

## Application Note

# Chromatographic Separation and Photodiode Array Identification of Synthetic Industrial Dyes in Foods, Beverages, Over the Counter (OTC) Drugs, and Cosmetics

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## Abstract

In this application note, the Waters Alliance™ iS HPLC System with Waters Photodiode Array (PDA) Detector is shown to separate synthetic food dyes formulated in a variety of foods, beverages, OTC drugs, and cosmetics. Three individual wavelengths were extracted from one HPLC injection to quantify yellow, red, and blue and green synthetic dyes. The PDA software determined chromatographic peak purity by UV spectral analysis, and identified dyes according to an Empower™ Software PDA Library.

## Benefits

- A single chromatographic method separated ten synthetic food dyes in a variety of consumer products and matrices
- Empower Software, when paired with the Alliance iS HPLC System with PDA Detector, can determine peak purity for chromatographic separations of food dye containing products
- Synthetic food dyes in complex matrices are spectral identified using the Empower Software PDA Library

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## Introduction

Food, drug, and cosmetic (FD&C) dyes are used to enhance visual appeal, reduce natural product variation, and increase overall product recognizability and desirability. Synthetic food dyes are typically less expensive than natural dyes, relatively shelf-stable, and blend easily to create a variety of hues of intense color.<sup>1</sup> In the US, the most popular colors are Red #40, Yellow #5, and Yellow #6.<sup>2</sup> Food dye ingredients are often in the news due to concerns regarding neurobehavioral impacts on children, particularly hyperactivity, inattentiveness, restlessness, and other behavioral problems.<sup>3</sup> As a result, some US states, such as California, prohibit the sale of products that contain synthetic food dyes in schools, while other states, such as West Virginia, have moved a step further by prohibiting synthetic dye containing food products throughout the state.<sup>4</sup> In addition to neurobehavioral concerns, Red #3, also known as erythrosine, was shown in a 2022 US Food and Drug Administration (FDA) petition to cause cancer in male laboratory rats. With this data, the FDA revoked the authorization for use of Red #3 in consumer products, issuing a complete phase out of the ingredient across the US by 2028.<sup>5</sup>

In the US, FDA-regulated food products, cosmetics, and OTC drugs require that package labels include a statement of identity, net quantity of contents, nutrition information, an ingredient list, and the identity of the manufacturer, packer, or distributor.<sup>6</sup> Synthetic food dyes are required to be listed as they appear in the Code of Federal Regulations (CFR), and the product manufacturer must use color additives that are Certificate of Analysis (CoA) batch-certified, which includes content and purity analysis.<sup>7</sup> The FDA works to continuously monitor adulterated or misbranded products, and, notably, color additive violations are a common reason for import refusals of food and cosmetic products offered for entry to the US.<sup>8</sup>

In this application note, a variety of common off-the-shelf snacks, drinks, OTC drugs, and cosmetics were analyzed using the Alliance iS HPLC System with PDA Detector. The Alliance iS HPLC System was used to

chromatographically separate, quantify, and identify ten synthetic food dyes, including Red #3, recently banned in the US. The PDA Detector, in combination with Empower Software, provided chromatographic purity analysis and identified synthetic food dye extracted from matrix using a PDA Library.

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## Experimental

### LC Conditions

LC system:	Alliance iS HPLC System with PDA Detector, Software version 1.4.0
Column:	XBridge™ Premier BEH™ C <sub>18</sub> 2.5 µm, 4.6 x 150 mm, p/n: 186009849
Column temperature:	40 °C
Sample temperature:	20 °C
Injection volume:	30 µL
Flow rate:	1.6 mL/min
Mobile phase A:	10 mM Ammonium acetate pH 7.0
Mobile phase B:	Methanol
Mobile phase C:	Acetonitrile
Sample filter:	0.2 µm PTFE CE Acrodisk Minispike Filter, p/n: WAT200556
Needle wash:	50/50 Methanol/Water

