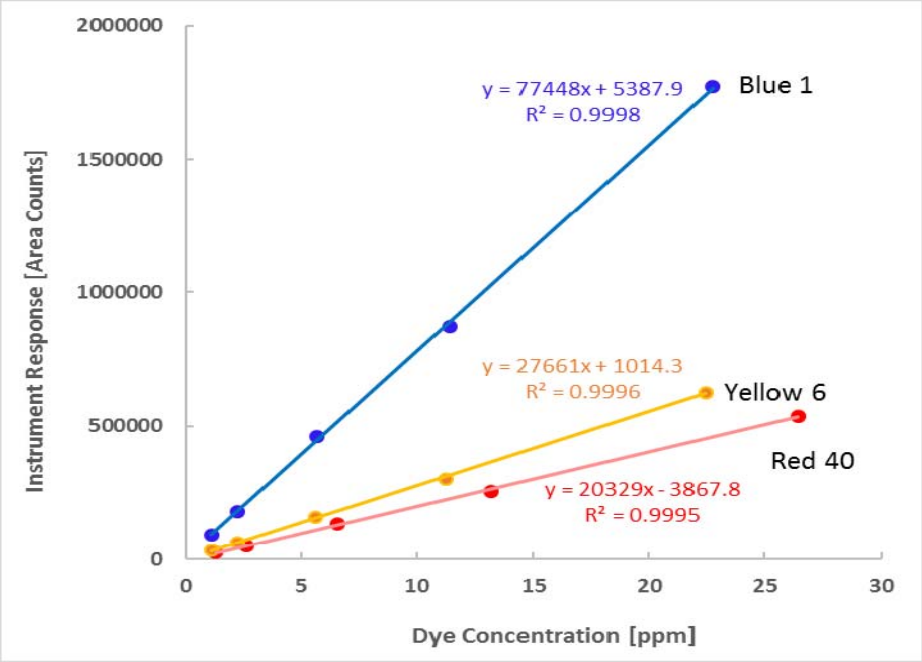


Figure 45. Examples of color standard calibration curves obtained during the validation process, for the calculation of spike recovery values. Shown are the curves for FD&C Blue No. 1 (top); FD&C Yellow No. 6 (middle); and FD&C Red No. 40 (bottom), demonstrating linear instrument response in the relevant concentration range.

9.2.4. Method validation

In addition to the recovery data provided in the text above, an extensive method validation packet is on file at the FDA Denver Laboratory, where a compilation of qualitative and some quantitative data is archived, testing various food and cosmetic matrices with the methods presented here. Each method section has been evaluated with appropriate sample types, along with side-by-side tests of samples run by these methods and TLC, to confirm that comparable (or better) results were obtained. In addition, an extensive limit study was performed and is included in the validation packet, examining the Limit of Detection (LOD) values for many commonly observed dyes using the UPLC instrument and detection method described. In addition to LOD analysis, the POD (Probability of Detection) and LOI (Limit of Identification) approaches were examined, which are relatively new metrics for qualitative analysis (20, 21). One method of estimating the LOD uses an MDL-style calculation, which is the standard deviation of seven low level (0.5 mg/kg) replicates multiplied by the one-tailed Student's t-test value for N-1 degrees of freedom at the 99% confidence interval. Using this method, the 18 dyes examined ranged from LOD values of 0.012 mg/kg (Red 22) to 0.112 mg/kg (Red 3), with a mean of 0.051 +/- 0.007 mg/kg.

Limit of Identification (LOI) values take into account not just the detection, but also the positive identification of a dye. This is an estimate of the minimum concentration where a dye is not only detected, but identified with high confidence (above a minimum threshold, as discussed below), based on the spectral library match. The LOI values for these 18 dyes appeared to fit into three distinct categories: Category A, which often included dyes with strong chromophores. This category includes Blue 1 and Green 3, and the estimated LOI values ranged from approximately 0.02 to 0.2 mg/kg. Category B dyes tended to be those with moderately absorbing chromophores, and contained the majority of dyes examined such as Yellow 6, Red 40, Ponceau 4R, Red 6, etc. The estimated LOI values for Category B dyes ranged from approximately 0.3 to 0.5 mg/kg. Category C dyes were primarily those with weakly absorbing chromophores (which depends somewhat on the solvent used), and includes such dyes as Carminic acid, Yellow 10, and Ext. Violet 2. The estimated LOI values for Category C dyes ranged from approximately 0.5 to 1.2 mg/kg.

