Automation of AOAC 988.13 for the Identification of FD&C Color Additives in Foods using Solid Phase Extraction

Application Note FB0112

Keywords

GX-274 ASPEC™, AOAC, Spectrophotometer, Food and Beverage, Solid Phase Extraction, SPE, Color Additives, FD&C, FDA, TRILUTION ® LH, Liquid Handling

Introduction

A color additive is any dye, pigment or other substance which imparts color to a food, drug or cosmetic or to the human body (1). Color additives for food are commonly found in expected places, such as candies and powdered drink mixes, but also can be added to fruit skins to make them look more appealing. The addition of synthetic color additives is regulated closely by the FDA and is examined from the manufacturing of the pigment itself, through to its use and appropriate product labeling. Color additives have come under scrutiny recently because of their potential adverse physical and mental health effects that may be linked to ingestion, especially in children.

AOAC method 988.13 qualitatively tests for the presence of eight synthetic color additives, one of which is now banned. These color additives are FD&C colors approved for use in food, drugs and cosmetics. Additives are extracted from the sample matrix using solid phase extraction (SPE), and then identified by spectrum analysis on a spectrophotometer. The color additives examined by the method – FD&C Red Nos. 3 and 40, Blue Nos. 1 and 2, Yellow Nos. 5 and 6, Green No. 3 and the now banned Red No. 2 – are listed in Table 1, along with the E Number used in the EU and UK, common chemical name and chemical structure.

In this application, AOAC method 988.13 was automated using a Gilson GX-274 ASPEC to perform the SPE process just prior to automated spectrum analysis using the Agilent 8453 UV-visible Spectrophotometer with the Agilent 8-position Multicell Transport. Automation of routine and tedious manual methods allows for consistent reproducibility and higher throughput, freeing laboratory personnel to perform analysis and interpretation of spectra as well as other laboratory applications.

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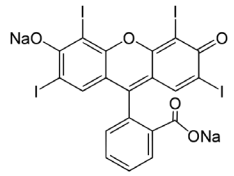
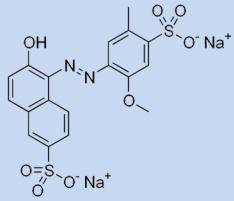
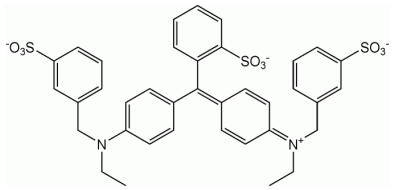
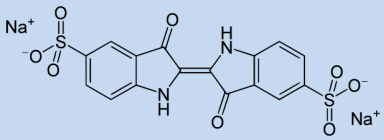
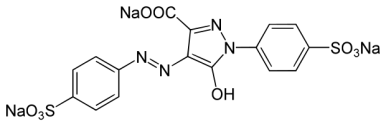
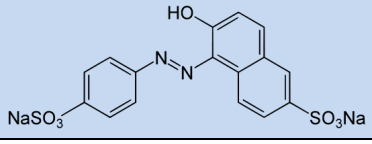
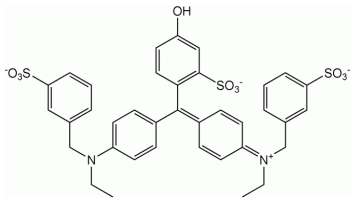
Table 1. FD&C Color Additives Identified in AOAC 988.13

FD&C Name	E Number	Common Name	Chemical Structure*
FD&C Red 3	E 127	Erythrosin B	
FD&C Red 40	E 129	Allura Red AC	
FD&C Blue 1	E 133	Brilliant Blue FCF Erioglaucine	
FD&C Blue 2	E 132	Indigo Carmine	
FD&C Yellow 5	E 102	Tartrazine	
FD&C Yellow 6	E 110	Sunset Yellow FCF	
FD&C Green 3	E 143	Fast Green FCF	
FD&C Red 2	E 123	Amaranth	Banned in the US in 1976

*Structures obtained from www.wikipedia.org

Materials & Methods

Samples & Solvents

- Allura Red AC (Sigma, P/N 458848)
- Tartrazine (Sigma, P/N T0388)
- Erioglaucine (Sigma, P/N 861146)
- Isopropanol (B&J, P/N 10071758)
    - 2.5, 13 and 20% solutions were prepared with NanoPure water
- Acetic Acid (Sigma, P/N 320099)
    - 1% solution was prepared with NanoPure water
- Sodium Hydroxide (EM Science, P/N SX0600-1)
    - 50% solution was prepared with NanoPure water
- Hydrochloric Acid (Sigma, P/N 258148)
- NanoPure Water
- Black Food Coloring (McCormick)
- Kool-Aid® (Grape and Orange, powder)

Apparatus

- Gilson GX-274 ASPEC™ with two 406 Dual Syringe Pump (Figure 1)
    - (4) 10 mL syringes
    - GX Transfer Port Assembly (Special 1785) (Figure 1 Insert)
    - Code 386 rack for 6 mL SPE cartridges

Figure 1. Gilson GX-274 ASPEC, Insert: GX Transfer Port Assembly

- Agilent 8453 UV-visible Spectrophotometer (Figure 2)
    - Multicell Transport (8-cell)
    - (8) Flow Cell (1 mm, 40 µL)
- Phenomenex Strata® C18-E SPE Cartridge (6 mL/1000 mg), P/N 8B-S001-JCH
- Grace Alltech® Extract-Clean™ Filter Columns (8.0 mL), P/N 211108

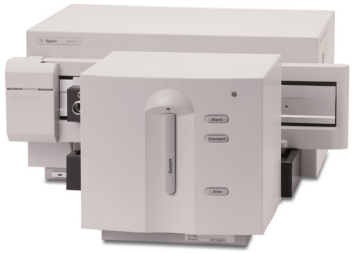


Figure 2. Agilent 8453 UV-visible Spectrophotometer with Multicell Transport

Method

All tests were run in quadruplicate.