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3D Wavelengths:	200–800 nm
Resolution:	1 nm
Data rate:	10 Hz
Extracted channels:	455 nm (Yellows), 520 nm (Reds), 628 nm (Blues and Greens)
CDS:	Empower Software, Version 3.8.0

## Gradient Table

Time (min)	Mobile Phase (%)		
	A	B	C
Start	97.0	2.0	1.0
3.00	97.0	2.0	1.0
3.50	90.0	9.0	1.0
10.00	50.0	25.0	25.0
12.00	2.0	49.0	49.0
15.00	2.0	49.0	49.0
16.00	97.0	2.0	1.0

## Sample Preparation

Yellow #5, Yellow #6, Blue #2, Red #40, Green S, Carmoisine, Green #3, Blue #1, Red #3, and Patent Blue V food dye certified reference standards were obtained from Sigma-Aldrich®. Standards were solubilized in water to equal a concentration of 1.0 mg/mL. A standard stock mixture was prepared to contain each dye at 0.1 mg/mL. From the mixture, serial dilutions were prepared in dye-free sports drink to equal HPLC column sample loads between 0.003 ug to 0.375 ug of each dye. From the linearity injections, a PDA Library was created in the Empower Software (Table 1). For solid samples, one serving was dissolved in a respective volume of water (i.e. 10–200 mL). Each extraction was sonicated or stirred until the matrices were dissolved. The extractions were

centrifuged for 10 minutes at 15,000 RPM to separate insoluble material, and the supernatant used for analysis. For analysis of sports drinks, each beverage was diluted 1:10 in water prior to HPLC injection.

Artificial dye	Structure <sup>a</sup>	PDA spectrum
Yellow #5 (Tartrazine, FD&C Yellow No. 5, E102, and Acid Yellow 2)		
Blue #2 (Indigo carmine, or 5,5'-indigodisulfonic acid sodium salt, E132)		
Yellow #6 (Sunset yellow, CI Food Yellow 3, Orange Yellow S, CI 1585, E110)		
Red #40 (Allura Red AC, E129, CI Food Red 17, and CI 16035)		
Green S (Acid green 50, Lissamine green B, Wool green S, FD&C green 4, C.I. 44090, and E142)		
Carmoisine (Azorubine, Acid Red 14, E122 or C.I. 14720)		
Green #3 (Fast Green FCF; E143)		
Blue #1 (Brilliant Blue, Acid Blue 9, C.I. 42090, E133)		
Red #3 (erythrosine, E127)		
Patent Blue V (Food Blue 5, Sulphan Blue, Acid Blue 3, L-Blau 3, C-Blau 20, Sky Blue, C.I. 42051, E131)		

Table 1. PDA spectrum index plot for A) yellow #5 B) Blue #2 C) Yellow #6 D) Red #40 E) Green S F)

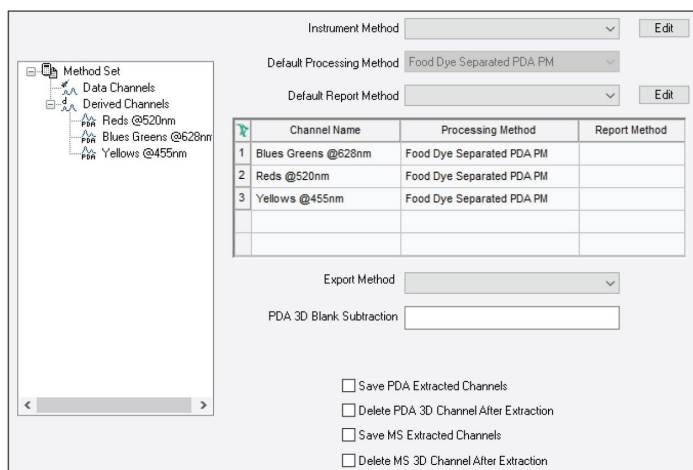
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*Carmoisine G) Green #3 H) Blue #1 I) Red #3 and J) Patent Blue V industrial food colorants.*

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## Results and Discussion

The HPLC method provided baseline separation of all reference standards in the dye mixture. Using the Empower Software Method Set (Figure 1), chromatograms at various wavelengths were visualized, and peaks quantified from the 3-D, 200-800 nm chromatogram. Yellow dyes were visualized by extracting a UV channel at 455 nm, while 520 nm was utilized for red dyes, and 628 nm utilized for both green and blue dyes (Figure 2). A PDA Library was created from the reference standard mixture injections after wavelength extraction. The chromatographic separation method showed linearity between 0.003 ug and 0.750 ug (Figure 3), mass on column.



