INTRODUCTION

Dyes have many applications in the food and beverage industries, such as being used to make food more appealing, hide defects, or to strengthen consumer perception of the association between color and flavor. For example, lime flavor is associated with the color green and thus, lime soft drinks are often colored with green food dye. Despite the existence of many dyes, only a few have been approved for use in foods and beverages. The U.S. FDA permits seven artificial colorings in food: Brilliant Blue FCF (FD&C Blue 1), Indigotin (FD&C Blue 2), Sunset Yellow FCF (FD&C Yellow 6), Tartrazine (FD&C Yellow 5), Allura Red AC (FD&C Red 40), Fast Green FCF (FD&C Green 3), and Erythrosine (FD&C Red 3).

Reversed-phase chromatography is an excellent technique for the analysis of dyes. Many dyes are hydrophobic, readily soluble in reversed-phase eluents, and have strong visible and UV absorbance properties. This application note (AN) demonstrates fast separation of 10 dyes in less than 5 min using an Acclaim® PA2 (3 µm) column in a 3 × 75 mm format.

The Acclaim PA2 column is ideal for resolving mixtures of compounds with a wide range of hydrophobicities, including very polar compounds. This method was used to determine the quantity of food dyes in six soft drinks and a gelatin dessert. The combination of an UltiMate® 3000 Rapid Separation LC (RSLC) system and an Acclaim PA2 column is suitable for the fast analysis of food and beverage samples that have both approved and illegal dyes.

EQUIPMENT

Dionex UltiMate 3000 RSLC system consisting of:

SRD-3600 Solvent rack with integrated vacuum degasser
HPG-3400RS Binary gradient pump with 400 µL static mixer kit
WPS-3000RS Split loop sampler with 100 µL sample loop
TCC-3000RS Thermostatted column compartment
DAD-3000RS Diode array detector, 5 µL flow cell
Chromeleon® Chromatography Data System, Version 6.80 SR7
**REAGENTS AND STANDARDS**
Deionized water (DI), Type I reagent grade, 18 MΩ-cm resistivity or better
Acetonitrile (CH₃CN), LAB-SCAN
Di-ammonium hydrogen phosphate ((NH₄)₂HPO₄), Ajax
8 N Potassium hydroxide solution (KOH), KANTO
Tartrazine (C₁₆H₉N₄Na₃O₉S₂), Fluka
Amaranth (C₂₀H₁₁N₂Na₃O₁₀S₃), Fluka
Indigo Carmine (C₁₆H₈N₂Na₂O₈S₂), Fluka
New Coccine (C₂₀H₁₁N₂Na₂O₁₀S₂), Fluka
Sunset Yellow FCF (C₁₆H₁₀N₂Na₂O₇S₂), Fluka
Fast Green FCF (C₃₇H₃₄N₂Na₂O₁₀S₃), Fluka
Eosin Y (C₂₀H₆Br₄Na₂O₅), Fluka
Erythrosin B (C₂₀H₆I₄Na₂O₅), Fluka
Phloxine B (C₂₀H₂Br₄Cl₄Na₂O₅), Fluka
Bengal Rose B (C₂₀H₂Cl₄I₄Na₂O₅), Fluka
Brilliant Blue (C₃₇H₃₄N₂Na₂O₉S₃), Fluka

**CHROMATOGRAPHIC CONDITIONS**
Column: Acclaim PA2, 3 µm, 3 × 75 mm
(P/N 066277)
Mobile Phase: A) 20 mM (NH₄)₂HPO₄ pH 8.8
B) 50% 20 mM (NH₄)₂HPO₄
pH 8.8 in CH₃CN (v/v)
Flow Rate: 0.709 mL/min
Gradient: 12% B from –3 to 0.00 min,
ramp to 100% B in 3.5 min,
hold 100% B for 1.0 min and
return to 12% B in 0.1 min.
Column Temp.: 30 ºC
Inj. Volume: 3 µL
Detection: UV 254 nm and wavelength scanning
200–800 nm
System
Backpressure: 2100 psi

**PREPARATION OF SOLUTIONS AND REAGENTS**
**Eluent A [20 mM (NH₄)₂HPO₄, pH 8.8]**
Weigh 2.64 g di-ammonium hydrogen orthophosphate in a 250 mL beaker. After dissolving with deionized water (used for all eluent and sample preparation), transfer to a 1 L volumetric flask. Add 850 µL of 8 N sodium hydroxide, dilute to 1 L with water, and mix. Filter with a 0.45 µm filter before use.