Sample Preparation

Reference Standard Solutions: (Stock) 100 mg of the reference material was diluted to 100 mL with NanoPure water. (Standard) 10 mL of stock solution was diluted to 100 mL with the appropriate isopropanol solution.

Note: The Reference Standard Solution was made at 10x concentration to account for the pathlength of the flow cell used versus what was referenced in the method.

Black Food Coloring: A 1:200 dilution of black food coloring was prepared with NanoPure water.

Kool-Aid®: 1 g powder was dissolved in 100 mL NanoPure water and filtered on bed.

Solid Phase Extraction

The SPE scheme from AOAC 988.13 was utilized, with some volume modifications to adjust for cartridge size. This original SPE scheme can be found below in Figure 3. The volumes used for the application can be found in Tables 2, 3 and 4. Variables were used in the TRILUTION® LH Method to allow for modification and method development at the Application level.

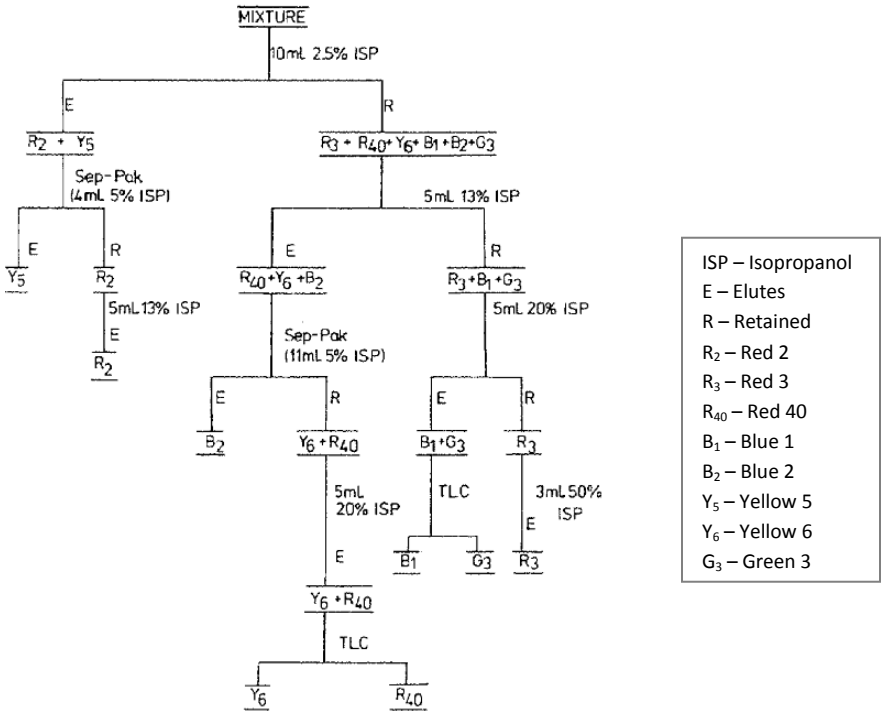


Figure 3. SPE Scheme for FD&C Color Identification from AOAC 988.13

Table 2. SPE Parameters used for the Separation of Black Food Coloring

Step	Solvent	Volume (µL)	Air Push (µL)
Condition #1	IPA	2000	1250
Condition #2	1% Acetic Acid	2500	1750
Load	Black Food Coloring	1500	600
Fractionate #1	2.5% IPA	2250	750
Fractionate #2	13% IPA	1500	1000
Fractionate #3	20% IPA	3000	2000

Table 3. SPE Parameters used for the Separation of Grape Kool-Aid®

Step	Solvent	Volume (µL)	Air Push (µL)
Condition #1	IPA	2000	1250
Condition #2	1% Acetic Acid	2500	1750
Load	Kool-Aid	2000	1200
Wash	2.5% IPA	3000	1500
Fractionate #1	13% IPA	2000	1000
Fractionate #2	20% IPA	3000	2000

Table 4. SPE Parameters used for the Separation of Orange Kool-Aid®

Step	Solvent	Volume (µL)	Air Push (µL)
Condition #1	IPA	2000	1500
Condition #2	1% Acetic Acid	2500	2000
Load	Kool-Aid	2000	1200
Fractionate #1	2.5% IPA	4000	2500
Fractionate #2	13% IPA	3000	2000

Automated SPE Fraction Preparation for Absorbance Reading

The fractions collected from the automatic SPE process were then prepared for identification using the GX-274 ASPEC™ controlled with TRILUTION® LH software.

1) The fraction was transferred to a clean test tube; volume transferred was 250 µL less than the amount of solvent used for elution in the Fractionate step
2) The fraction was diluted to 6 mL with appropriate IPA solution and volume
3) 2 mL was transferred to each of two sets of clean tubes
4) 1500 µL was transferred to the flow cells via the transfer ports, and an absorbance reading was taken on the neutral diluted fraction
5) A drop (23 µL) of concentrated hydrochloric acid was added to the second set of test tubes and the solution was mixed
6) 1500 µL was transferred to the flow cells via the transfer ports, and an absorbance reading was taken on the acidic fraction solution
7) A drop (23 µL) of 50% sodium hydroxide solution was added to the third set of test tubes and the solution was mixed
8) 1500 µL was transferred to the flow cells via the transfer ports, and an absorbance reading was taken on the basic fraction solution

A blank of the appropriate IPA solution was taken prior to each set of absorbance readings. The readings were taken from 190 to 1100 nm; however the spectra were only analyzed from 350 to 750 nm, as specified in AOAC 988.13. The flow cells and lines were rinsed with 5 mL NanoPure water after each reading to eliminate carryover between samples.

