Method 28.0

Determination of oil soluble dyes in capsicum and turmeric samples and products by high performance liquid chromatography

Purpose: To determine the presence of Para Red, Sudan I, Sudan II, Sudan III, Sudan IV, Sudan Orange G, Sudan Red B, Dimethyl Yellow, and cis-Bixin dyes in capsicum and turmeric samples.

A. Apparatus:

1. HPLC equipped with gradient pump, PDA detector, autosampler and computer data workstation
2. Chromatography column, Symmetry C18, 250 mm x 4.6 mm i.d., 5 μm – Waters Corp., Part # WAT054275 or equivalent
3. Balance, readable to 0.0001 g.
4. 50 ml culture tubes with Teflon lined cap.
5. Vortex mixer
6. Wrist action shaker
7. 5 ml Luer-Lok disposable syringe.
8. Whatman Nylon 0.45 μ filter, or equivalent.
9. Fisherbrand PrepSep Silica SPE columns, 500 mg load, 3 cc or equivalent
10. SPE Vacuum Manifold
11. Evaporator / Concentrator
12. Volumetric pipets, various sizes
13. Volumetric flasks, various sizes
14. 25 mL Graduated cylinder

B. Reagents:

1. Methylene Chloride (HPLC grade)
2. Acetonitrile (HPLC grade)
3. Acetic Acid, Glacial (HPLC grade)

4. Acetone (HPLC grade)

5. Methanol (HPLC grade)

6. Sodium Chloride (A.C.S. grade or better)

7. Solution A: Prepare a 200 g/L sodium chloride solution

8. Solution B: Prepare a 12.5 % methanol in sodium chloride solution using Solution A

9. Ethyl Ether (HPLC grade)

10. Hexane (HPLC grade)

11. Sudan I (CAS 842-07-9), Aldrich Chemical Company, Catalog # 10362-4, purity 97%

12. Sudan II (CAS 3118-97-6), Aldrich Chemical Company, Catalog # 19965-6, purity 90%

13. Sudan III (CAS 85-86-9), Aldrich Chemical Company, Catalog # 19811-0, purity 87%

14. Sudan IV (CAS 85-83-6), Aldrich Chemical Company, Catalog #19810-2, purity 88%

15. Sudan Orange G (CAS 2051-85-6), Acros Organics, Catalog # AC190170250

16. Sudan Red B (CAS 3176-79-2), Aldrich Chemical Company-Fluka, Product # 86010

17. Dimethyl Yellow (CAS 60-11-7), Aldrich Chemical Company-Sigma, Product # D6760

18. Para Red (CAS 6410-10-2), Aldrich Chemical Company, Catalog # 100994-25, purity 95%

19. *cis*-Bixin (CAS 6983-79-5), Chromadex, Order # ASB-0002320, purity 88.5 %

C. Preparation of Calibration Standards

1. Prepare a stock standard of Sudan 1-4, Para Red, and Dimethyl Yellow dyes by accurately weighing 0.025 g of each dye into a 100 mL volumetric flask. Dissolve the dyes with methylene chloride. This is Stock Standard A.

2. Prepare a stock standard of Sudan Orange G, and Sudan Red B dyes by accurately weighing 0.025 g of each dye into a 100 mL volumetric flask. Dissolve the dyes with methylene chloride. This is Stock Standard B.

3. Prepare a stock standard of *cis*-Bixin by accurately weighing 0.025 g of the dye into a 100 mL volumetric flask. Dissolve the dye with methylene chloride. This is stock standard C.

   NOTE: *cis*-Bixin will isomerize to its *trans* configuration in solution. A new stock *cis*-Bixin standard and working standards must be prepared when this occurs.

4. Prepare four calibration standards from Stock Standard A, and four calibration standards from Stock Standards B and C, containing the following concentration of dyes: 0.1 µg/mL, 1.0 µg/mL, 5.0 µg/mL and 10.0 µg/mL. Dilute the eight calibration standard solutions to volume with acetonitrile.

5. Transfer the calibration standards to autosampler vials and inject on the HPLC instrument.

NOTE: Correct each standard weight to pure dye content based on the declared purity of the dye.

NOTE: Store all standards in a freezer when not in use.

NOTE: Standard should be injected after each 4 to 6 sample injections.

NOTE: After the lab has established the instrument linearity by running the calibration standard series, then a single point standard calibration can be run with the 1.0 µg/mL standard. However, the PDA detector must be capable of detecting a 0.10 µg/mL solvent standard.

D. Acquisition Parameters:

1. Mobile Phase A – 1.0 % Acetic acid

2. Mobile Phase B – Acetonitrile

3. Mobile Phase C – Acetone

4. Gradient time program

Time (min.)	Mobile Phase A (%)	Mobile Phase B (%)	Mobile Phase C (%)
0	30	70	0
13.3	5	95	0
20.0	0	100	0
23.3	0	100	0
24.0	0	0	100
28.0	0	0	100
28.7	30	70	0
32.7	30	70	0

a. Flow Rate: 1.5 ml/min
b. Detection wavelength: 300 nm to 650 nm.
c. Injection volume: 20 µl.
d. Column Temperature: 25 °C

E. Preparation of Sample Control and Spikes

1. Accurately weigh 4.0 to 8.0 g of a control sample into five separate 50 mL culture tubes. To one of the samples, pipet 10 µL of the Stock Standard A solution (the concentration of each dye in this spike sample will be between approximately 0.3 – 0.6 mg/kg). To a second sample, pipet 100 µL of the Stock Standard A solution (the concentration of each dye in this spike sample will be between approximately 3 – 6 mg/kg). To a third sample, pipet 10 µL of Stock Standard B and 10 µL of Stock Standard C solutions (the concentration of each dye in this spike sample will be between approximately 0.3 – 0.6 mg/kg). To a fourth sample, pipet 100 µL of Stock Standard B and 100 µL of Stock Standard C solutions (the concentration of each dye in this spike sample will be between approximately 3 – 6 mg/kg).

