Determination of dyes in capsicum and turmeric samples and products by high performance liquid chromatography with MS/MS detection

Purpose: To determine the presence of various dyes in samples of paprika, capsicum, turmeric and oleoresin paprika.

Principle: This method uses HPLC separation with tandem mass spectrometer (MS/MS) detection. MS/MS detection is employed using electrospray ionization (ESI) in positive and negative-ion modes. Dyes are detected using dye-specific MRM (multiple reaction monitoring) transitions.

Safety concerns: dyes are known hazardous chemicals and appropriate care in handling such standards should be exercised. Consult the MSDS documentation for best handling practices. HPLC waste should also be properly disposed off according to local laws and regulations.

A. Apparatus

1. HPLC equipped with gradient pump, tandem mass spectrometer, autosampler and computer data workstation
2. Chromatography column, Symmetry C18, 150 mm x 2.1 mm i.d., 3.5 µm (Waters Corp., Part # WAT106005) or equivalent
3. Balance, readable to 0.0001 g
4. 50 mL culture tubes with Teflon lined cap
5. Vortex mixer
6. Glass 4 mL vials
7. Wrist action (or equivalent) shaker
8. Centrifuge capable of 3,000 RPMs
9. 5 ml Luer-Lok disposable syringe
10. PTFE 0.45 µm pore syringe filter
11. Volumetric pipets, various sizes
12. Volumetric flasks, various sizes
13. 25 mL graduated cylinder
14. Micropipetter or microliter syringes of various sizes.

B. Reagents

Dyes (analytes of interest)

1. Sudan I, [842-07-9], F.W. = 248.30
2. Sudan II, [3118-97-6], F.W. = 276.34
3. Sudan III, [85-86-9], F.W. = 352.40
4. Sudan IV, [85-83-6], F.W. = 380.40
5. Sudan Red 7 B, [6368-72-5], F.W. = 379.47
6. Sudan Red G, [1229-55-6], F.W. = 278.31
7. Sudan Orange G, [2051-85-6], F.W. = 214.22
8. Para Red, [6410-10-2], F.W. = 293.27
9. Dimethyl Yellow, [60-11-7], F.W. = 225.30

10. Rhodamine B, [81-88-9], F.W. = 479.02*
11. Orange II, [633-96-5], F.W. = 350.33*
12. Sudan Red B, [3176-79-2], F.W. = 380.44
13. Fast Garnet GBC Base, [97-56-3], F.W. = 225.30
14. Sudan Black B, [4197-25-5], F.W. = 456.55
15. Toluidine Red, [2425-85-6], F.W. = 307.30
16. 4-Nitroaniline, [100-01-6], F.W. = 138.13
17. Metanil Yellow, [587-98-4], F.W. = 375.38*
18. Auramine O, [2465-27-2], F.W. = 321.84

Notes:

a. CAS numbers are shown between square brackets
b. Formula Weight = F.W.
c. Sigma-Aldrich is a vendor for these dyes. Dyes are best if ordered by CAS numbers. Seek higher dye purity options.
d. Sodium salt, indicated by an asterisk (*) following F.W. values

Solvents

1. Methylene Chloride (LC/MS grade or equivalent)
2. Acetonitrile (LC/MS grade or equivalent)
3. Acetic Acid, Glacial (LC/MS grade or equivalent)
4. Acetone (LC/MS grade or equivalent)
5. Methanol (LC/MS grade or equivalent)
6. Water (LC/MS grade or equivalent)

Note: If LC/MS grade solvent or equivalent is not available, a purity check should be performed to establish the solvent purity before use.

C. Preparation of Sample

Preparation of Calibration Standards

1. Stock Standard A (methylene chloride-soluble dyes). Prepare a stock standard of Sudan I-IV, Para Red, Sudan Orange G, Sudan Red B, Fast Garnet GBC Base, Toluidine Red, Sudan Red 7B, Sudan Black B, 4-Nitroaniline, Sudan Red G and Dimethyl Yellow dyes by accurately weighing 0.0250 g of each dye into a 100 mL volumetric flask. Dissolve the dyes with methylene chloride. This is Stock Standard A.
   Note: Correct each standard weight to pure dye content based on the declared purity of the dye.
2. Stock Standard B (methanol-soluble dyes). Prepare a stock standard of Auramine O, Metanil Yellow, Orange II and Rhodamine B by accurately weighing 0.0250 g of the dye into a 100 mL volumetric flask. Dissolve the dye with methanol. This is stock standard B.
3. Prepare a working standard solution by pipeting 0.400 mL of each stock standard solution to a 100 mL volumetric flask. Dilute to volume with Acetonitrile and mix well. The concentration of the intermediate standard solution will be 1 µg/mL.

Preparation of matrix Standards

A detailed scheme for preparation of Matrix Standards is provided in section E under Calibration

Notes:

a. Run acetonitrile as a blank (0.0 ng/mL) standard solution.
b. Store all standard in a freezer wrapped in aluminum foil to minimize exposure to light when not in use.
c. Inject a standard corresponding to the level of action periodically to show instrument stability.
d. After the lab has established the instrument linearity by running the matrix calibration standard series, a single point standard calibration can be run with the 1.0 ng/mL.