*Figure 1. Empower Software, Method Set derived channels used to extract PDA chromatograms for quantitation of yellow, red, blue and green dyes.*

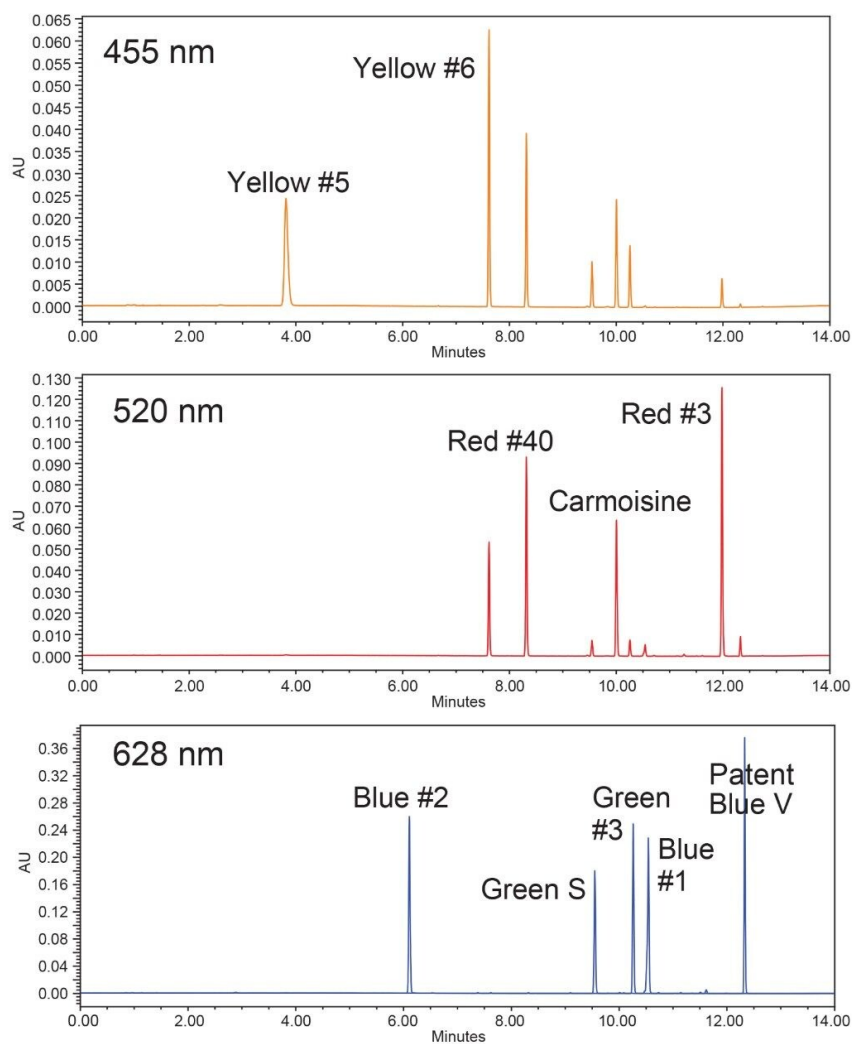


Figure 2. Synthetic food dye Reference Standard Mixture PDA spectrum derived at 455 nm, 520 nm, and 628 nm using the Empower Software Method Set.



