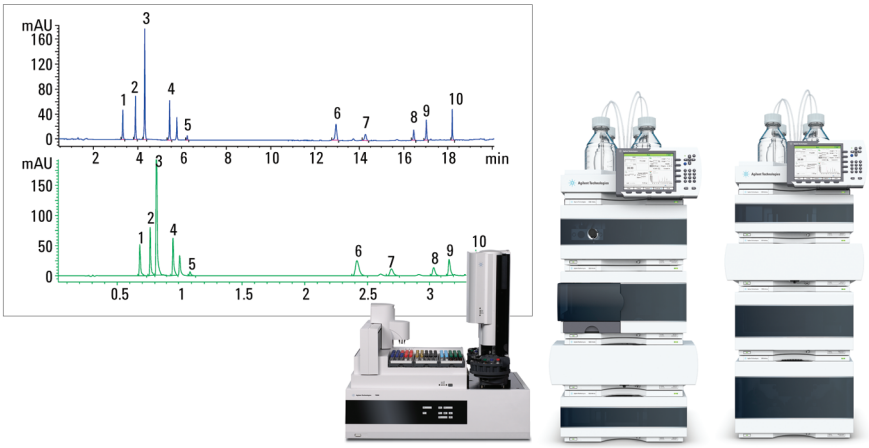
Agilent Application Solution

Analysis of color additives in sweets

Application Note

Food Testing



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Abstract

Synthetic or artificial colors are used as additives in food and drinks to improve the appearance of the product. In this study, a robust reverse phase high performance liquid chromatographic (RP-HPLC) method for simultaneous determination of 10 synthetic colorants was developed. Separation and quantification was achieved by an Agilent 1260 Infinity LC System using an Agilent Poroshell EC-C18 column. Robustness of the method was established by partial validation. Suitability of this method to quantify artificial colorants from food matrix is demonstrated by analyzing color additives from sweets. Finally, this HPLC method was effectively transferred to a short Ultra High Pressure Liquid Chromatographic (UHPLC) method using an Agilent 1290 Infinity LC System for faster analysis without compromising resolution. With the Agilent 1290 Infinity Diode Array Detector (DAD), various wavelengths were selected to quantify different colorants at their absorbance maxima. The limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, and linearity of each colorant were established using both methods. Sample preparation for LOD, LOQ and linearity studies was facilitated by incorporating the Agilent 7696A Sample Prep WorkBench into the analytical workflow.

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Introduction

A color additive is defined as any dye, pigment, or substance which, when added to food, is capable of imparting color[1]. There are natural and synthetic color additives which mainly originate from plants or animals. Turmeric and saffron are two examples of this. Synthetic colors are chemically synthesized colors like tartrazine and indigo carmine[2]. There are many reasons for adding color in food. Adjusting the color loss due to long term storage conditions, correcting the natural variations in color, and providing color to colorless foods are some of them. In fact, color additives are an unavoidable part of most packed foods on the market[1]. It is proven that overexposure to artificial colors beyond the allowed daily intake limit can provoke hyperactivity and other disturbed behavior in children[3]. The Food and Drug Administration (FDA) has regulations to control and ensure the usage of only permitted color additives in food. This underlines the importance of precise analytical techniques to identify and quantify the colorants.

In this Application Note, we developed a reverse phase high pressure liquid chromatography method on an Agilent Poroshell 120 EC-C18 column. The water-solubility of food colorants makes reverse phase HPLC the ideal analysis technique for these substances.

Method

Instruments and software

An Agilent 1260 Infinity Quaternary LC System, consisting of the following modules was used:

- Agilent 1260 Infinity Quaternary Pump and vacuum degasser (G1311B)
- Agilent 1260 Infinity High-Performance Autosampler (G1367E)
- Agilent 1260 Infinity Thermostatted Column Compartment (G1316A)
- Agilent 1260 Infinity Diode Array Detector (G4212B) with Max-Light flow cell (60 mm path length) (G4212-60007)
- Agilent Poroshell 120 EC-C18 column 4.6 x 150 mm, 2.7 µm (693975-902)

The UHPLC analysis was developed and performed using the Agilent 1290 Infinity LC System consisting of:

- Agilent 1290 Infinity Binary Pump with integrated vacuum degasser (G4220A) and 100 µL Jet Weaver mixer.
- Agilent 1290 Infinity High Performance Autosampler (G4226A)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1290 Infinity Diode Array Detector (G4212A) with Max-Light flow cell (1.0 µL dispersion volume, 10 mm path length) (G4212-60008)
- Agilent Poroshell 120 EC-C18 columns with internal diameters of 2.1 mm and lengths of 75 mm, packed with 2.7-µm particles (697775-902)

Both systems were controlled using the Agilent ChemStation revision B.04.02.

The dilution series for the linearity levels were prepared using the Agilent 7696A Sample Prep WorkBench.

