## Quantitation and Identification of 13 Azo-dyes in Spices using LC-MS/MS

**Instrument:** Applied Biosysyem QSTAR Elite Hybrid LC/MS/MS System

### Chemicals

Solvents, reagents and dye standards were obtained at highest available purity from Sigma-Aldrich (dye content 80-98%). Internal standards (D5-Sudan I and D6-Sudan IV) were obtained from WITEGA laboratories (Berlin, Germany). Stock solutions were prepared in acetonitrile freshly due to degradation of some azo-dyes. Solvent standards were diluted in the starting mobile phase.

### Spice Samples

Spice samples were purchased on local markets in India (Garam Masala), Korea (Red Chili), and Egypt (Saffron) and analyzed by LC-MS/MS. Not one of the 13 investigated azo-dyes was detected in the selected spice samples. Matrix matched standards were prepared in Garam Masala extract. In addition every matrix was spiked with known concentrations of a mix of azo-dyes prior to analysis. These samples were used to investigate standard addition.



### Sample Preparation

The goal was to develop a generic sample preparation procedure that is easy extendable to other emerging azo-dyes.

1. Weigh 1 g of homogenized sample (multiple times for standard addition).

2. Add 20 µL of internal standard solution (1 µg/mL of D5-Sudan I and D6-Sudan IV).

3. Add standard solution(s) in case of standard addition.

4. Add 10 mL of acetonitrile.

5. Shake for 10 min.

6. Add 10 mL of water.

7. Shake and centrifuge (or filtrate) before injection.

### HPLC

The goal was to develop a flexible HPLC method to separate a variety of emerging dyes. A gradient of 30 min was chosen to allow sufficient separation of analytes from matrix components. This method can be shortened easily, but matrix effects might increase significantly. No HPLC conditions could be identified for the separation of the two isomeric dyes Sudan IV and Sudan Red B, although various columns (C8 and C18), mobile phases (water, methanol, acetonitrile), buffers (ammonium formate, ammonium acetate, formic, and acetic acid), and pH values were investigated.

An Agilent 1100 HPLC system with binary pump (without static mixer), well plate autosampler, and column oven was used. A Phenomenex LUNA 5u C8,150x2 mm column and a gradient of eluent A: water + 0.2% formic acid + 2 mM ammonium formate and eluent B: water/acetonitrile (10/90) + 0.2% formic

acid + 2 mM ammonium formate was used at a flow rate of 300 µL/min. Details of the gradient are given in Table 1. The column oven temperature was set to 30°C. A volume of 50 µL of each sample was injected

**Table 1.** HPLC gradient

| Step | Total Time (min) | A (%) | B (%) |
|---|---|---|---|
| *0* | 10 | 80 | 20 |
| *1* | 15 | 0 | 100 |
| *2* | 29 | 0 | 100 |
| *3* | 30 | 80 | 20 |

**MS/MS**

A 3200 QTRAP®LC/MS/MS System equipped with Turbo V™ Source and Electrospray Ionization (ESI) probe was used. ESI was found to be suitable for the ionization of azo-dyes. The ion source temperature (450°C) was optimized for the highest sensitivity of Orange II and Para Red, the two compounds showing lowest sensitivity in positive polarity. Two MRM transitions were monitored per analyte to allow quantitation and identification using ion ratios (Table 2). Two additional MRM transitions were detected for Sudan IVand Sudan Red B to allow differentiating between both co-eluting and isomeric compounds.



**Table 2.** MRM transitions, retention times ($t_R$), of detected azo-dyes and signal-to-noise (S/N) of the qualifier MRM transition at a concentration of 10 ng/mL

| Analyte Name | CAS | Q1 (amu) | Q3-1 (amu) | Q3-2 (amu) | Q3-3 (amu) | Q3-3 (amu) | $t_R$ (min) | S/N at 10ng/mL |
|---|---|---|---|---|---|---|---|---|
| *Dimethyl Yellow* | 60-11-7 | 226.1 | 120.1 | 105.1 | - | - | 14.5 | 980 |
| *Fast Garnet GBC* | 97-56-3 | 226.1 | 91.1 | 107.1 | - | - | 13.5 | 300 |
| *Orange II (positive)* | 633-96-5 | 329.1 | 156.0 | 128.0 | - | - | 13.0 | 30 |
| *Orange II (negative)* | 633-96-5 | 327.0 | 171.0 | 80.0 | - | - | 13.0 | 220 |
| *Para Red* | 6410-10-2 | 294.1 | 156.1 | 128.1 | - | - | 14.2 | 300 |
| *Rhodamine B* | 81-88-9 | 443.2 | 399.1 | 355.1 | - | - | 8.7 | 10600 |
| *Sudan I* | 842-07-9 | 249.1 | 93.0 | 156.1 | - | - | 15.0 | 500 |
| *Sudan II* | 3118-97-6 | 277.1 | 121.1 | 106.1 | - | - | 16.6 | 1090 |
| *Sudan III* | 85-86-9 | 353.1 | 197.1 | 128.1 | - | - | 17.4 | 200 |
| *Sudan IV* | 85-83-6 | 381.1 | 224.1 | 225.1 | 143.1 | 104.1 | 18.8 | 80 |
| *Sudan Orange G* | 2051-85-6 | 215.1 | 93.1 | 122.1 | - | - | 11.8 | 310 |
| *Sudan Red 7B* | 6368-72-5 | 380.2 | 183.1 | 115.1 | - | - | 18.9 | 1860 |
| *Sudan Red B* | 3176-79-2 | 381.2 | 224.1 | 225.1 | 156.1 | 134.1 | 18.8 | 140 |
| *Sudan Red G* | 1229-55-6 | 279.1 | 123.1 | 108.1 | - | - | 14.7 | 1910 |
| *D5-Sudan Red I* | | 254.1 | 156.0 | - | - | - | 14.9 | - |
| *D6-Sudan Red IV* | | 387.1 | 106.0 | - | - | - | 18.7 | - |

**Reference:** André Schreiber, Kristin von Czapiewski AB SCIEX Concord, Ontario (Canada), AB SCIEX Darmstadt (Germany)
http://www.absciex.com/Documents/Downloads/Literature/mass-spectrometry-Azo-dyes-API3200-1281510.pdf