Software

The entire automated AOAC 988.13 application, from the solid phase extraction to the absorbance readings, was controlled and coordinated by Gilson's liquid handling software TRILUTION® LH v2.0. TRILUTION LH was programmed to coordinate with Agilent ChemStation for UV-visible spectroscopy software to control the absorbance readings and movement of the Multicell Transport on the 8453 Spectrophotometer. All spectra analysis was performed using the ChemStation software.

Application Format in TRILUTION LH

For flexibility and efficiency, the application was separated into several TRILUTION LH Methods, and contained variables to permit adaptation from the Application screen. All of the Methods were run sequentially from the TRILUTION LH Sample List. A screenshot of the Sample List for the black food coloring samples can be seen in Figure 4. The Sample List has been labeled to identify which step of the application each Method is associated with. The variable matrix is not shown.

	Method Name	
1	Conditions and Load	Solid Phase Extraction
2	Fractionate	
3	Fractionate	
4	Fractionate	
5	Sample Prep	Yellow Fraction Preparation and Analysis
6	2_5 Blank Injection	
7	Acidify	
8	2_5 Blank Injection	
9	Basify	
10	2_5 Blank Injection	
11	Sample Prep	Red Fraction Preparation and Analysis
12	13 Blank Injection	
13	Acidify	
14	13 Blank Injection	
15	Basify	
16	13 Blank Injection	
17	Sample Prep	Blue Fraction Preparation and Analysis
18	20 Blank Injection	
19	Acidify	
20	20 Blank Injection	
21	Basify	
22	20 Blank Injection	

Figure 4. Sample List for AOAC 988.13 Analysis of Black Food Coloring

Coordination with the Agilent 8453 UV-visible Spectrophotometer
The Multicell Transport for the spectrophotometer was established to use 4 constant blank cells and 4 cells connected to the transfer ports on the GX-274 ASPEC™ for sample introduction, as seen below in Figure 5.

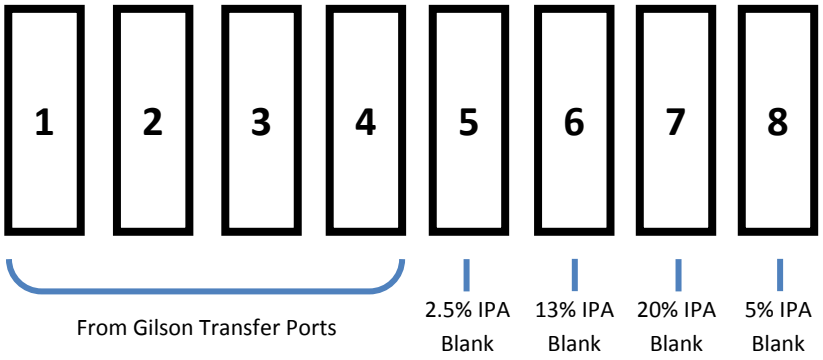


Figure 5. Arrangement of Flow Cells in the Multicell Transport

The Method in TRILUTION® LH transferred the sample to the flow cells via the transfer ports, ran an executable file which triggered the blank and sample absorbance readings in the Agilent ChemStation software, rinsed the liquid handler probes and then transferred water to the flow cells to rinse out the cells and lines prior to the next set of samples. A different version of the Method was made for each blank required, as there were different executable files for each blank cell location (Figure 5). The tasks used in the TRILUTION LH Method can be seen below in Figure 6.

Figure 6. TRILUTION LH Method to Trigger the Agilent 8453 Spectrophotometer

Analysis of Spectra

All spectra were analyzed by Spectrum/Peaks Analysis in the Standard Mode of ChemStation for UV-visible Spectroscopy. The wavelength range was from 350 to 750 nm and the software annotated 2 peaks within that range (Figure 7). Spectra were overlaid in the software to create the neutral, acid and base comparison spectra required for the identification.