Reagents and materials

All the chemicals and solvents used were HPLC grade and highly purified water from a Milli Q water purification system (Millipore Elix 10 model, USA) was used. Methanol was of super gradient grade and was purchased from Lab-Scan (Bangkok, Thailand). Disodium hydrogen phosphate and o-phosphoric acid were purchased from Fluka (Germany). Dimethyl sulphoxide (DMSO) was purchased from Qualigens (India). Standards of tartrazine, amaranth, indigo carmine, ponceau 4R, sunset yellow FCF, carmoisine, fast green FCF, acid blue/eryoglaucine, ponceau 3R, and erythrosine B were purchased from Aldrich (India). The sweets for recovery and quantification analysis were purchased locally.

Chromatographic parameters

Chromatographic parameters used for reverse phase liquid chromatography and UHPLC are shown in Table 1.

Colorant standard solution

Standard stock solutions of tartrazine, amaranth, indigo carmine, ponceau 4R, sunset yellow FCF, carmoisine, fast green FCF, acid blue/eryoglaucine, ponceau 3R, and erythrosine B were prepared individually by weighing approximately 20 mg of the standard and transferring it to a 10-mL volumetric standards flask. A 300-µL amount of DMSO was added to each flask and a premixed solution of mobile phase A and B in the ratio 80:20 was used as diluent. Sonication was used when required.

Mixed standard solution and linearity levels

About 100 µL of each standard were precisely mixed with diluent to get a 2,000 µL standard mix of colorants at a concentration of 200 ppm each. Linearity levels were prepared by subsequent serial dilution of this 200 ppm standard mix solution using the Agilent 7696A Sample Prep WorkBench. The linearity standard solutions were covering a range of 0.01 ng/µL to 200 ng/µL (10 levels and 6 replicates).

Parameter	Agilent 1260 Infinity Quaternary LC System	Agilent 1290 Infinity LC System
Column	Agilent Poroshell 120 EC-C18, 4.6 x 150 mm, 2.7 µm, (p/n N693975-902)	Agilent Poroshell 120 EC-C18, 2.1 x 75 mm, 2.7 µm (p/n 697775-902)
Column oven	45 °C	45 °C
Injection volume	5 µL (Needle with wash, flush port active for 5 seconds)	1 µL (Needle with wash, flush port active for 5 seconds)
Sample thermostat	5 °C	5 °C
Mobile phase A	10 mM Na_2HPO_4, pH 7	10 mM Na_2HPO_4, pH 7
Mobile phase B	Methanol	Methanol
Gradient	At 0 min: 5% B	At 0 min: 5% B
	At 4 min: 30% B	At 0.15 min: 5% B
	At 10 min: 40% B	At 0.5 min: 30% B
	At 14 min: 40% B	At 2.3 min: 40% B
	At 18 min: 95% B	At 2.6 min: 40% B
	At 22 min: 95% B	At 3.25 min: 95% B
	At 22.1 min: 5% B	At 4.00 min: 95% B
		At 4.01 min: 5% B
Post run time	5 minutes	1 minute
Flow rate	1.2 mL/min	0.7 mL/min
Flow cell	60 mm path (p/n G4212-60007)	10 mm path (p/n G4212-60008)
Data acquisition	288 nm: Indigo carmine	288 nm: Indigo carmine
	428 nm: Tartrazine	428 nm: Tartrazine
	484 nm: Sunset yellow FCF	484 nm: Sunset yellow FCF
	511 nm: Ponceau 4R and Ponceau 3R	511 nm: Ponceau 4R and Ponceau 3R
	520 nm: Amaranth and Carmoisine	520 nm: Amaranth and Carmoisine
	530 nm: Erythrosin B	530 nm: Erythrosin B
	626 nm: Fast green FCF and Acid blue	626 nm: FastGreen FCF and Acid blue
Aquisition rate	20 Hz, 0.013 min peak width, (0.25 s response time)	80 Hz, 0.003 min peak width, (0.062 s response time)

Table 1
Chromatographic parameters used for the Agilent 1260 Infinity System and the Agilent 1290 Infinity LC System.

Sample preparation for color quantification and recovery studies

Five different types of samples, sweets containing various colors, were used for color quantification and recovery studies. Colors from 2 g sweets were extracted by a simple process using sequential addition of 400 µL DMSO and 20 mL diluent. After sonication and centrifugation at 8,300 rcf for 10 minutes using C0650 rotor on a Beckman Coulter Allegra X22R centrifugation system, the solution was filtered through a 0.25-µm PTFE Agilent Econofilter syringe filter membrane, and used for analysis. Recovery studies were performed using spiked and unspiked samples of sweets. An on-column concentration of 25 ng standard mix was used for sample spiking. The extraction procedure was the same as before.

Precautions

To extend the stability of compounds in solution, all the prepared solutions were wrapped in aluminum foil and stored in a refrigerator at 4 °C in the dark, when not in use. The thermostatted autosampler tray was maintained at 5 °C during the analysis.