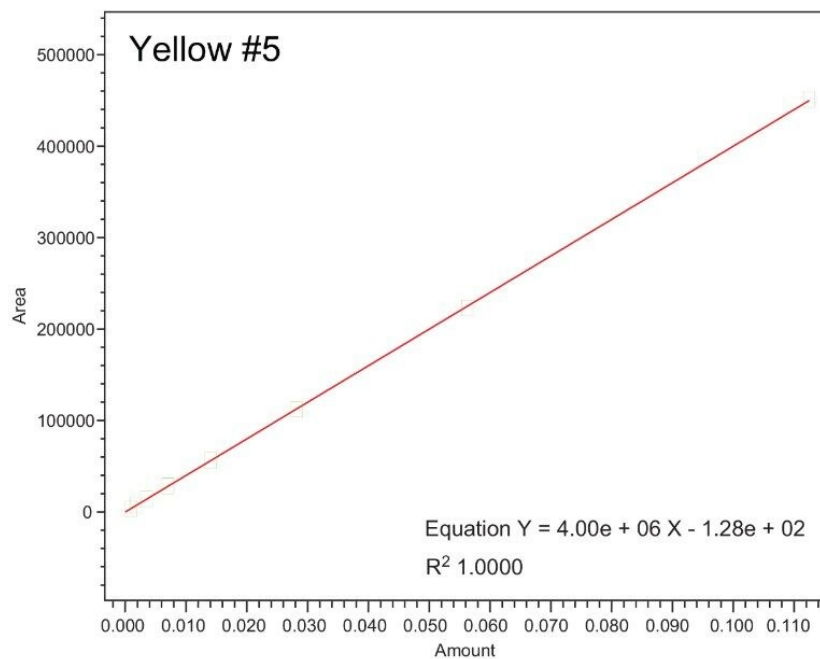
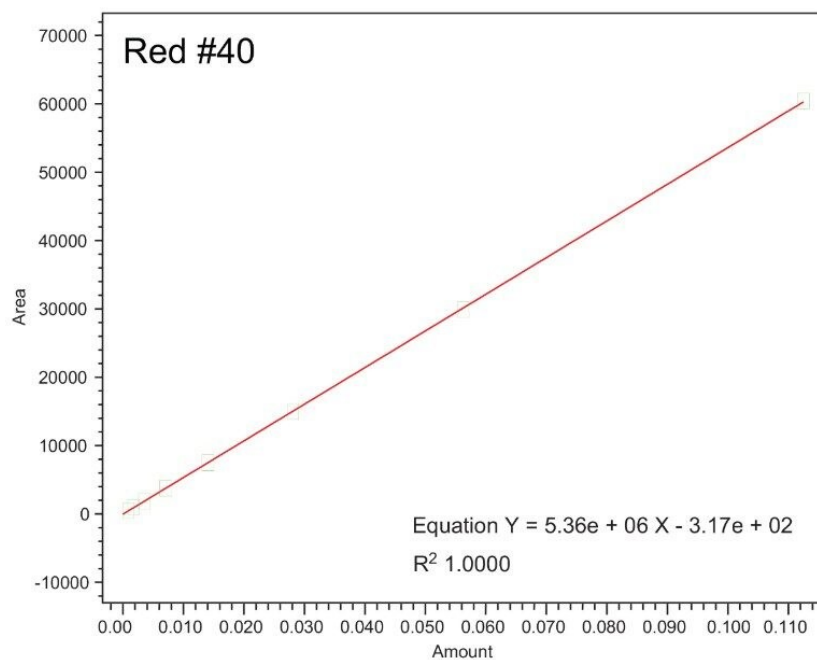


Figure 3. Chromatographic linearity of the two most common synthetic food dyes in the US.

Peak purity analysis was performed for each dye peak in sample solutions using the Empower Software Processing Method. As a representative example, dyes in fruit flavored candy were visualized at the respective wavelengths, as shown in Figure 4. Peak purity for dye peaks was determined by the software through automated PDA spectral Purity Angle and Purity Threshold comparison (Figure 5). In the fruit flavored candy, the Purity Angle was below the spectral Purity Threshold of a selected matrix-only region of the baseline, therefore chromatographic peaks were spectrally pure and absent of co-eluting constituents. Dye peaks in the fruit flavored candy were identified as Yellow #5, Yellow #6, Red #40, Blue #1, and Blue #2 using the Match Angle of reference spectra stored in the PDA Library (Figure 6).