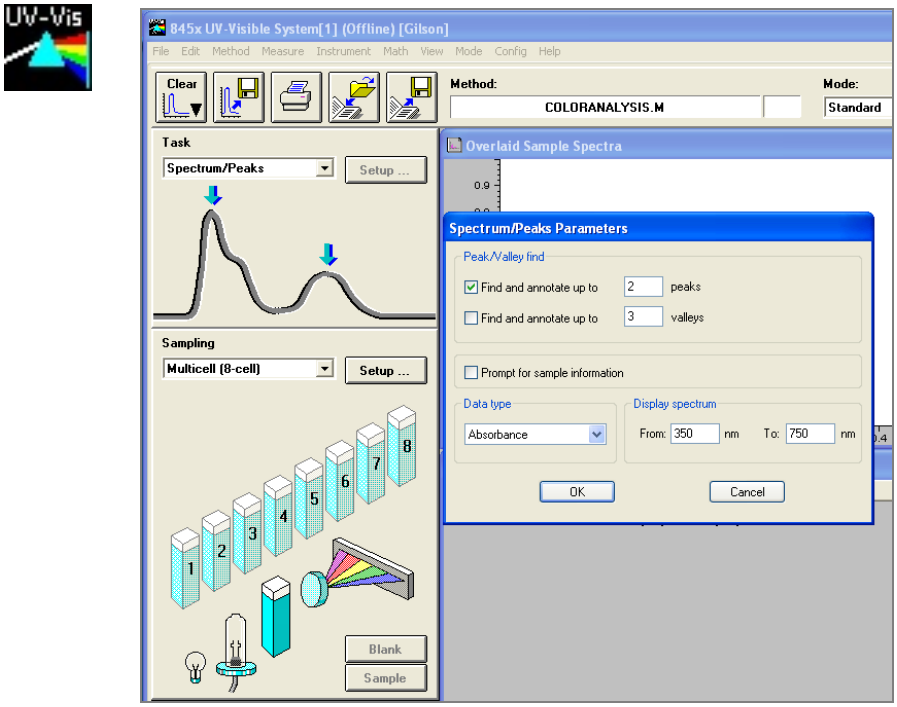


Figure 7. ChemStation for UV-visible Spectroscopy Analysis Parameter

Results

Prior to running samples, equivalency between manual and automated procedures was established. First, it was established that adding only one drop of 50% sodium hydroxide solution was equivalent to the stated procedure of adding one drop of concentrated hydrochloric acid and two drops of 50% sodium hydroxide for the basic spectra. These drops were added manually with a glass Pasteur pipette to the blue standard solution before being analyzed. Representative spectra can be seen below in Figures 8 and 9. Second, it was established that adding the drop manually via a glass pipette was equivalent to adding the corresponding volume (23 µL) by the GX-274 ASPEC™ from a capped 2 mL vial having a pierceable septa. A representative spectrum can be seen in Figure 10.

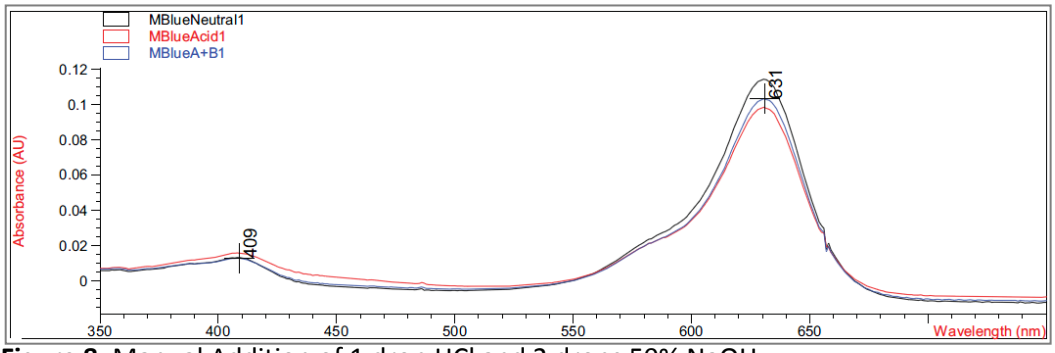


Figure 8. Manual Addition of 1 drop HCl and 2 drops 50% NaOH

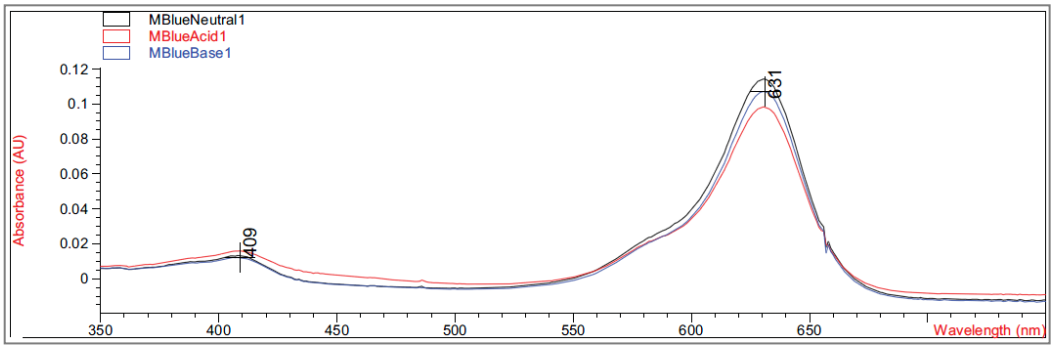


Figure 9. Manual Addition of 1 drop 50% NaOH

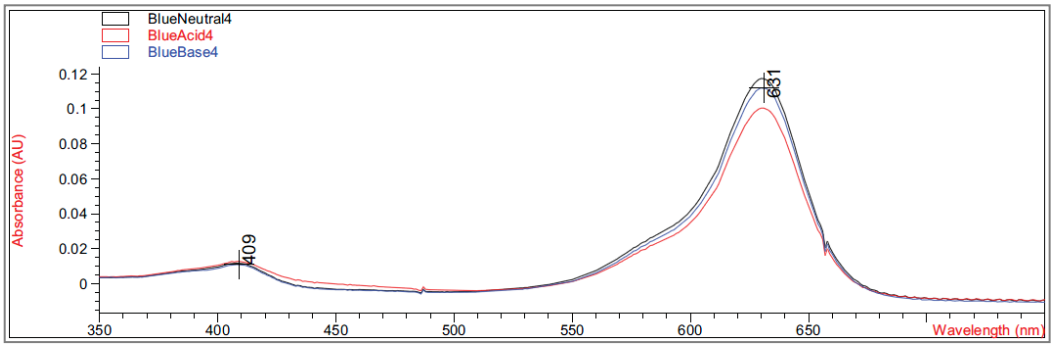


Figure 10. Automated Addition of 23 µL 50% NaOH

It was concluded that the automated acidification and basification of the samples were equivalent to the manual methods described in AOAC 988.13. The differences in absorbance from one method to another were within the %CV for the comparison between the two manual methods. When comparing the manual method to the automated method, the automated acid addition was within 1.5% of the manual absorbance values, while the base addition was within 2.5 % of the manual absorbance values.