Procedure

Calibration levels shown in Table 2, were prepared by subsequent dilution of 200 ng/µL standard mix solution with diluent. The Agilent 7696A Sample Prep WorkBench equipped with a 500 µL syringe was operated in two subsequent sequences to create the linearity levels. In the first sequence, a fixed amount of diluent was added to each vial and in the second sequence, 250 µL of 200 ng/µL solution was added to the vials and vortexed for 15 seconds. Note that instead of running two sequences, the steps can also be programmed in one method and run in one sequence. Serial dilutions were carried out by taking 250/100 µL from the previous level and adding to the next level vial. The syringe parameters used in the setup of the Agilent 7696A Sample Prep WorkBench are given in Table 3. The Agilent 7696A Sample Prep WorkBench[4] setup is well described in the Agilent Application Note, publication number 5990-6850EN.

A 5-µL solution of diluent with DMSO was injected as a blank and followed by each calibration level in six replicates. Area and retention time (RT) information of each level were used to calculate standard deviation (SD) and relative standard deviation (RSD) values. LOD and LOQ were established from the lower linearity level injections. The average area of colorant peaks in each linearity level was plotted against the concentration to construct linearity curves.

Initial concentration (ppm or ng/µL)	Volume taken (µL) (second sequence)	Diluent (µL) prepared (first sequence)	Total vol. (µL)	Concentration of resulting liquid (ng/µL)	On-column with 5 µL injection volume (ng)	Level name
200	250	250	500	100	500	10
100	100	400	500	20	100	9
20	250	250	500	10	50	8
10	100	400	500	2	10	7
2	250	250	500	1	5	6
1	100	400	500	0.2	1	5
0.2	250	250	500	0.1	0.5	4
0.1	100	400	500	0.02	0.1	3
0.02	250	250	500	0.01	0.05	2
0.01	100	400	500	0.002	0.01	1

Table 2
Dilution details for calibration level preparation.

Parameter	Solvent prewash 1	Dispense wash	Dispense pumps	Dispense settings
Number pumps or washes	1	1	2	
Wash volume (µL)	250	250	50	
Draw speed (µL/min)	500	500	500	500
Dispense speed (µL/min)	2500	2500	2500	2500
Needle depth offset (mm)	-1	-1	-1	-1
Viscosity delay (s)	1	1	1	1
Turret solvent	A			
Air gap (%syr.vol)	0			0

Table 3
Agilent 7696A Sample Prep WorkBench syringe parameters.

Six critical method parameters were changed to evaluate the robustness of the method. A standard mix of about 30 ng (on-column) of each colorant was injected in six replicates and data was used for studying the robustness of the method. Recovery studies were performed by injecting with and without spiking 25 ng color additive standard to 2 g sweets. Using the characteristic spectra of all ten color standards, a UV spectral library was created. Along with the retention times this library was used to identify color additives in sweets.

The method was effectively transferred to UHPLC. LOD, LOQ, and linearity of each colorant were evaluated and precision of the method was established by Area and RT RSD. Linearity curves for all colors using the UHPLC method were also plotted. The UHPLC method allows the analysis to be performed much faster without compromising on resolution.

Results and discussion

Separation and detection

Excellent separation of 10 colorants in 20 minutes was achieved using an Agilent Poroshell 120 EC-C18 (150 mm x 4.6 mm, 2.7 µm) column. The absorbance maximum was found to be different for different colors. The chromatographic elution patterns of 10 colors are shown in Figure 1 and the list of colors with individual absorbance maxima are shown in Table 4. We used the peak purity feature in the ChemStation software to check the purity of each peak and thus the specificity of the method was evaluated. Precision, linear range, accuracy, specificity, recovery, and robustness studies were done to validate the method.

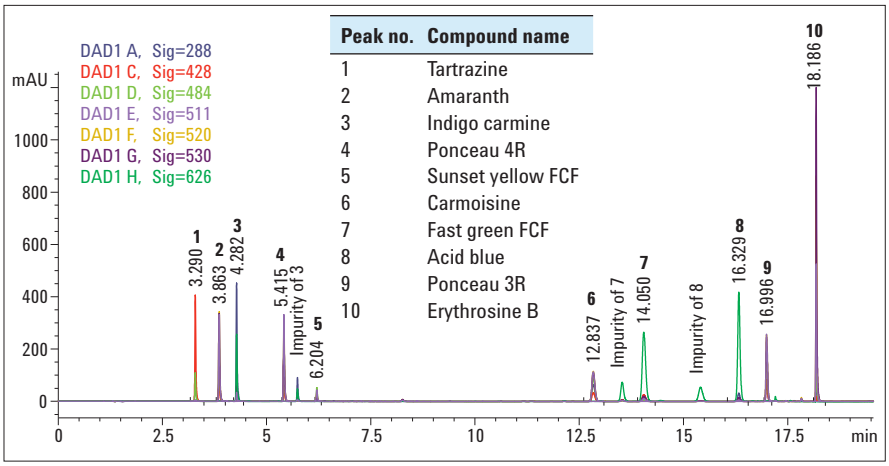

Figure 1
Separation of 10 colorants using a 15-cm Agilent Poroshell 120 EC-C18 column. Traces from seven different wavelengths are overlaid.