Note, that in these tests, though somewhat subjective depending on the observer, the solutions measured appeared to the human eye to contain color (with a clear background, in a UPLC vial) at approximately 0.1 to 0.5 mg/kg, though this depends on the dye. One of the critical aspects of regulatory color analysis is the consideration of whether a given color additive "imparts color" to a product. This depends on the natural color context of the product, as well as the dye strength, and can be a difficult assessment. For instance, dyes added to a hot sauce would need to be at substantially higher concentrations to impart color compared to dyes in a white sugar candy.

As part of the analysis, the "Match Angle" parameter was examined that is reported for automated spectral matches in the Waters Empower® 3 Software. The Match Angle (MA) is a composite quality measure of the dissimilarity of two spectra across the entire wavelength range specified, and ranges in value from 0 (best possible match) to 90 degrees (worst match). Examination of match angles between different dyes in the library, based (somewhat subjectively) on the overall match quality resulted in the selection of a Match Angle threshold of 4.0. Thus, when two spectra are compared and produce an MA value less than 4.0, it is considered an acceptable overall match. Further examination, however, revealed that some highly similar spectra in the library (such as Yellow 6 and Red 9) will give 'false positive' matches between MA values of 3 and 4, which meet the acceptable overall comparison criterion but are from two distinct dyes. No such false positive matches were observed below a Match Angle of 3.0, so this was determined to be the threshold of identification (also used in the LOI calculations above). Thus, when evaluating a potential spectral

match, a Match Angle below 4.0 indicates a high quality match, but if it is above 3.0, the Analyst should use caution and verify Retention Times with standards and use any other available data to confirm dye identity.

10. Conclusions

Colors Manual 2016 is intended to be a framework that presents relatively straightforward and robust methods which work on many product matrices, for a wide variety of synthetic and natural dyes and pigments. It is not, however, able to specifically address every type of issue and matrix problem that will be encountered. Along these lines, several tips, tricks, and suggestions are provided in each section that will assist the experienced color analyst in troubleshooting difficult matrices. No single method or series of methods will work with every sample given the tremendous variety of food and cosmetic products on the market. The successful color analyst will make careful observations on the behavior of the sample, the colors that do or do not retain on the SPEs, and how the extracted colors compare to the appearance of the product, then use judgment and experience on how to proceed in their analysis. It can be a challenging process.

As new and more complicated matrices are received for analysis, it is important to try the individual methods offered here to identify which extraction method is most effective for the unique matrix of each sample. One or more of the extractions may prove effective. For instance, for dry milk-based samples, it may make sense to dissolve the sample in water, then apply Method 3.1, rather than just use methanol. Multiple extraction attempts, altering extraction solvents or conditions, may be necessary to obtain the desired result. Every attempt should be made, using all the tools available, as part of the effort to extract, detect, and identify the colors present in the foods, drugs and cosmetics offered to American consumers.

These methods build on previous techniques and take advantage of newer technologies to improve accuracy, sensitivity, and efficiency in color additive identification. The identification of an SPE column (the Avantor CBX) that can retain most of the commonly used dyes while dissolved in methanol was an incredible find, cutting out several steps and potential losses in the methods it was designed to replace. It has allowed for more straightforward extractions using less product with more sensitive results and producing less solvent waste.

The Waters Corporation discusses the use of the extended-wavelength PDA detector for dye analysis in foods (22), and provides a “dilute-and-shoot” method for water-soluble dyes. However, samples received for color analysis have matrices that are interesting, varied, and often are very difficult to extract, precluding the use of the “dilute-and-shoot” approach. This paper offers various new techniques that can be used in an attempt to simplify the extraction and cleanup for the variety of products received in the course of regulatory analysis. The UPLC system, with an extended PDA system from 270-790 nm and Maxplot software that optimizes the absorbance wavelength for a given peak, offers the best opportunity for identification of known and unknown dyes over the traditional methods used for color additive analysis.

Colors Manual 2016 is the second in a series of the FDA Denver Laboratory’s ongoing research project studying color methodology for food and cosmetic products. The first paper detailed the analysis of Butter Yellow in bean curd by liquid chromatography-triple quadrupole and quadrupole time-of-flight tandem mass spectrometry at part-per-billion levels (23).

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Flowchart 1. Colors Manual 2016 Method Selection (see text for details).