Preparation of Sample and Spike Extracts

1. For spices, accurately weigh 4.0 g of sample into a 50 mL culture tube. For oleoresin samples, accurately weigh 2.0 g of sample into a 50 mL culture tube. For spikes and mixed standard, add at an appropriate level. Do same extraction as done for samples.
2. For spices, pipette 20 mL of acetonitrile into each tube, cap and shake on a wrist action shaker for 30 min.
3. For oleoresins, pipette 20 mL of acetone into each tube, cap and shake on a wrist action shaker for 30 min.
4. Filter through a 0.45 mm PTFE filter into a 4 mL vial. If the extract solution contains insoluble oils, it may be helpful to centrifuge the extract for 5 minutes at 3,000 RPMs before filtration of the supernatant.
5. Transfer exactly 1 mL to autosampler vial.
6. To make sample concentration equivalent to the matrix standards add 20 µL of acetonitrile to the autosampler vial.

D. Procedure

1. Mobile Phase A: 0.1 % acetic acid
2. Mobile Phase B: acetonitrile
3. Mobile Phase C: acetone
4. Flow Rate: 0.30 mL/min
5. Injection Volume: 20 µL
6. Column Temperature: 25 - 35°C
7. Gradient Program:

Time (min.)	Mobile Phase A (%)	Mobile Phase B (%)	Mobile Phase C (%)
0.0	75	25	0
2.0	75	25	0
5.0	30	70	0
15.0	0	100	0
20.5	0	100	0
21.0	0	0	100

22.8	0	0	100
23.2	75	25	0
30.0	75	25	0

Notes:

a. The acetone wash is intended to clear the chromatography column of late eluting matrix components. Frequency and duration necessary for this step may be reduced according to the nature of the samples injected. Analysis of concentrates and extracts, for example, will generally require more frequent washing to maintain column performance. If only a binary pump is used in the HPLC instrumentation, perform a "bake out" of the column with an acetonitrile flush at higher temperature/ higher flow rate for several minutes to eliminate late eluters)

b. For capsicum and paprika samples, column flow should be switched to waste after the last eluting analyte of interest, in order to preserve MS instrument sensitivity by minimizing MS instrument contamination with natural color pigments. Column flow should be returned back to MS instrument at the end of the HPLC gradient.

c. For turmeric samples, due to similar sensitivity reasons, in order to minimize MS source contamination with natural yellow curcuminoid pigments, column flow should be switched to waste right after the eluting time for rhodamine B, and returned back to MS instrument just before the eluting time of Sudan orange G.

MS/MS Detection

1. MS/MS detection is employed using electrospray ionization (ESI) in positive and negative-ion modes.
2. The MRM (multiple reaction monitoring) transitions in the following table are used to detect the dyes of interest.
3. Since instrument parameters are instrument dependent, cone voltages and collision energies (coll. energy) are given for reference only. Each user should determine the optimum parameters based upon their instrument performance. Additionally, mass analyzer calibration differences will require that the actual molecular ion and product ion masses be determined for each instrument; these values should also be verified each time the mass spectrometer is calibrated.
4. Retention times will also vary based upon the overall HPLC setup and are given only as a guide to retention order.

Dye	CAS #	Cone (V)	RT (min)	MRM transitions (coll. energy, eV)		
Positive-Ion				MRM Transition 1	MRM Transition 2	MRM Transition 3
Auramine O	2465-27-2	40	2.6	268.1 → 122.0 (25)	268.1 → 147.0 (30)	268.1 → 252.0 (30)
4-Nitroaniline	100-01-6	25	5.7	139.0 → 122.0 (15)		
Rhodamine B	81-88-9	45	7.2	443.2 → 355.0 (60)	443.2 → 398.9 (45)	443.2 → 412.9 (45)
Sudan Orange G	2051-85-6	25	9.4	215.1 → 93.1 (20)	215.1 → 122.0 (20)	215.1 → 197.9 (20)
Fast Garnet GBC Base	97-56-3	30	11.0	226.1 → 91.0 (20)	226.1 → 121.0 (25)	226.1 → 133.0 (20)
Dimethyl Yellow	60-11-7	30	12.4	226.1 → 77.0 (20)	226.1 → 121.0 (20)	226.1 → 134.0 (20)
Sudan Red G	1229-55-6	30	13.0	279.1 → 123.0 (30)	279.1 → 156.0 (30)	279.1 → 262.0 (30)
Toluidine Red	2425-85-6	25	13.1	308.1 → 152.0 (20)	308.1 → 156.0 (15)	308.1 → 290.9 (15)
Sudan I	842-07-9	25	13.6	249.1 → 93.1 (25)	249.1 → 156.0 (15)	249.1 → 232.0 (15)
Sudan II	3118-97-6	25	16.8	276.9 → 121.0 (20)	276.9 → 156.0 (15)	276.9 → 260.0 (10)
Sudan Black B	4197-25-5	40	18.1	457.2 → 105.0 (25)	457.2 → 194.0 (35)	457.2 → 210.9 (25)
Sudan III	85-86-9	30	19.3	353.1 → 120.0 (25)	353.1 → 156.0 (20)	353.1 → 197.0 (20)
Sudan Red 7B	6368-72-5	25	21.0	380.1 → 156.9 (15)	380.1 → 168.9 (25)	380.1 → 182.9 (15)
Sudan Black B	4197-25-5	40	21.2	457.2 → 245.9 (25)		
Sudan Red B	3176-79-2	40	21.9	381.1 → 91.0 (25)	381.1 → 133.8 (25)	381.1 → 224.0 (25)
Sudan IV	85-83-6	30	22.0	381.2 → 91.0 (30)	381.2 → 225.0 (15)	381.2 → 276.0 (20)
Negative-Ion						
Para Red	6410-10-2	35	12.3	292.1 → 122.0 (25)	292.1 → 138.0 (20)	292.1 → 263.8 (20)
Orange II	633-96-5	30	16.1	327.0 → 170.7 (25)		
Metanil Yellow	587-98-4	45	16.5	352.1 → 155.8 (25)		