After establishing equivalency, the standard solutions were analyzed to provide comparison spectra for the samples. The representative standard spectra for FD&C Yellow No. 5, FD&C Red No. 40 and FD&C Blue No. 1 can be found below in Figures 11, 12 and 13 respectively.

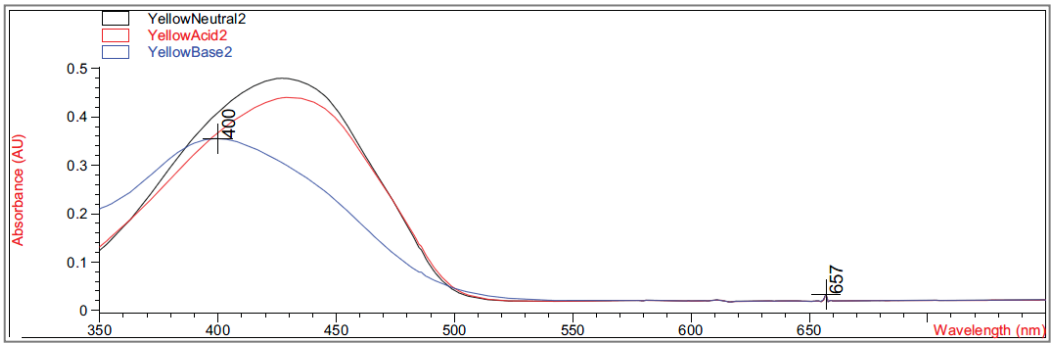


Figure 11. Standard Spectra for FD&C Yellow No. 5

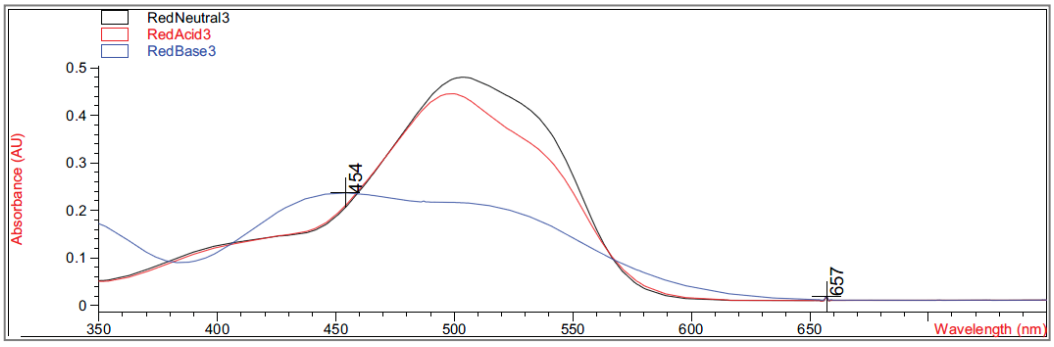


Figure 12. Standard Spectra for FD&C Red No. 40

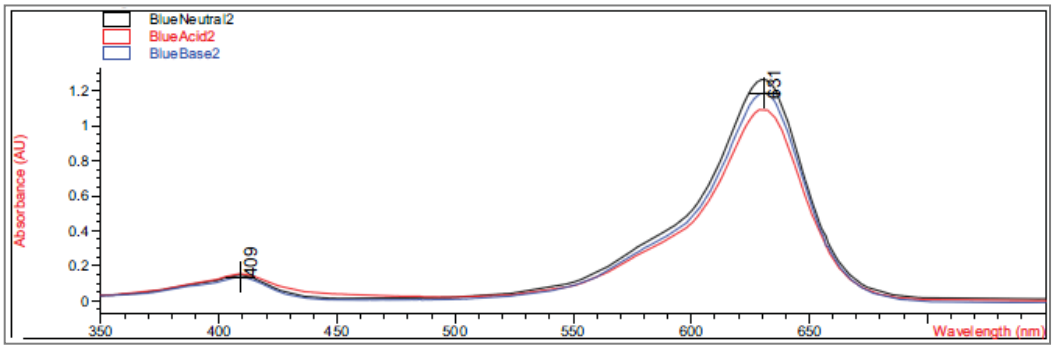


Figure 13. Standard Spectra for FD&C Blue No. 1

McCormick brand black food coloring (Figure 14) was used as a primary test sample as it has a relatively clean matrix associated with it. The ingredient labeling for the black food coloring indicated it contained FD&C Yellow No. 5, FD&C Red No. 40 and FD&C Blue No. 1. The resultant spectra confirmed the presence of these color additives. Representative spectra from each of the fractions can be found below in Figures 15, 16 and 17. A slight carryover of FD&C Red No. 40 into the FD&C Blue No. 1 fraction can be observed in Figure 17.

Absorbance and wavelength information for each of the black food coloring samples can be found below in Tables 5, 6 and 7. It should be noted that although the absorbance values from each sample could have up to a 5.8% CV, the %CV of the ratio of the neutral peak to the acidic or basic peak was much more consistent, with the largest difference being 2.2% CV. Grape and Orange Kool-Aid® powder were used as secondary test samples for this application (Figure 18).