Sl no.	Compound name	Molecular formula	Molecular weight	Retention time	Absolute maximum
1	Tartrazine	$C_{16}H_9N_4Na_3O_9S_2$	534.36	3.29	428
2	Amaranth	$C_{20}H_{11}N_2Na_3O_{10}S_3$	604.47	3.86	522
3	Indigo carmine (Indigotine)	$C_{16}H_8N_2Na_2O_8S_2$	466.35	4.28 (imp 5.74)	288 and 612
4	Ponceau 4R (Ponceau SX)	$C_{20}H_{11}N_2Na_3O_{10}S_3$	604.47	5.41	510
5	Sunset yellow FCF	$C_{16}H_{10}N_2Na_2O_7S_2$	452.37	6.20	482
6	Carmoisine	$C_{20}H_{12}N_2Na_2O_7S_2$	502.43	12.83	518
7	Fast green FCF	$C_{37}H_{34}N_2O_{10}S_3Na_2$	808.85	14.04 (imp 13.52)	622
8	Acid blue / Eryoglaucine	$C_{37}H_{34}Na_2N_2O_9S_3$	792.85	16.32 (imp 15.40)	628
9	Ponceau 3R	$C_{19}H_{16}N_2Na_2O_7S$	494.45	16.99	512
10	Erythrosine B	$C_{20}H_8I_4O_5$	835.89	18.18	530

Table 4
List of colors and observed absorbance maxima for each color.

Limit of detection (LOD) and Limit of quantitation (LOQ)

The analyte concentration that provides a signal-to-noise ratio (S/N) of greater than three was considered as LOD and analyte concentration with S/N greater than 10 was considered as LOQ. Observed LOD and LOQ values of each color are shown in Table 5. As an example, the overlay of LOQ chromatograms of ponceau 4R (0.1 ng on-column) with blank is shown in Figure 2.

Linearity

All the prepared linearity levels were injected in six replicates and linearity curves for each color were constructed from the LOQ level to a highest concentration level using area response and concentration values. The observed regression coefficients for all colors are shown in Table 5.

Peak number	Compound name	LOD (ng)	LOQ (ng)	Total levels (n=6)	On–column linearity range (ng)	Linearity equation	R^2 value
1	Tartrazine	0.05	0.1	8	0.1 to 100	y = 15.477x - 5.7137	0.9993
2	Amaranth	0.1	0.25	7	0.25 to 100	y = 12.686x - 5.8682	0.9993
3	Indigo carmine	0.05	0.1	8	0.1 to 100	y = 16.723x - 5.9163	0.9993
4	Ponceau 4R	0.05	0.1	8	0.1 to 100	y = 13.168x - 5.0258	0.9993
5	Sunset yellow FCF	0.25	0.5	8	0.5 to 1000	y = 1.8621x + 7.2227	0.9992
6	Carmoisine	0.25	0.5	8	0.5 to 1000	y = 10.018x + 41.05	0.9993
7	Fast green FCF	0.1	0.25	7	0.25 to 100	y = 31.981x - 14.22	0.9993
8	Acid blue	0.05	0.1	8	0.1 to 100	y = 36.351x - 12.193	0.9994
9	Ponceau 3R	0.1	0.25	9	0.25 to 1000	y = 11.324x + 39.972	0.9992
10	Erythrosine B	0.05	0.1	8	0.1 to 100	y = 40.628x - 10.168	0.9997

Table 5
LOD, LOQ and linearity results of all 10 colors. A 0.25 ng on-column concentration was achieved by injecting 2.5 µL of 0.1 ng/µL standard solution.

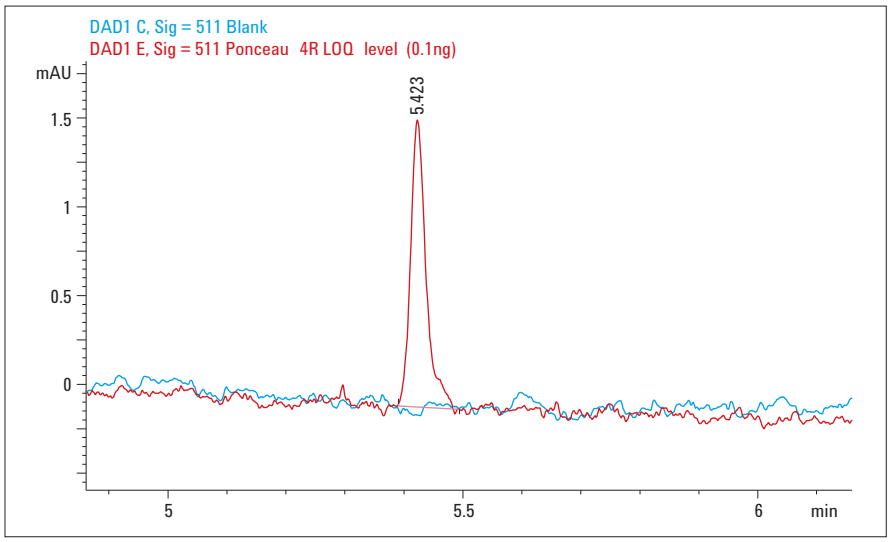

Figure 2
LOQ (0.1 ng) chromatograms of ponceau 4R overlaid with blank.