**Eluent B [50% 20 mM (NH₄)₂HPO₄ pH 8.8 in CH₃CN]**
Mix eluent A and CH₃CN 1:1. Filter with a 0.45 µm filter before use.

**Standard Solutions**
All 1000 mg/L stock standard solutions were prepared separately in water and used to prepare four mixtures of the 10 dyes that were the working standards for method calibration. The dye concentrations in the working standard solutions are shown in Table 3.

**Sample Preparation**
All samples were purchased from a supermarket in Bangkok, Thailand.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Designation</th>
<th>Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrolyte sports drink</td>
<td>1</td>
<td>Yellow</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Yellow</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Orange</td>
</tr>
<tr>
<td>Carbonated drink</td>
<td>1</td>
<td>Orange</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Orange</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Green</td>
</tr>
<tr>
<td>Gelatin dessert</td>
<td>1</td>
<td>Red</td>
</tr>
</tbody>
</table>

The electrolyte sports drinks were filtered with a 0.45 µm filter before analysis. The carbonated drinks were placed in an ultrasonic bath for 5 min to degas, then diluted with water (1:2 for sample 1 and 1:1 for samples 2 and 3), and filtered with a 0.45 µm filter. Then, half a gram of the gelatin dessert was placed in a 25 mL beaker, mixed with 7 mL water, and placed in hot water for 5 min or until it completely dissolved. After cooling to room temperature, the sample was transferred to a 10 mL volumetric flask, and diluted to 10 mL with water.
RESULTS AND DISCUSSION

Figure 1 shows the separation of a mixture of 10 dyes, including the US FDA-permitted food dyes Tartrazine, Sunset Yellow, Fast Green, and Erythrosine, in less than 5 min. This separation uses an ammonium phosphate/acetonitrile eluent at pH 8.8, a pH value that would pose a problem for most silica-based, reversed-phase columns. The Acclaim PA2 column is tolerant of this high pH. The separation is achieved in less than 5 min by using a 3 µm particle size and a 3 × 75 mm column format.

When the food dye Brilliant Blue was added to the standard mixture, complete resolution between Fast Green and Brilliant Blue was not achieved. Though few food samples will contain both dyes, a method for the fast separation of the 10 dyes (listed in Figure 1) and Brilliant Blue using the Acclaim PA column is presented in Figure 2. Similar to the Acclaim PA2 column, the PA column provides a polar-embedded phase that can be used to separate compounds with a wide range of hydrophobicities. Figure 2 shows that Brilliant Blue is resolved from Fast Green (peaks 6 and 7). Because the Acclaim PA column does not have the high pH tolerance of the PA2 column, the eluent pH was lowered to 7.3.

The separation on the Acclaim PA column is also less than 5 min because it uses the 3 µm particle size resin and the 3 × 75 mm column format. The eluents used in both the PA and PA2 separations are compatible with MS detection. In both figures, the dyes are detected at 254 nm. Given that both these dyes absorb at other wavelengths, a more selective wavelength can be chosen for each dye. The PA2 separation was used for the rest of the analysis but both methods can be used to analyze the samples. Because the last compound elutes at about 0.5 min earlier on the PA2 column and the resolution between peaks 2 and 3 is better, it is possible to make the PA2 separation slightly faster than the PA separation using the 3 × 75 mm column format, but this was not evaluated.

Spectral scanning was used for the analysis of the standard mix (Figure 1). Table 2 displays the match and PPI values from the spectral scanning. The high match values suggest that the peaks are pure and the peak spectra were loaded in the spectral library for use in identifying dye peaks in samples. Table 2 also displays resolution values, with no resolution values less than 2.8.
METHOD CALIBRATION

Prior to sample analysis, the method was calibrated for each of the 10 dyes between 1 and 30 mg/L. Four concentrations, 1, 10, 20, and 30 mg/L, were used and the curves were forced through the origin. Table 3 shows that for this range, calibration for each of the 10 dyes was linear.

SAMPLE ANALYSIS

Seven samples were purchased from a local supermarket for analysis. Three different electrolyte sports drinks, two yellow and one orange, were analyzed (Table 1). Three carbonated drinks, two orange and one green, were also analyzed. The seventh sample was a red gelatin dessert. All samples were labeled to contain a dye, but none listed the dye or dyes used. Figures 3–9 show the chromatography for each sample. Using the spectral library and retention time, the two yellow sports drinks were found to contain Tartrazine (Figures 3 and 4). The samples were found to have similar concentrations of the dye (Table 4). The same approach identified the allowed food dye Sunset Yellow FCF in the orange sports drink (Figure 5). The dye was found in both orange carbonated drinks (Figures 6 and 7). The green carbonated drink contained two dyes, Tartrazine and Fast Green FCF (Figure 8). More importantly, the red dye New Coccine was found in the red gelatin dessert (Figure 9). This dye is banned for use in foods in the United States and some other countries.