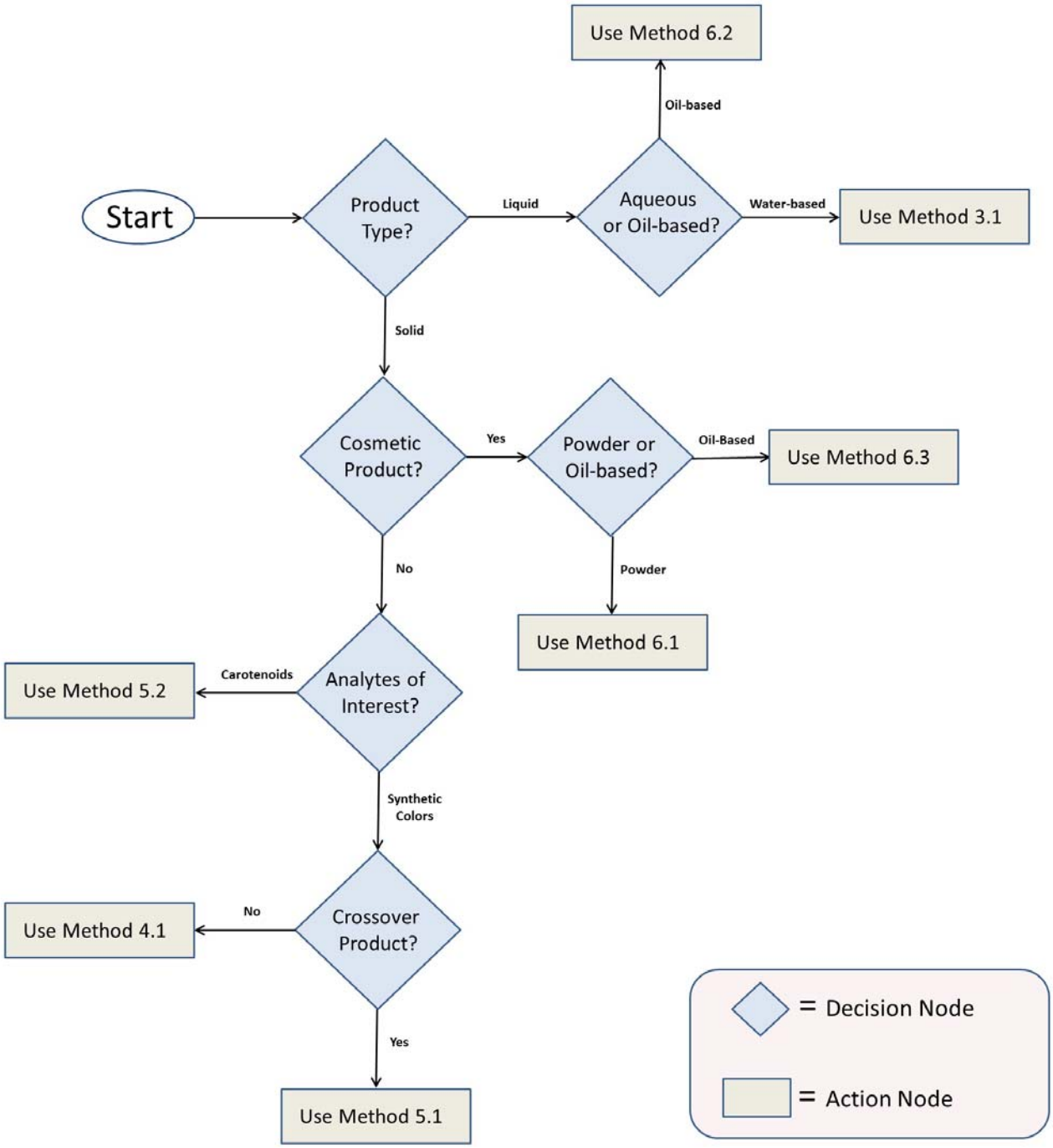

Table 9. Summary of Colors Manual 2016 method steps (see text for details)