Precision of retention time and area

To establish the method precision, relative standard deviation (RSD) values for retention time (RT) and area of all 10 colors at 1, 10, and 100 ng (on-column) concentration were calculated. The highest observed area RSD value was 1.19% (for Carmoinsine at 1 ng) and RT RSD was 0.09% (for Tartrazine at 1 ng). Graphical representation of area RSD values of 10 colors is shown in Figure 3 and RT RSD values are shown in Figure 4.

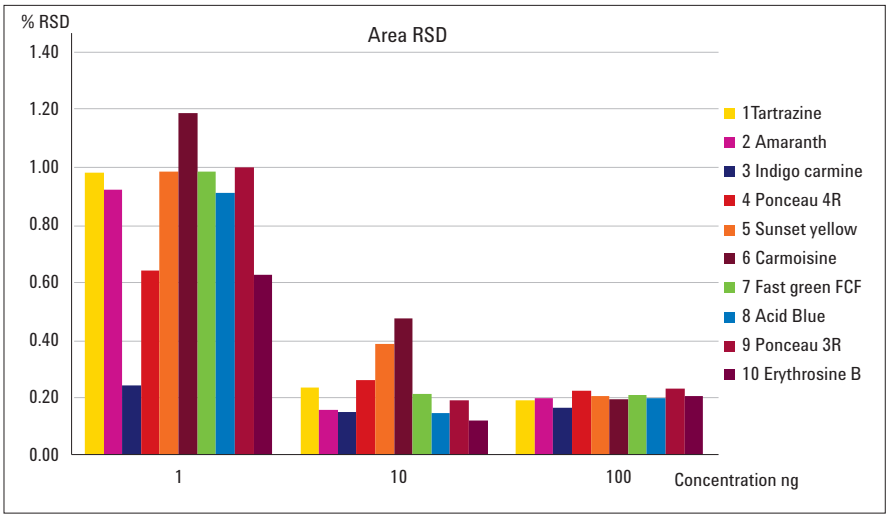

Figure 3
Excellent area RSD values for all colors at 1 ng, 10 ng, and 100 ng (on-column) concentration.

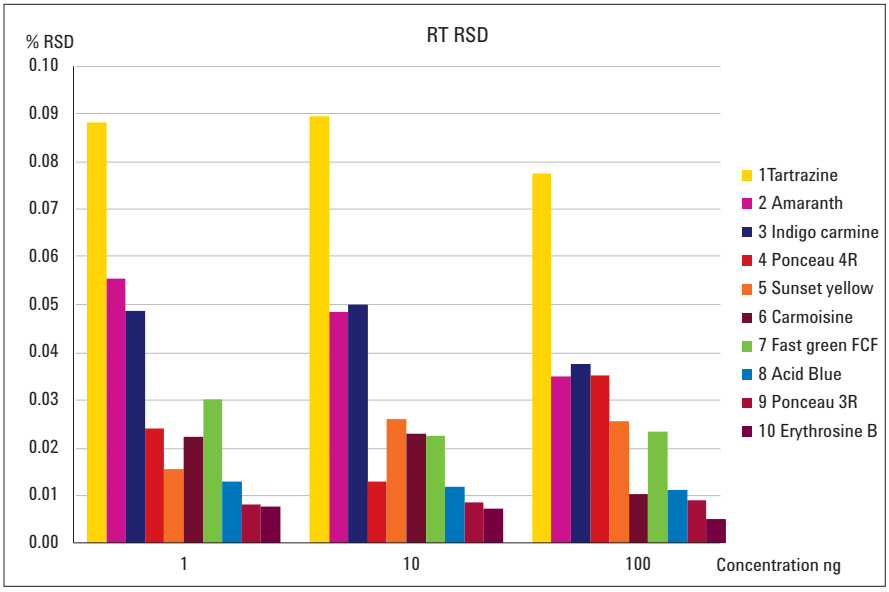

Figure 4
Excellent RT RSD values for all colors at 1 ng, 10 ng, and 100 ng (on-column) concentration.

Robustness

Robustness of the method was evaluated by deliberately varying six critical method parameters. The resulting deviation in area and retention time was calculated and compared to the original method. A standard spike mix solution of color standards was injected in six replicates. Allowed deviations for retention time and area were set to ±3% and ±5% respectively. The robustness test conditions used in this study are noted in Table 6 and results from robustness study are summarized in Figures 5 and 6.

Sl no.	Parameter (actual value)	Measured deviation	Modified value
1	Flow rate (1.2)	2%	1.224 mL/min 1.176 mL/min
2	Injection volume (5 µL)	2%	5.1 µL 4.9 µL
3	Wavelength (288, 428, 484, 511, 520, 530, 626 nm)	(±) 3 nm	Wavelength (291, 431, 487, 514, 523, 533, 629 nm) Wavelength (285, 425, 481, 508, 517, 527, 623 nm)
4	Ph (7.0)	(±) 0.15	10 mm Buffer pH 7.15 10 mm Buffer pH 6.85
5	Column temperature (45 °C)	(±) 2 °C	47 °C 43 °C
6	Gradient steepness (6.25, 5 to 30 in 4 minutes and 13.75, 40 to 95 in 4 minutes)	~10%	6.75, 5 to 32 in 4 minutes and 14.25 for 38 to 95 in 4 minutes 5.75, 5 to 28 in 4 minutes and 13.25 for 42 to 95 in 4 minutes

Table 6
Robustness test conditions used in this study.