All samples were spiked with the standard or standards identified by spectral matching and retention time to assess the accuracy of the determination. Table 4 shows the concentration of each dye in each sample, the spectral match, and the results of the spiking studies. Good recoveries were observed in each sample, suggesting that the method is accurate. Method reproducibility was evaluated by making five injections of each sample and each spiked sample, and concentrations of dyes in each sample were determined. Table 5 shows that the method has good short-term reproducibility.
Column: Acclaim PA2 3 µm, 3 × 75 mm
Eluent: A) 20 mM (NH₄)₂HPO₄, pH 8.8
B) 50% 20 mM (NH₄)₂HPO₄, pH 8.8 in CH₃CN
Eluent Gradient: 12% B at -3 min to 0.00 min, ramp to 100% B in 3.5 min, hold 100% B for 1 min, return to 12% B in 0.1 min
Temperature: 30 °C
Flow Rate: 0.709 mL/min
Inj. Volume: 3 µL
Detection: UV, 254 nm
Sample: Orange carbonated drink 1
Peaks:
1. Sunset yellow FCF 85.6 mg/L

Figure 4. Chromatogram of electrolyte sports drink 2.

Column: Acclaim PA2 3 µm, 3 × 75 mm
Eluent: A) 20 mM (NH₄)₂HPO₄, pH 8.8
B) 50% 20 mM (NH₄)₂HPO₄, pH 8.8 in CH₃CN
Eluent Gradient: 12% B at -3 min to 0.00 min, ramp to 100% B in 3.5 min, hold 100% B for 1 min, return to 12% B in 0.1 min
Temperature: 30 °C
Flow Rate: 0.709 mL/min
Inj. Volume: 3 µL
Detection: UV, 254 nm
Sample: Yellow electrolyte sports drink 2
Peaks:
1. Tartrazine 4.63 mg/L

Figure 5. Chromatogram of electrolyte sports drink 3.

Column: Acclaim PA2 3 µm, 3 × 75 mm
Eluent: A) 20 mM (NH₄)₂HPO₄, pH 8.8
B) 50% 20 mM (NH₄)₂HPO₄, pH 8.8 in CH₃CN
Eluent Gradient: 12% B at -3 min to 0.00 min, ramp to 100% B in 3.5 min, hold 100% B for 1 min, return to 12% B in 0.1 min
Temperature: 30 °C
Flow Rate: 0.709 mL/min
Inj. Volume: 3 µL
Detection: UV, 254 nm
Sample: Orange carbonated drink 3
Peaks:
1. Sunset yellow FCF 14.3 mg/L

Figure 6. Chromatogram of carbonated drink 1.

Column: Acclaim PA2 3 µm, 3 × 75 mm
Eluent: A) 20 mM (NH₄)₂HPO₄, pH 8.8
B) 50% 20 mM (NH₄)₂HPO₄, pH 8.8 in CH₃CN
Eluent Gradient: 12% B at -3 min to 0.00 min, ramp to 100% B in 3.5 min, hold 100% B for 1 min, return to 12% B in 0.1 min
Temperature: 30 °C
Flow Rate: 0.709 mL/min
Inj. Volume: 3 µL
Detection: UV, 254 nm
Sample: Orange carbonated drink 2
Peaks:
1. Sunset yellow FCF 33.8 mg/L

Figure 7. Chromatogram of carbonated drink 2.
Table 4. Sample and Recovery Results