Figure 14. McCormick Black Food Coloring

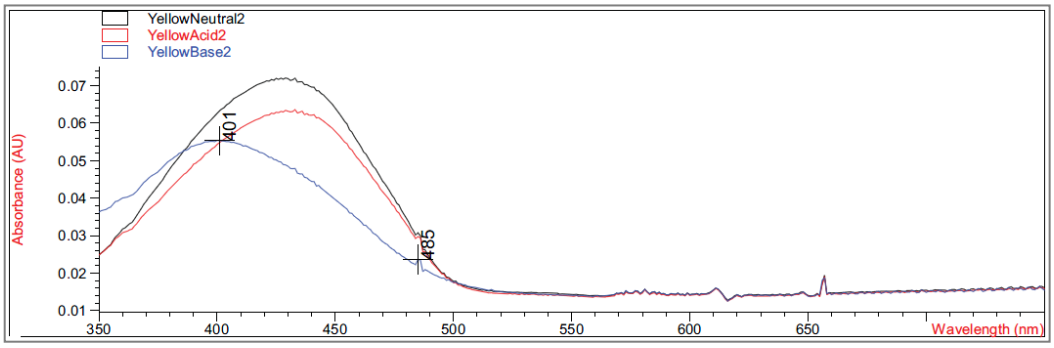


Figure 15. FD&C Yellow No. 5 from Black Food Coloring

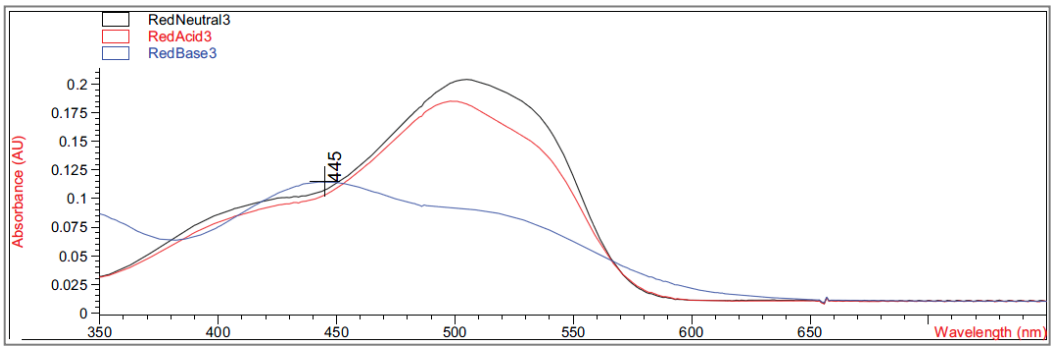


Figure 16. FD&C Red No. 40 from Black Food Coloring

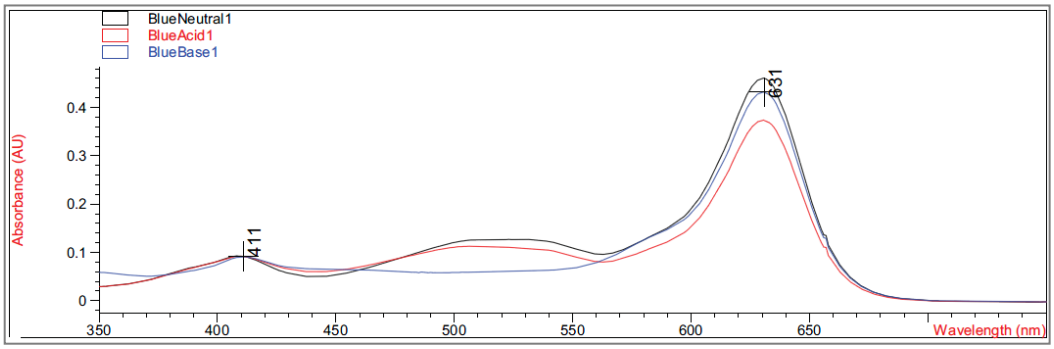


Figure 17. FD&C Blue No. 1 from Black Food Coloring

Table 5. FD&C Yellow No. 5 Absorbance Values for Black Food Coloring

Sample	λ (Neutral)	AU (Neutral)	λ (Acid)	AU (Acid)	Acid/ Neutral	λ (Base)	AU (Base)	Base/ Neutral
1	426	0.06627	432	0.05819	0.87813	400	0.05200	0.78459
2	427	0.07198	433	0.06358	0.88332	401	0.05533	0.76863
3	432	0.06992	432	0.06380	0.91243	400	0.05381	0.76953
4	432	0.06462	432	0.05887	0.91103	400	0.04833	0.74797
Ave	429.25	0.06820	432.25	0.06111	0.89623	400	0.05237	0.76784
%CV	0.75	4.93	0.12	4.90	2.01	0.12	5.76	1.96

Table 6. FD&C Red No. 40 Absorbance Values for Black Food Coloring

Sample	λ (Neutral)	AU (Neutral)	λ (Acid)	AU (Acid)	Acid/ Neutral	λ (Base)	AU (Base)	Base/ Neutral
1	505	0.23225	499	0.20725	0.89236	447	0.12522	0.53916
2	505	0.22156	498	0.19814	0.89429	444	0.12479	0.56323
3	505	0.20416	499	0.18510	0.90664	445	0.11468	0.56172
4	505	0.21741	499	0.19816	0.91146	447	0.11824	0.54386
Ave	505	0.21885	498.75	0.19716	0.90119	445.8	0.12073	0.55199
%CV	0.00	5.31	0.10	4.62	1.03	0.34	4.26	2.22