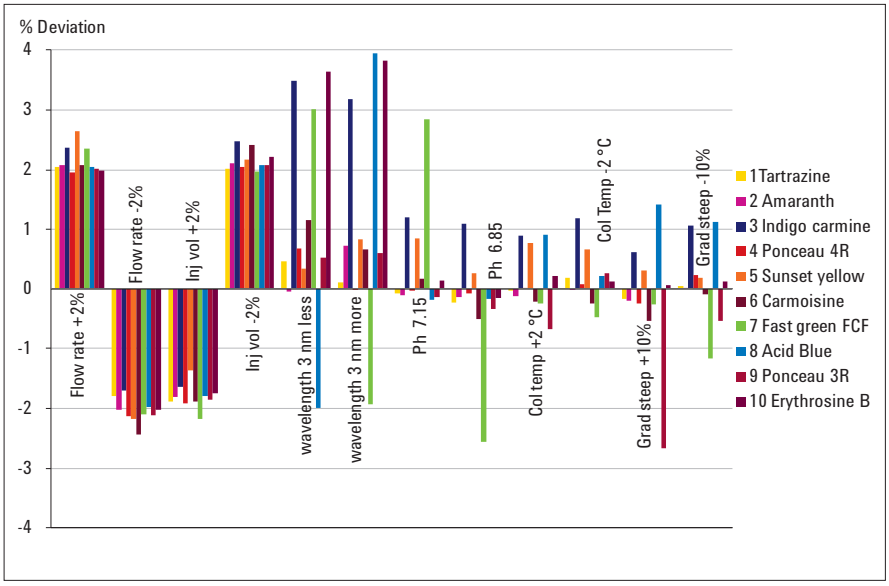

Figure 5
Robustness test result summary for area.

The area deviations for all 10 colors were found to be within the allowed limit for all the varied parameters. Also, retention time deviation for flow rate, injection volume, and pH of mobile phase was found to be within the allowed limit for this robustness study. However, the impact of increased column temperature on RT deviation exceeded the allowed limit for two compounds. With a decrease in column temperature, RT deviation for three compounds crossed the allowed limit. One critical parameter which has considerable impact on retention time was found to be gradient slope. We observed that more than five compounds were showing a RT deviation beyond the allowed limit with a ±10% change in gradient slope. Robustness results indicate that the method is reliable to use for normal usage and the performance remains unaffected to a great extent by deliberate change in parameters.

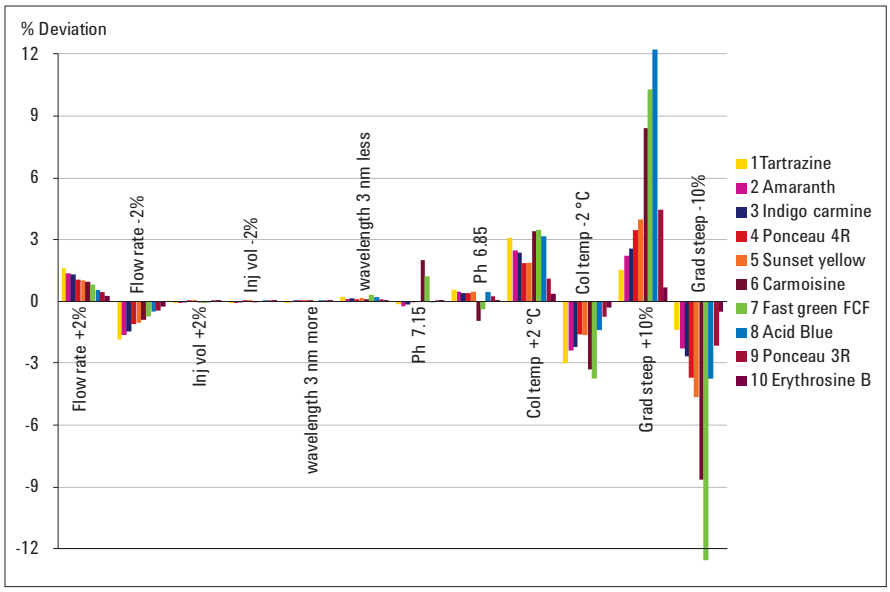

Figure 6
Robustness test result summary for retention time.

Recovery of colorants from sweets

Recovery analyses for various colorants from five different colored sweets were carried out by a standard addition method[5]. A standard mix solution of all ten colorants at 25 ng (on-column) was used for this analysis. The peak area of the individual colorants in the spiked sample, unspiked sample, and standard chromatogram were measured separately. The difference in detector response between spiked and unspiked sample was compared against response observed in standard chromatogram and expressed in percentage as recovery. The recovery for all colorants from sweets were greater than 98%. Chromatograms observed for spiked or unspiked extracted samples from red sweets and standard mix solutions are shown in Figure 7.