<table>
<thead>
<tr>
<th>Sample</th>
<th>Color</th>
<th>Dye Spiked into the Sample</th>
<th>Spiked Conc. (mg/L) Added to Sample</th>
<th>Average Dye Concentration in Sample (mg/L)</th>
<th>Average Dye Concentration in Spiked Sample (mg/L)</th>
<th>%Recovery</th>
<th>Peak Purity Match</th>
<th>Match with Spectra Library</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrolyte sports drink 1</td>
<td>Yellow</td>
<td>Tartrazine</td>
<td>1</td>
<td>4.22</td>
<td>5.18</td>
<td>96.0</td>
<td>997</td>
<td>996</td>
</tr>
<tr>
<td>Electrolyte sports drink 2</td>
<td>Yellow</td>
<td>Tartrazine</td>
<td>1</td>
<td>4.63</td>
<td>5.57</td>
<td>94.0</td>
<td>999</td>
<td>996</td>
</tr>
<tr>
<td>Electrolyte sports drink 3</td>
<td>Orange</td>
<td>Sunset yellow FCF</td>
<td>4</td>
<td>14.3</td>
<td>18.3</td>
<td>100</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>Carbonated drink 1</td>
<td>Orange</td>
<td>Sunset yellow FCF</td>
<td>10</td>
<td>85.6</td>
<td>93.8</td>
<td>82</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>Carbonated drink 2</td>
<td>Orange</td>
<td>Sunset yellow FCF</td>
<td>10</td>
<td>33.8</td>
<td>43.3</td>
<td>95.0</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>Carbonated drink 3</td>
<td>Green</td>
<td>Tartrazine</td>
<td>10</td>
<td>54.8</td>
<td>63.1</td>
<td>83.0</td>
<td>1000</td>
<td>997</td>
</tr>
<tr>
<td>Carbonated drink 3</td>
<td>Green</td>
<td>Fast Green FCF</td>
<td>2</td>
<td>4.29</td>
<td>6.12</td>
<td>91.5</td>
<td>1000</td>
<td>999</td>
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<tr>
<td>Gelatin dessert</td>
<td>Red</td>
<td>New Coccine</td>
<td>40</td>
<td>507</td>
<td>545</td>
<td>95.0</td>
<td>1000</td>
<td>1000</td>
</tr>
</tbody>
</table>

**FASTER ANALYSIS**

While the method presented in this application note is fast, it is possible to make it faster. Figure 10 shows that by switching to a shorter column with a smaller particle size, it is possible to reduce the separation time from 4.5 min to 2.5 min while still resolving all 10 dyes. To accomplish this, the injection volume was reduced to 1 μL, the 400 μL static mixer was replaced with a 200 μL static mixer (P/N 6040.5150), and the flow cell was changed to a semi-micro 2.5 μL flow cell. The data collection rate was also set to 25 Hz and the response time to 0.5 s. The backpressure of this separation was 3150 psi.
### Table 5. Reproducibility of Five Injections of Samples and Spiked Samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Color</th>
<th>Dyes Found in Samples and Spiked Samples</th>
<th>Concentrations Found in Samples and Spiked Samples (mg/L)</th>
<th>RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Injection #</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Electrolyte sports drink 1</td>
<td>Yellow</td>
<td>Tartrazine in sample</td>
<td>4.20</td>
<td>4.27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tartrazine in spiked sample</td>
<td>5.19</td>
<td>5.16</td>
</tr>
<tr>
<td>Electrolyte sports drink 2</td>
<td>Yellow</td>
<td>Tartrazine in sample</td>
<td>4.62</td>
<td>4.64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tartrazine in spiked sample</td>
<td>5.58</td>
<td>5.56</td>
</tr>
<tr>
<td>Electrolyte sports drink 3</td>
<td>Orange</td>
<td>Sunset yellow FCF in sample</td>
<td>14.3</td>
<td>14.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sunset yellow FCF in spiked sample</td>
<td>18.3</td>
<td>18.3</td>
</tr>
<tr>
<td>Carbonated drink 1</td>
<td>Orange</td>
<td>Sunset yellow FCF in sample</td>
<td>85.6</td>
<td>85.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sunset yellow FCF in spiked sample</td>
<td>93.7</td>
<td>93.8</td>
</tr>
<tr>
<td>Carbonated drink 2</td>
<td>Orange</td>
<td>Sunset yellow FCF in sample</td>
<td>33.8</td>
<td>33.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sunset yellow FCF in spiked sample</td>
<td>43.3</td>
<td>43.3</td>
</tr>
<tr>
<td>Carbonated drink 3</td>
<td>Green</td>
<td>Tartrazine in sample</td>
<td>54.8</td>
<td>54.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tartrazine in spiked sample</td>
<td>63.0</td>
<td>63.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fast Green FCF in sample</td>
<td>4.29</td>
<td>4.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fast Green FCF in spiked sample</td>
<td>6.09</td>
<td>6.14</td>
</tr>
<tr>
<td>Gelatin dessert</td>
<td>Red</td>
<td>New Coccine in sample</td>
<td>507</td>
<td>506</td>
</tr>
<tr>
<td></td>
<td></td>
<td>New Coccine in spiked sample</td>
<td>546</td>
<td>546</td>
</tr>
</tbody>
</table>

Note: The results were multiplied by the appropriate dilution factor in the Chromeleon software.

### Figure 10. Faster separation of the 10 dyes standard.

**CONCLUSION**

This application note presents a fast HPLC method (< 5 min) for the accurate determination of dyes in food and beverage samples. This method can be used to quantify permitted dyes and identify illegal dyes in food and beverage samples.

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