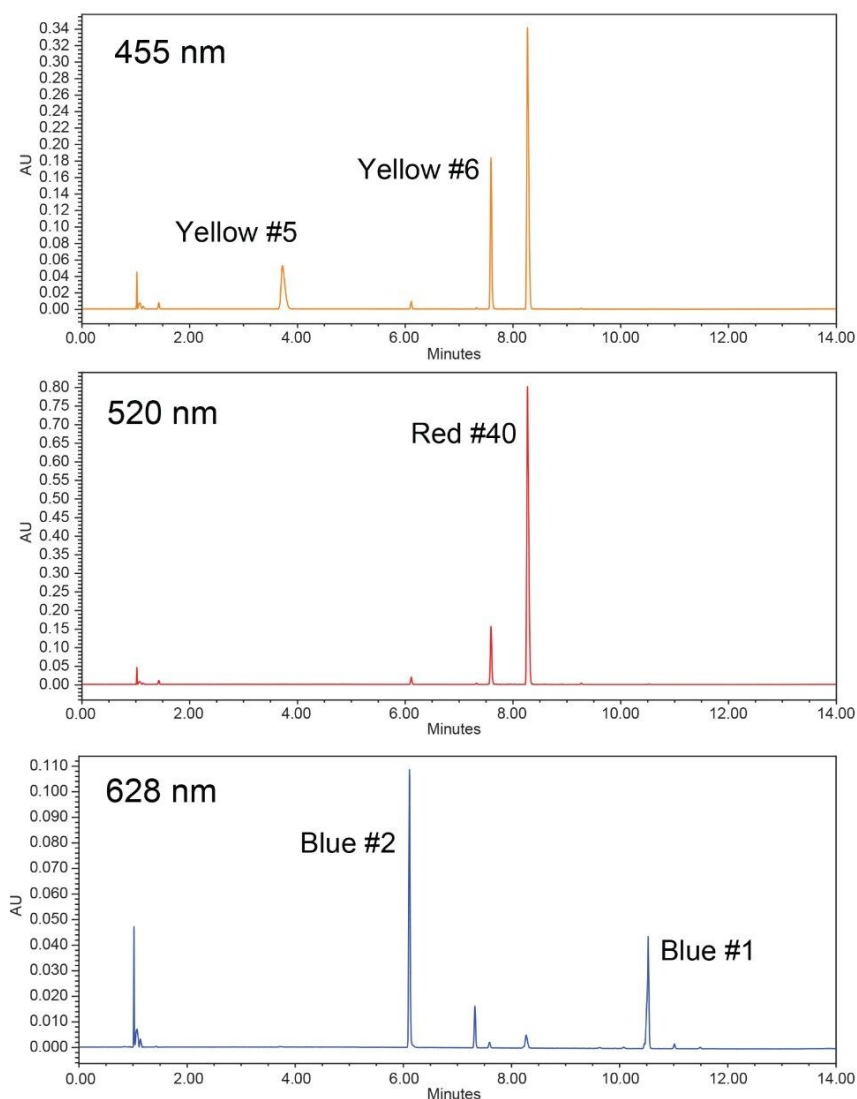


Figure 4. Chewy fruit candy dyes at 455 nm (yellow), 520 nm (red), and 628 nm (green and blue) extracted from the 3-D PDA spectrum (200–800 nm).