Method No.	Matrix Type	SPE Type	Sample Preparation	Column Preparation	Wash Method	Elution Method
3.1	**Liquid; Aqueous**	CBX	1-5g in 3-15mL MeOH	3mL MeOH; 1mL 1% HCl/MeOH; Apply 1-5mL	6mL H_2O; 3mL MeOH	3-4mL 10% NH_4OH in MeOH
4.1	**Solid; Dry Foods**	CBX	3-5g in 20mL MeOH:ACN:H_2O: HCl (50:20:29:1)	3mL MeOH; 1mL 1% HCl/MeOH; Apply 1-5mL	6mL H_2O; 3mL MeOH	3-4mL 10% NH_4OH in MeOH
	(Colors that pass through CBX)	HLB	Collect water wash; Dilute w/H_2O to 10mL	3mL MeOH; 3mL H_2O; Apply 10 mL	6mL H_2O; 3mL 40% MeOH	3-4mL MeOH
	(Additional colors remaining on HLB (i.e. Sudans))	HLB	-	-	-	3-4mL 10% NH_4OH in MeOH or DCM
5.1	**High protein, fat, salt, carbs & Seafood** (For H_2O-Soluble Colors)	CBX	3-5g in 20mL MeOH	3mL MeOH; 1mL 1% HCl/MeOH; Apply 1-5mL	6mL H_2O; 3mL MeOH	3-4mL 10% NH_4OH in MeOH
5.2	**Fish/Seafood** (For Carotenoids)	CBX	3-5g in 10mL hexane	5mL hexane; Apply 5mL	5mL hexane	4-5mL DCM or MeOH
6.1	**Cosmetics: Powders/ Lipsticks**	CBX	0.2g in 4-5mL 1% HCl in MeOH	3mL MeOH; 1mL 1% HCl/MeOH; Apply 1-5mL	3mL MeOH; 3mL H_2O; 3mL MeOH	3-4mL 10% NH_4OH in MeOH
6.2	**Oil-based products**	SiOH	0.5-3g in 5-10mL hexane	5mL hexane; Apply 2-5mL	4mL hexane (twice)	4-5mL ACN
6.3	**Cosmetics: Pomades and Waxes**	SiOH	1-3g in 5-10mL hexane	5mL hexane; Apply (heated) 2-4mL	4mL hexane (twice)	4-5mL ACN
7.1	**Imitation Crab** (Carmine)	CBX	3-5g in 15-20mL 10% HCl/MeOH	3mL MeOH; 1mL 1% HCl/MeOH; Apply 2-4mL	6mL H_2O; 3mL MeOH	3-4mL 10% NH_4OH in MeOH
	(Colors that pass through CBX)	HLB	Collect water wash; Dilute w/H_2O to 10mL	3mL MeOH; 3mL H_2O; Apply 10 mL	6mL H_2O; 3mL 40% MeOH	3-4mL 10% NH_4OH in MeOH
7.2	**Bar Soaps**	CBX	3-5g in 20mL MeOH; Dil. 2mL w/4mL 1% HCl/MeOH	3mL MeOH; 1mL 1% HCl/MeOH; Apply diluted 6mL	6mL H_2O; 3mL MeOH	3-4mL 10% NH_4OH in MeOH
7.3	**Hair Conditioners**	CBX	2g in 3-4mL THF	3mL MeOH; 1mL 1% HCl/MeOH; Apply entire extract	3mL MeOH; 6mL H_2O; 3mL MeOH	3-4mL 10% NH_4OH in MeOH
7.4	**Alkaline Products**	CBX	1-5g in 3-15mL 1% HCl/ MeOH	3mL MeOH; 1mL 1% HCl/MeOH; Apply 1-5mL	6mL H_2O; 3mL MeOH	3-4mL 10% NH_4OH in MeOH
7.5	**Products w/Waxy Coatings**	CBX	Pre-treat with 5mL Hexane, then proceed with regular method	-	-	-

Abbreviations: ACN = Acetonitrile; DCM = Dichloromethane; HCl = Hydrochloric Acid; MeOH = Methanol; NH_4OH = Ammonium hydroxide; THF = Tetrahydrofuran

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Appendix 1. Partial list of standards in Colors Manual 2016 spectral library