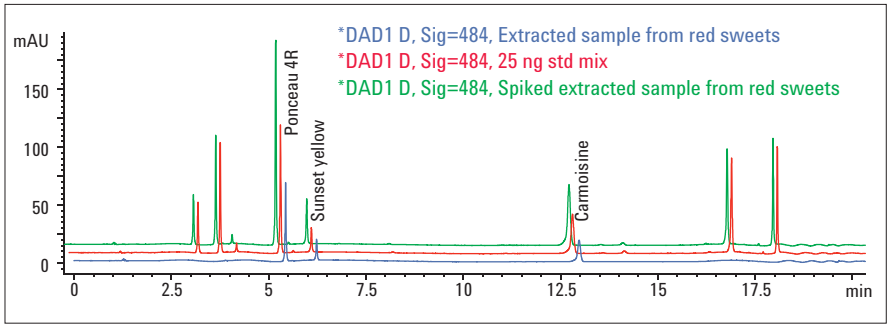

Figure 7
Overlay of spiked, unspiked extracted sample from red sweets and standard mix.

Quantitation of color additives in sweets

Color additives present in various colored sweets were determined using the area response. Linearity equations originating from linearity curves were used for the calculation. In addition, the in-house created UV spectral library was used to identify the compounds using spectral matching. The calculated amounts of colorants from 1 g of five different sweets are tabulated in Table 7. The observed spectral match for Ponceau 4R peak from red sweet with library spectra is shown in Figure 8.

Item number	Color of the sweets	Components	Amount present (µg/g)
Sweet_1	Blue	Acid blue	44.7
Sweet_2	Yellow	Tartrazine	61.7
Sweet_3	Green	Tartrazine	52.5
		Acid blue	10.9
Sweet_4	Orange	Tartrazine	24.8
		Ponceau 4R	26.9
		Sunset yellow FCF	43.3
Sweet_5	Red	Ponceau 4R	27.5
		Sunset yellow FCF	38.3
		Carmoisine	20.6

Table 7
The calculated amounts of colorants from 1 g of sweets.

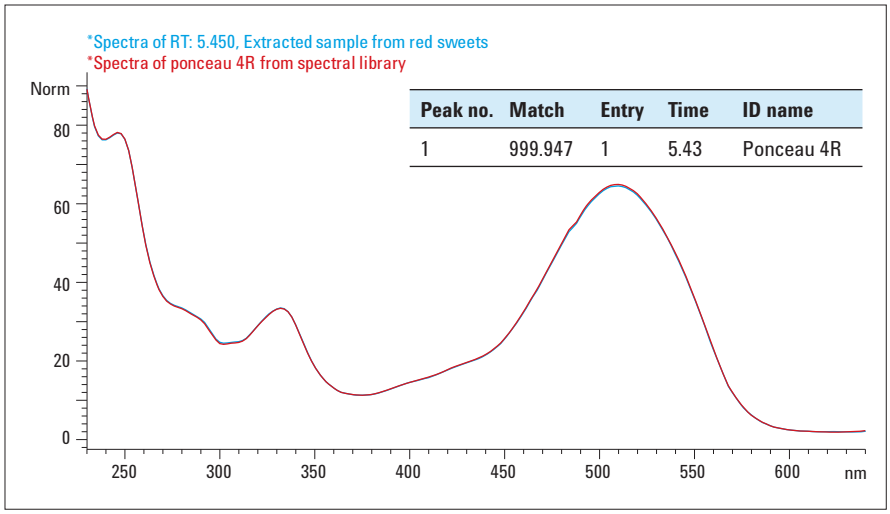

Figure 8
Spectral match for Ponceau 4R peak from red sweet with library spectra.

UHPLC method

A UHPLC method was developed for the separation of ten colorants with diode array detection. The UHPLC method shows excellent resolution and saves about 81% analysis time and 89% solvent compared to the 21-minute HPLC gradient (Figure 9). The resolution value between the fast green FCF peak and its impurity (peak at 13.526) was found to be the lowest of all peaks in the HPLC method, so this resolution was monitored in the UHPLC results to evaluate the overall resolution of peaks in a short run time. With the HPLC method, this resolution was 3.71 and with the short UHPLC method this value was greater than 1.8. The observed LOD, LOQ, and linearity results obtained with the UHPLC method are shown in Table 8. To evaluate the precision of the method, RSD values for RT and area for an on-column concentration of 10 ng were calculated. The highest observed Area RSD was 0.84% and the RT RSD was 0.04% —both for sunset yellow. The results are shown in Figure 10. Low RSD values for area and RT confirmed the precision of the method. These results prove the reliability of the developed UHPLC method. Quick quantification of colors from sweet samples is possible using this method.