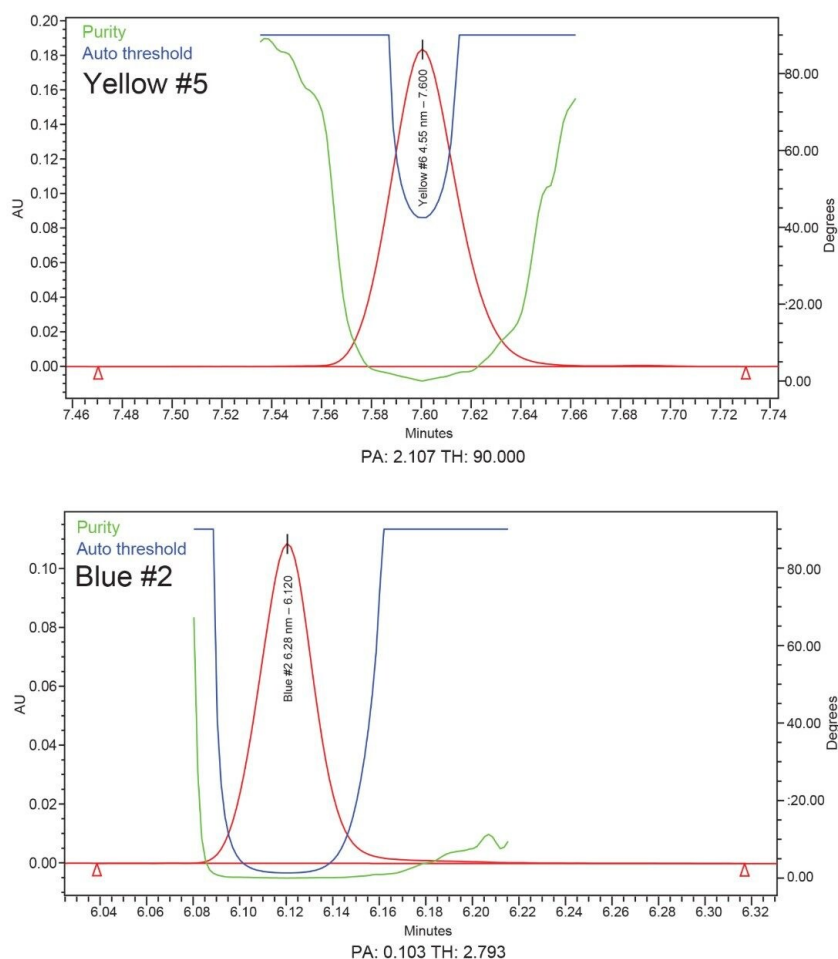


Figure 5. Empower Software representative Purity Angle and Purity Threshold comparison plots for Yellow #5 and Blue #2 observed in fruit flavored candy.