2. Pipet 20 mL of acetonitrile into each tube, cap and shake on a wrist action shaker for 1 hour. Allow the solids to settle or centrifuge.

3. Filter through 0.45 µ nylon filter into autosampler vials and inject on the HPLC instrument.

NOTE: Spike recovery should be between 75-125% of calculated amounts for each dyes.

NOTE: Spikes should be prepared and run with each different sample matrix to identify co-eluting or interfering peaks from the sample matrix. For similar sample matrices, a spiked sample should be run with every 10 sample extracts.

F. Preparation of Sample

1. Accurately weigh 4.0 to 8.0 g of sample into a 50 mL culture tube.

2. Pipet 20 mL of acetonitrile into each tube, cap and shake on a wrist action shaker for 1 hour. Allow the solids to settle or centrifuge.

3. Filter through 0.45 µ nylon filter into autosampler vials and inject on the HPLC instrument

NOTE: A sample clean-up step may be necessary for concentrated or complex products in order to remove some of the compounds that interfere with the chromatographic peaks of interest. To perform this clean-up step, proceed as in section G below.
Please be advised that this clean-up step may not recover *cis*-Bixin.

NOTE: When testing for Sudan Orange G, sample clean-up must be performed to eliminate matrix interferences which co-elute with the Sudan Orange G peak in capsicum samples.

G. Sample and Spike Sample Cleanup

1. Pipet 5.0 mL of the acetonitrile layer (from step 2 in sections E and F) into a 50 mL centrifuge tube.

2. Pipet 5.0 mL of hexane into the centrifuge tube.

3. Use a 25 mL graduated cylinder to add 25 mL of Solution B to the centrifuge tube.

4. Shake gently for 30 seconds then centrifuge for 3 minutes.

5. Prepare a silica SPE column for each sample. Initially prewash the silica SPE with one column volume of ethyl ether followed by two column volumes of hexane. Discard the eluted solvent wash.

   NOTE: Prewash each silica SPE column prior to use. Keep the silica bed wet with solvent and do not store prewashed SPE columns for more than 30 minutes. Mild vacuum may be applied to the SPE columns to pull the solvents through the column.

6. Pipet 2.0 mL of the hexane (top) layer from step 4 into the solvent washed silica SPE columns. Drain the sample extract into the column bed at 1-2 drips per second.

7. Wash with one column volume of hexane and discard the hexane wash.

3. Place clean collection tubes below the silica SPE columns and elute the dyes into the collection tubes with two column volumes of 10% acetone in hexane.

4. Evaporate the solvent in the collection tubes to dryness under a stream of dried nitrogen or other inert gas.

5. Redissolve the residue in each collection tube with 2.0 mL of acetonitrile.

6. Filter through 0.45 µ Nylon filter into autosample vials and inject on the HPLC instrument.

H. Processing/Data Analysis Parameters:

1. Set-up a processing/data analysis method to process the calibration standards containing Sudan 1-4, Para Red, and Dimethyl Yellow. Use the following wavelengths: Dimethyl Yellow – 450 nm; Para Red – 450 nm; Sudan 1 – 505 nm; Sudan 2 – 505 nm; Sudan 3 – 530 nm; Sudan 4 – 505 nm (550 nm for paprika oleoresin).

2. Process the corresponding four calibration standards and samples for these six dyes.

3. Set-up a processing/data analysis method to process the calibration standards containing Sudan Orange G, *cis*-Bixin, and Sudan Red B. Use the following wavelengths: Sudan Orange G – 377 nm; *cis*-Bixin – 460 nm; Sudan Red B – 505 nm (550 for paprika oleoresin).

4. Process the corresponding four calibration standards and samples for these three dyes.

I. Calculations:

1. Using the data processing technique in section H above, perform a linear regression analysis for each dye to determine the slope, m, of the dye's calibration curve. Force the line through the origin. Let the peak areas be the y-variable and the concentration, µg/mL, be the x-variable.

2. Calculate the concentration of dye(s) in the samples with the following formula:

$$C_x = (A_x * 20) / (m * W)$$

where

C_x: concentration of dye (x) found, µg/g (mg/kg)
A_x: peak area of dye (x) in sample
m: slope of the calibration curve for dye (x)
20: sample extraction volume in mL
W: sample weight in g

J. Notes:

1. Limit of quantitation is estimated to be 1.0 mg/kg for each dye.

2. Confirmation of dyes present in a sample can be performed by LC/MS. A photodiode array (PDA) detector can provide some limited confirmation by comparing the visible spectrum and wavelength of maximum absorbance of the dye in question to the visible spectrum and wavelength of maximum absorbance of a reference standard.

3. There can be a peak that co-elutes with Sudan III in paprika. The identification and confirmation of Sudan III in these samples should be done with another detector, such as a mass spectrometer.

4. The order of elution of Para Red, Dimethyl Yellow, and Sudan I through IV is as follows: Para Red, Dimethyl Yellow, Sudan I, Sudan II, Sudan III and Sudan IV. The order of elution of Sudan Orange G, *cis*-Bixin, and Sudan Red B is as Follows: Sudan Orange G, *cis*-Bixin, Sudan Red B.

K. 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

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