Table 7. FD&C Blue No. 1 Absorbance Values for Black Food Coloring

Sample	λ (Neutral)	AU (Neutral)	λ (Acid)	AU (Acid)	Acid/ Neutral	λ (Base)	AU (Base)	Base/ Neutral
1	630	0.46048	630	0.37377	0.81170	631	0.43197	0.93809
2	630	0.47513	630	0.40056	0.84305	630	0.45019	0.94751
3	630	0.47172	630	0.38937	0.82543	631	0.44424	0.94175
4	630	0.46956	630	0.38524	0.82043	631	0.44575	0.94929
Ave	630	0.46922	630	0.38724	0.82515	630.75	0.44304	0.94245
%CV	0.00	1.33	0.00	2.86	1.60	0.08	1.76	0.50

Figure 18. Kool-Aid Unsweetened Drink Mix in Grape and Orange Flavors

The drink mixes provided a slightly more complex sample matrix, containing chemicals such as citric acid, calcium phosphate and natural flavorings. The Grape Kool-Aid® powder was labeled as having FD&C Red No. 40 and FD&C Blue No. 1. The Orange Kool-Aid listed both FD&C Yellow No. 5 and FD&C Red No. 40, as well as FD&C Red No. 40 Lake. Some overlapping of color bands was apparent in the spectra for both Kool-Aid flavors.

Representative spectra for the Grape Kool-Aid can be found in Figures 19 and 20. Crossover between the FD&C Red No. 40 and FD&C Blue No. 1 fractions is apparent in both spectra, but does not hinder the identification of the color additive.

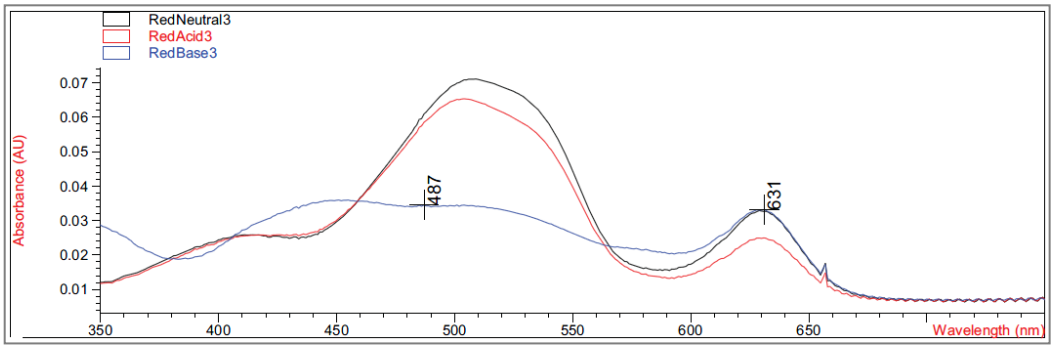


Figure 19. FD&C Red No. 40 from Grape Kool-Aid

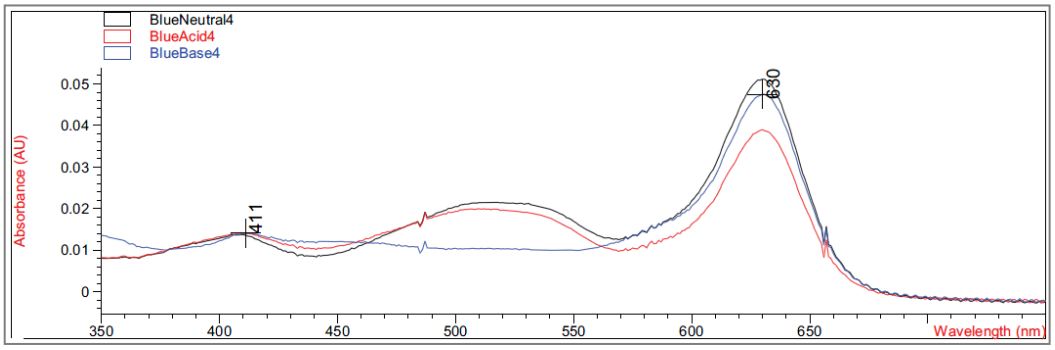


Figure 20. FD&C Blue No. 1 from Grape Kool-Aid

The absorbance and wavelength information for the FD&C Red No. 40 and FD&C Blue No. 1 in the Grape Kool-Aid samples can be found in Tables 8 and 9 respectively. It can be noted that the %CV is higher for these samples than in the black food coloring. This is not unexpected as the concentration is significantly lower. Slight differences with lower concentration and absorbance values have a greater impact.

Table 8. FD&C Red No. 40 Absorbance Values for Grape Kool-Aid®

Sample	λ (Neutral)	AU (Neutral)	λ (Acid)	AU (Acid)	Acid/ Neutral	λ (Base)	AU (Base)	Base/ Neutral
1	508	0.06693	503	0.05848	0.87363	449	0.03020	0.45116
2	508	0.07696	503	0.06832	0.88768	449	0.03816	0.49583
3	507	0.07105	504	0.06534	0.91961	451	0.03595	0.50593
4	508	0.06844	504	0.06166	0.90094	451	0.03024	0.44184
Average	507.75	0.07085	503.5	0.06345	0.89547	450.0	0.03364	0.47369
%CV	0.10	6.24	0.11	6.76	2.19	0.26	12.03	6.73

Table 9. FD&C Blue No. 1 Absorbance Values for Grape Kool-Aid

Sample	λ (Neutral)	AU (Neutral)	λ (Acid)	AU (Acid)	Acid/ Neutral	λ (Base)	AU (Base)	Base/ Neutral
1	630	0.05307	630	0.04037	0.76054	630	0.04889	0.92109
2	630	0.05872	630	0.04312	0.73439	630	0.04811	0.81932
3	630	0.06036	630	0.04868	0.80655	630	0.05682	0.94135
4	630	0.05111	630	0.03895	0.76201	630	0.04752	0.92970
Average	630	0.05582	630	0.04278	0.76587	630	0.05033	0.89392
%CV	0.00	7.93	0.00	10.05	3.91	0.00	8.66	7.32

Representative spectra for the Orange Kool-Aid can be found in Figures 21 and 22. Due to low concentration in the Orange Kool-Aid, as well as the observance of some FD&C Yellow No. 5 remaining in the fraction, the absorbance for the FD&C Red No. 40 was not quantifiable. The characteristic shapes seen in the standard are, however, apparent in the 475 to 575 nm range and the fraction can thus still be qualitatively identified.