Shade	Common Name	Other Names and Designations	E #	C.I. #
Yellows	Auramine O	C.I. Basic Yellow 2		41000
	Brilliant Yellow	C.I. Acid Yellow 270		10317
	Brilliant Yellow 2G	C.I. Acid Yellow 17		18965
	Butter Yellow	C.I. Solvent Yellow 2		11020
	Flavazin	C.I. Acid Yellow 11, former Ext. D&C Yellow 3		18820
	HC Yellow No. 4	HC Yellow 4 (HC = Hair Color)		
	Metanil Yellow	C.I. Acid Yellow 36, former Ext. D&C Yellow No. 1		13065
	Milling Yellow O	C.I. Acid Yellow 38		25135
	Milling Yellow P2J	C.I. Acid Yellow 94		N/A
	Naphthol Yellow S	C.I. Acid Yellow 1, Ext. D&C Yellow No. 7		10316
	Polar Yellow 5G	C.I. Acid Yellow 40, former Ext. D&C Yellow No. 4		18950
	Quinoline Yellow	C.I. Food Yellow 13, D&C Yellow No. 10	E104	47005
	Quinoline Yellow SS	C.I. Solvent Yellow 33, D&C Yellow No. 11		47000
	Sunset Yellow FCF	C.I. Food Yellow 3, FD&C Yellow No. 6	E110	15985
	Tartrazine	C.I. Food Yellow 4, FD&C Yellow No. 5	E102	19140
	Uranine	C.I. Acid Yellow 73, D&C Yellow No. 8		45350
Reds	Acid Fuchsine	C.I. Acid Red 33, D&C Red No. 33		17200
	Allura Red AC	C.I. Food Red 17, FD&C Red No. 40	E129	16035
	Amaranth	C.I. Food Red 9, former FD&C Red No. 2	E123	16185
	Azorubine/Carmoisine	C.I. Food Red 3, former Ext. D&C Red No. 10	E122	14720
	Croceine Scarlet MOO	C.I. Acid Red 73, former Ext. D&C Red No. 13		27290
	Eosin	C.I. Acid Red 87, D&C Red No. 22		45380
	Erythrosine	C.I. Food Red 14, FD&C Red No. 3	E127	45430
	Helindone Pink CN	C.I. Pigment Red 181, D&C Red No. 30		73360
	Lake Bordeaux BN	C.I. Pigment Red 63:1, D&C Red No. 34		15880:1
	Lake Red C	C.I. Pigment Red 53, former D&C Red No. 8		15585
	Lake Red CBA	C.I. Pigment Red 53:1, former D&C Red No. 9		15585:1
	Lithol Red	C.I. Pigment Red 49, former D&C Red No. 10		15630
	Lithol Red CA	C.I. Pigment Red 49:2, former D&C Red No. 11		15630:2
	Lithol Red SR	C.I. Pigment Red 49:3, former D&C Red No. 13		15630:3
	Lithol Rubine B	C.I. Pigment Red 57, D&C Red No. 6		15850
	Lithol Rubine BCA	C.I. Pigment Red 57:1, D&C Red No. 7	E180	15850:1
	Oil Red N	C.I. Solvent Red 26		26120
	Phloxine B	C.I. Acid Red 92, D&C Red No. 28		45410
	p-Rosaniline Hydrochloride	C.I. Basic Red 9		42500
	Red 2G	C.I. Food Red 10, former Ext. D&C Red No. 11	E128	18050
	Sirius Red F3B	C.I. Direct Red 80		35780
	Tetrabromofluorescein	C.I. Solvent Red 43, D&C Red No. 21		45380:2
Blues	Alizarine Blue B	C.I. Acid Blue 27, former D&C Blue No. 5		61530
	Azure Blue	C.I. Pigment Blue 29, Ultramarine Blue		77007
	Brilliant Blue FCF	C.I. Food Blue 2, FD&C Blue No. 1	E133	42090
	Brilliant Blue FCF	C.I. Acid Blue 9, D&C Blue No. 4 (ammonium salt)		42090
	Calcosyn Sapphire Blue	C.I. Disperse Blue 3		61505
	HC Blue No. 2	HC Blue 2		
	Indigo	C.I. Pigment Blue 66, D&C Blue No. 6		73000
	Indigotine	C.I. Food Blue 1, FD&C Blue No. 2	E132	73015
	Methasol Blue 2B	C.I. Solvent Blue 38		74180
	Nile Blue A	C.I. Basic Blue 12		51180