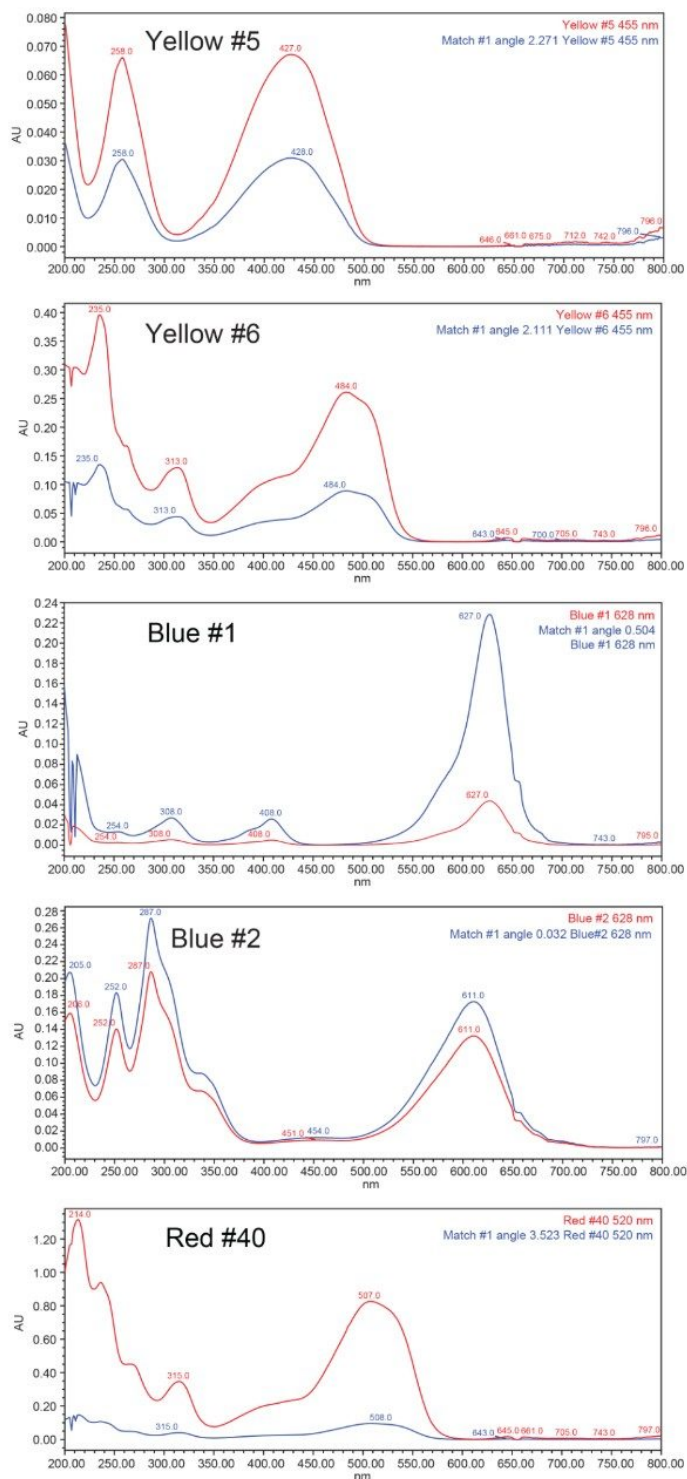


Figure 6. Match Angle PDA Library identification for mixed fruit candy. The

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*library reference spectrum (red trace) is compared by the software against the sample peak (blue trace).*

Samples were ranked according to the total quantity of synthetic food dye per serving (Table 3). The highest amount was found in the spicy hot snack sticks. The product contained primarily Yellow #6, combined with a relatively low quantity of Red #40. The visual appearance, a deep yellow, flour derived product heavily coated in red powder, correlated with the dye ratio reflected by the chromatographic results. Additionally, upon extraction, the product showed the most intense color by visual inspection when compared with other samples.

The second highest dye content per serving was observed in beverages. The single serving size of sports drinks was 360 mL. As a result, the mass of dye per serving was relatively high compared to small serving size products. Chewy fruit candy, coated chocolate, and jellybeans, packaged in approximately 2 oz bags per serving, showed the highest combination of different dyes (i.e. Red #40, Blue #1, Blue #2, Yellow #5, and Yellow #6) when compared to other samples. The dye content in the candy ranged between 4.8 mg and 18.6 mg per serving.

The quantity of dye per serving in individually wrapped, single flavor, sour hard candy ranged between 0.1 mg and 0.6 mg per serving. Each individually wrapped, sour hard candy weighed approximately 3.0 g, and was 1.0 cm<sup>2</sup> in diameter, which is a relatively small serving size. OTC drugs and cosmetics were the lowest of the samples tested, again due to the relatively small serving size. Over the counter syrup, gelcaps and tablets, contained 0.5 mg of dye or less per serving, which was equivalent to mouthwashes and toothpastes, which are not intended for ingestion by the consumer.