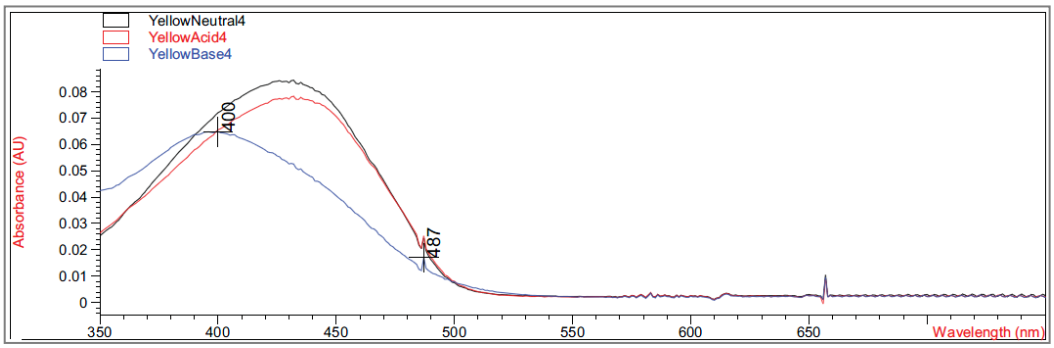


Figure 21. FD&C Yellow No. 5 from Orange Kool-Aid

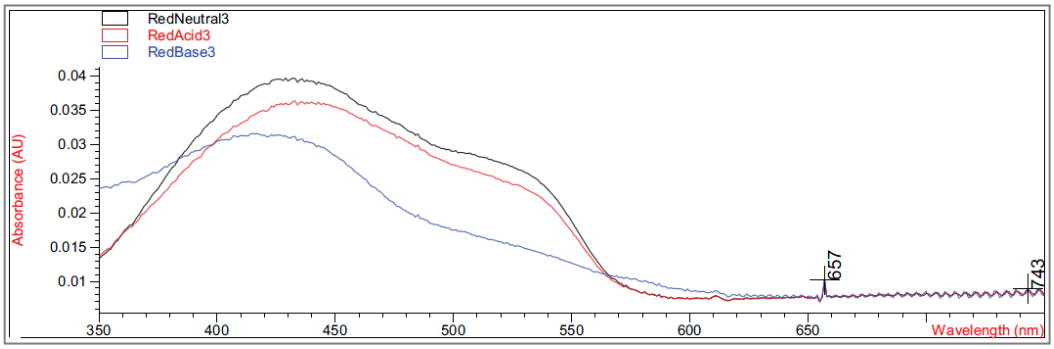


Figure 22. FD&C Red No. 40 from Orange Kool-Aid®

The absorbance and wavelength information for the FD&C Yellow No. 5 fraction of the Orange Kool-Aid can be found below in Table 10. As stated above, the FD&C Red No. 40 fraction was not quantifiable due to low concentration and overlap with the FD&C Yellow No. 5 peaks. The FD&C Yellow No. 5 has a slightly higher absorbance that resulted in a more consistent reading than the color additives in the Grape Kool-Aid and %CV values near 5%. The %CV for the ratio of the neutral peak to the acidic or basic peak was improved over the Grape Kool-Aid sample, with values less than 3% CV.

Table 10. FD&C Yellow No. 5 Absorbance Values for Orange Kool-Aid

Sample	λ (Neutral)	AU (Neutral)	λ (Acid)	AU (Acid)	Acid/ Neutral	λ (Base)	AU (Base)	Base/ Neutral
1	429	0.09544	432	0.08661	0.90745	399	0.07062	0.73993
2	429	0.09108	430	0.08315	0.91295	401	0.06745	0.74059
3	432	0.08857	432	0.08515	0.96141	400	0.06936	0.78311
4	432	0.08430	432	0.07823	0.92802	400	0.06483	0.76906
Average	430.5	0.08985	431.5	0.08329	0.92746	400.0	0.06807	0.75817
%CV	0.40	5.19	0.23	4.39	2.61	0.20	3.70	2.83

Summary

Finding viable automation solutions to tedious and manual methods creates efficiency and day-to-day consistency. By automating standard lab practices such as spectrophotometer readings and sample preparation, a typical laboratory is able to increase sample throughput, eliminate personnel to personnel variation and use laboratory personnel for other more important laboratory work such as data analysis. The basic process of how the manual method was automated can also be applied to other manual methods requiring both qualitative, as in AOAC 988.13, and quantitative spectrophotometric sample readings, expanding its application into many different sample categories.

This application shows equivalency between established manual methodology and its automated counterpart across different sample matrices. Through additional development and optimization the overlapping of color bands could be eliminated. Using flow cells with longer pathlengths could also assist with obtaining higher absorbance readings for low concentration samples.

References

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