Notes:

a. RT = retention time in minutes for each of the dyes.
b. The two isomers of Sudan Black B are chromatographically separated and thus the presence of both should be confirmed.
c. Response for the MRM transition 381.2 → 276.0 occurs only for Sudan IV, not for Sudan Red B. Therefore this transition is key for positively identifying these two closely eluting isomeric dyes.

E. Calculation

Calibration

1. Quantitate by standard addition on the extract fortified as follows with a 1 ppm mixed working standard solution (Section C), with the instructions presented in the following tables, depending on the nature of the samples.

a. Spice Samples

Sample extract/ Matrix control (µL)	1 ppm mixed std. (µL)	Acetonitrile (µL)	Total vol. (µL)	Added concentration on extract basis (ng/mL)	Added concentration on sample basis (ng/g)
1000	0	20	1020	0	0
1000	1	19	1020	1.00	5
1000	2	18	1020	2.00	10
1000	3	17	1020	3.00	15
1000	4	16	1020	4.00	20
1000	8	12	1020	8.00	40

b. Oleoresins Samples

Sample extract/ Matrix control (µL)	1 ppm mixed std. (µL)	Acetonitrile (µL)	Total vol. (µL)	Added concentration on extract basis (ng/mL)	Added concentration on sample basis (ng/g)
1000	0	20	1020	0	0
1000	2.0	18	1020	2.00	20
1000	5.0	15	1020	5.00	50
1000	8.0	12	1020	8.00	80
1000	10.0	10	1020	10.00	100
1000	20.0	0	1020	20.00	200

2. Plot Response on y-axis and Added Concentration on x-axis. Then (Response – y-intercept)/Slope gives concentration of analyte in the extract (Equation 1). The calculation then gives concentration of analyte in the sample extract directly (Equation 2).

Quantification

Let:

C_{Fx}= Concentration of analyte x in extract (ng/mL)
R_x = Response for analyte x
$Slope_x$ = Slope of the standard addition curve for analyte x
$Intercept_x$ = y-intercept of the standard addition curve for analyte x
V = Final volume of extract (mL)
C_{Sx} = Concentration of analyte x on sample basis (ng/g)
W = Weight of sample (g)

1. Perform a linear regression analysis for each dye to determine the slope of the dye's calibration curve. Do not include the origin (0,0) in the calibration curve. Determine slope (x) for each analyte.
2. For a standard addition curve developed as specified above, concentration of analyte in a sample extract is given by:

$$C_{Fx} = (R_x - Intercept_x) / Slope_x \qquad \text{(Equation 1)}$$

$$C_{Sx} = C_{Fx} * V / W \qquad \text{(Equation 2)}$$

Notes:

a. Since there is a great deal of variation in responses/matrix suppression/enhancement across spices and oleoresins, the requirement to prepare matrix matched standards for every unique matrix may be difficult in a production environment.
b. The use of a single point calibration (at MRL), following a one-time linearity study is a valid approach, while some laboratories may prefer the use of multi-point calibrations for routine testing to ensure accuracy.

F. Statistics

Due to the variety of MS instruments used for previous collaborative study, resulting in different analytical capabilities and sensitivities, it was decided to not include LOQ and LOD values in ASTA 29.0. Instead, LOD and LOQ would have to be determined by the method end-users, based on instrumentation, sample matrices, etc…

G. Notes

See notes provided within each section for clarity

H. References

1. European Commission NEWS notification 03/99
2. Chinese National Quality Assurance and Inspection Bureau, GB/T 19681 – 2005
3. Fedemet, Decision of 2004/92/CE Commission of 21 January 2004
4. ASTA Method 28.0, August 2005

I. Revision History

10/17/2013 New method.