Type	Product	Red #40 (520nm)	Red #3 (520nm)	Green #3 (628nm)	Blue #1 (628nm)	Blue #2 (628nm)	Yellow #5 (455nm)	Yellow #6 (455nm)	Dye per serving (mg)
Snack Food	Snack sticks, spicy hot	0.63	–	–	–	–	–	26.68	27.3
Beverage	Sports drink, apple	–	–	–	0.81	–	19.68	–	20.5
Beverage	Sports drink, fruit punch	20.07	–	–	–	–	–	–	20.1
Candy	Fruit candy, chewy assorted	4.14	–	–	0.07	12.65	0.73	1.06	18.6
Candy	Coated chocolate, assorted	2.97	–	–	0.91	–	1.78	7.08	12.7
Beverage	Sports drink, orange	–	–	–	–	–	–	11.18	11.2
Breakfast cereal	Flakes, mixed berry	1.11	–	–	0.45	–	2.28	0.6	9.7
Breakfast cereal	Rice puffs, fruit flavored	2.21	–	–	0.21	–	3.91	1.88	8.2
Candy	Jellybeans, assorted	1.14	–	–	0.22	2.28	0.35	0.82	4.8
Breakfast cereal	Puffed wheels, mixed fruit	1.11	–	–	0.45	–	2.28	0.60	4.4
Beverage	Sports drink, blue cherry	–	–	–	3.14	–	–	–	3.1
Breakfast cereal	Marshmallows and grains, assorted	1.02	–	–	0.24	–	1.69	0.17	3.1
Beverage	Sports drink, lemon-lime	–	–	–	–	–	1.82	–	1.8
Candy	Hard candy, sour cherry	0.80	–	–	0.36	–	–	–	1.2
Cake decor	Sugar, red	0.75	0.49	–	–	–	–	–	1.2
Cake decor	Sugar, green	–	–	0.002	1.03	–	–	–	1.0
Candy	Hard candy, sour raspberry	–	–	–	0.62	–	–	–	0.6
OTC drug	Ibuprofen, red tablet	0.47	–	–	–	–	–	0.02	0.5
Cosmetic	Mouthwash, blue	–	–	–	0.38	–	–	–	0.4
Snack food	Blue corn chips	–	–	–	0.50	–	–	–	0.5
OTC drug	Cough syrup, honey	0.10	–	0.02	–	–	–	0.06	0.2
Candy	Hard candy, sour watermelon	0.22	–	–	–	–	–	–	0.2
Candy	Hard candy, sour apple	–	–	–	–	–	0.17	–	0.2
Cosmetic	Mouthwash, green	–	–	0.11	0.01	–	0.05	–	0.2
Cosmetic	Toothpaste, red gel	0.18	–	–	–	–	–	–	0.2
Cosmetic	Mouthwash, purple	0.04	–	–	0.02	–	–	–	0.1
Cake decor	Sprinkles, assorted	0.04	0.01	–	0.02	–	0.002	0.01	0.02
OTC drug	Acetaminophen, red gelcap	0.01	–	–	–	–	–	–	0.1
Candy	Sugar buttons, assorted	–	0.07	0.004	0.06	–	–	–	0.1
Candy	Liquid candy, sour cherry	0.05	–	–	–	–	–	–	0.1
Candy	Sugar tablet, grape	–	0.01	–	–	0.08	–	–	0.1
OTC drug	Ibuprofen, blue gelcap	–	–	0.04	0.001	–	–	–	0.04
Cosmetic	Toothpaste, blue gel	–	–	–	0.04	–	–	–	0.04
OTC drug	Naproxen, blue gelcap	–	–	–	–	0.03	–	–	0.03
Cosmetic	Toothpaste, green gel	–	–	–	0.01	–	0.01	–	0.02

*Table 3. Summary of snack foods, sports drinks, candy, breakfast cereals, and cosmetics analyzed ranked by synthetic food dye per serving.*

## Conclusion

In the work presented here, we showed a single chromatographic method for separation of ten synthetic food dyes in a variety of consumer products. Empower Software peak purity analysis confirmed that the Alliance iS HPLC System with PDA Detector successfully resolved dyes from matrix constituents. Detector linearity and peak purity analysis provided accurate dye quantification, while PDA Matching identified dyes against reference

spectra stored in the PDA Library. With the method presented, synthetic dyes in a variety of food matrices can be quantified and identified.

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## Featured Products

Alliance iS HPLC System <

<https://www.waters.com/nextgen/global/products/chromatography/chromatography-systems/alliance-is-hplc-system.html>>

Empower Chromatography Data Software (CDS) <

<https://www.waters.com/nextgen/global/products/informatics-and-software/chromatography-software/empower-software-solutions/empower-cds.html>>

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