Shade	Common Name	Other Names and Designations	E #	C.I. #
Blues (cont.)	Patent Blue V	C.I. Food Blue 3	E131	42051
	Victoria Blue BO	C.I. Basic Blue 7		42595
	Victoria Blue R	C.I. Basic Blue 11		44040
Oranges	Acid Orange G	C.I. Food Orange 4, former D&C Orange No. 3		16230
	Alizarine	C.I. Pigment Red 83, former D&C Orange No. 15		58000
	Annatto	C.I. Natural Orange 4	E160b	75120
	Crocein Orange G	C.I. Food Orange 1		15970
	Dibromofluorescein	C.I. Solvent Red 72, D&C Orange No. 5		45370:1
	Diiodofluorescein	C.I. Solvent Red 73, D&C Orange No. 10		45425:1
	Erythrosine Yellowish K	C.I. Acid Red 95, former D&C Orange No. 12		45425
	Methyl Orange	C.I. Acid Orange 52		13025
	Orange 1	C.I. Acid Orange 20, former FD&C Orange No. 1		14600
	Orange B	C.I. Acid Orange 137		19235
	Orange II	C.I. Acid Orange 7, D&C Orange No. 4		15510
	Orange SS	C.I. Solvent Orange 2, former FD&C Orange No. 2		12100
	Tropaeolin O	C.I. Acid Orange 6		14270
Greens	Acid Fast Green B	C.I. Acid Green 9, former D&C Green No. 7		42100
	Alizarin Cyanine Green F	C.I. Acid Green 25, D&C Green No. 5		61570
	Brilliant Green	C.I. Basic Green 1		42040
	Fast Green FCF	C.I. Food Green 3, FD&C Green No. 3	E143	42053
	Green S	C.I. Food Green 4	E142	44090
	Guinea Green B	C.I. Acid Green 3, former FD&C Green No. 1		42085
	Light Green SF Yellowish	C.I. Acid Green 5, former FD&C Green No. 2		42095
	Malachite Green	C.I. Basic Green 4		42000
	Quinizarin Green SS	C.I. Solvent Green 3, D&C Green No. 6		61565
Violets, Blacks	Acid Violet 6B	C.I. Acid Violet 49, former FD&C Violet No. 1		42640
	Alizarine Fast Grey	C.I. Acid Black 48		65005
	Alizarine Violet	C.I. Acid Violet 43, Ext. D&C Violet No. 2		60730
	Alizurol Purple SS	C.I. Solvent Violet 13, D&C Violet No. 2		60725
	Brilliant Black BN	C.I. Food Black 1, Brilliant Black PN	E151	28440
	Ethyl Violet	C.I. Basic Violet 4		42600
	Fast Violet RR	C.I. Solvent Violet 2		16055
	Methyl Violet 2B	C.I. Basic Violet 3, Gentian Violet		42555
	Naphthol Blue Black	C.I. Acid Black 1, former D&C Black No. 1		20470
	Nigrosine B	C.I. Acid Black 2		50420
Ponceau dyes	Ponceau 2R	C.I. Acid Red 26, former D&C Red No. 5		16150
	Ponceau 3R	C.I. Food Red 6, former FD&C Red No. 1		16155
	Ponceau 4R	C.I. Food Red 7, C.I. Acid Red 18	E124	16255
	Ponceau S	C.I. Acid Red 112, Ponceau Red S		27195
	Ponceau SX	C.I. Food Red 1, FD&C Red No. 4	E125	14700
Sudans	Sudan I	C.I. Solvent Yellow 14		12055
	Sudan II	C.I. Solvent Orange 7, former FD&C Red No. 32		12140
	Sudan III	C.I. Solvent Red 23, D&C Red No. 17		26100
	Sudan IV	C.I. Solvent Red 24		26105
	Sudan Black B	C.I. Solvent Black 3		26150
	Sudan Blue	C.I. Solvent Blue 59		61552
	Sudan Blue II	C.I. Solvent Blue 35		61554

Shade	Common Name	Other Names and Designations	E #	C.I. #
Sudans (cont.)	Sudan Orange G	C.I. Food Orange 3		11920
	Sudan Red 7B	C.I. Solvent Red 19		26050
	Sudan Yellow G	C.I. Solvent Yellow 18, Oil Yellow XP		12740
Carotenoids	Astaxanthin	CAS Reg. No. 472-61-7		
	Canthaxanthin	C.I. Food Orange 8	E161g	40850
	Lutein	C.I. Natural Yellow 29	E161b	75137
	Zeaxanthin	CAS Reg. No. 144-68-3		
	β-Carotene	C.I. Food Orange 5	E160a	40800
Others	Beetroot	C.I. Natural Red 33, Beetroot Red	E162	75840
	Carmine	C.I. Natural Red 4:1	E120	75470:1
	Carminic acid	C.I. Natural Red 4, Cochineal extract	E120	75470
	Chocolate Brown HT	C.I. Food Brown 3	E155	20285
	Ethyl Eosin	C.I. Solvent Red 45		45386
	Resorcin Brown	C.I. Acid Orange 24, D&C Brown No. 1		20170
	Rhodamine 6G	C.I. Basic Red 1		45160
	Rhodamine B	C.I. Food Red 15, former D&C Red No. 19		45170
	Riboflavin	C.I. Food Yellow 15	E101	50900
	Rose Bengal	C.I. Acid Red 94		45440
	Saffron	C.I. Natural Yellow 6	E164	75100
	Turmeric	C.I. Natural Yellow 3, Curcumin	E100	75300