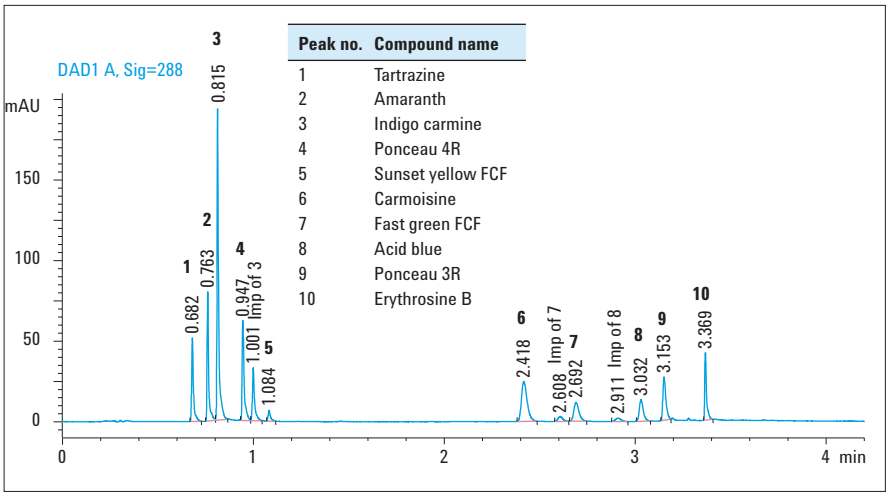

Figure 9
Separation of ten colorants using UHPLC method on the Agilent 1290 Infinity LC System.

Peak number	Compound name	LOD (ng)	LOQ (ng)	Total levels (n=6)	On–column linearity range (ng)	Linearity equation	R^2 value
1	Tartrazine	0.05	0.1	9	0.1 to 200	y = 4.6746x + 2.5573	0.9998
2	Amaranth	0.1	0.25	8	0.25 to 200	y = 3.7682x + 0.585	0.9996
3	Indigo carmine	0.05	0.1	9	0.1 to 200	y = 4.3278x + 3.0266	0.9998
4	Ponceau 4R	0.1	0.25	8	0.25 to 200	y = 3.9616x + 1.4427	0.9997
5	Sunset yellow FCF	0.5	1	6	1 to 200	y = 0.6479x + 0.8958	0.9993
6	Carmoisine	0.25	1	6	1 to 200	y = 3.8231x + 0.5447	0.9996
7	Fast green FCF	0.1	0.25	8	0.25 to 100	y = 9.008x + 3.0979	0.9998
8	Acid blue	0.1	0.25	8	0.25 to 100	y = 10.083x + 14.681	0.9991
9	Ponceau 3R	0.1	0.25	8	0.25 to 200	y = 4.1461x + 0.4156	0.9995
10	Erythrosine B	0.05	0.1	9	0.1 to 100	y = 11.354x + 11.912	0.9996

Table 8
LOD and LOQ values derived from the UHPLC method using the Agilent 1290 Infinity LC System.

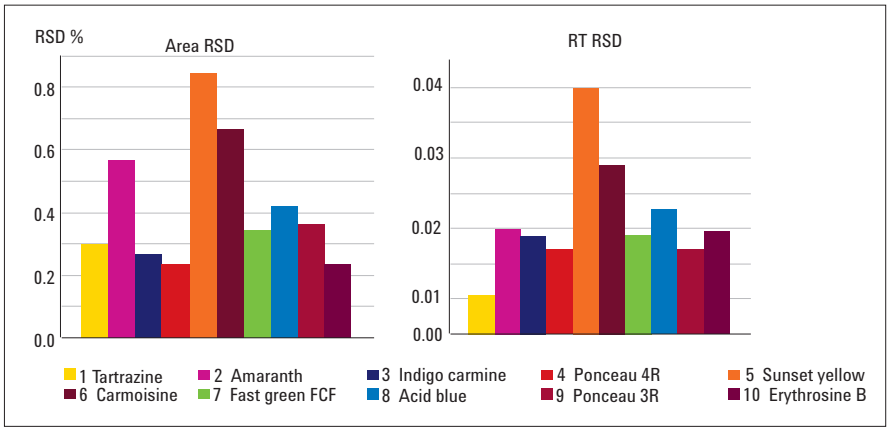

Figure 10
Area and RT RSD values from UHPLC results for all 10 colors at an on-column concentration of 10 ng level. Injection volume is 1 µL (six replicates).

Conclusion

Ten colorants were separated and quantified using an Agilent Poroshell 120 EC-C18 column. With the Agilent 1260 Infinity LC System, a robust, 20-minute HPLC gradient method was developed. The method was partially validated to demonstrate the usability to quantify colors such as tartrazine, amaranth, indigo carmine, ponceau 4R, sunset yellow FCF, carmoisine, fast green FCF, acid blue/eryoglaucine, ponceau 3R, and erythrosine B. The method is simple, specific, sensitive, rapid and also provides good precision, linearity, and recovery values. Efficient usage of this method was established by quantifying colorants from five different colored sweet matrices. Later, this method was transferred to a short 4-minute UHPLC method using the Agilent 1290 Infinity LC System, which saves about 81% analysis time and 89% solvent. These methods using the Agilent 1260 and 1290 Infinity LC systems can be used for accurate routine analysis of colorants. The Agilent 7696A Sample Prep WorkBench simplified the sample preparation for linearity studies. The excellent linearity results confirm that, the result obtained from the Agilent 7696A Sample Prep WorkBench is very precise, and reduces operator error.

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