Promulgated, Nov 19, 2012
Amended, Sep 6, 2013
MOHWA0016.01

Method of Test for Colorants in Foods

1. Scope

This method is applicable to the determination of colorants in foods.

2. Method

After extraction and wool dyeing, analytes are determined by paper chromatography (PC), thin layer chromatography (TLC) and high performance liquid chromatography (HPLC).

2.1. Sample preparation

2.1.1. Chemicals

Ethanol, reagent grade;
Acetic acid, reagent grade;
Diethyl ether, reagent grade;
Petroleum ether, reagent grade;
Ammonium hydroxide (25%), reagent grade;
Sodium chloride, reagent grade;
Deionized water, resistivity ≥ 18 MΩ · cm (at 25 ℃).

2.1.2. Reagents

2.1.2.1. 80% ethanol

Dilute ethanol with deionized water at the ratio of 80:20 (v/v).

2.1.2.2. 70% ethanol

Dilute ethanol with deionized water at the ratio of 70:30 (v/v).

2.1.2.3. 1% ammonium hydroxide in 70% ethanol

Dilute 4 mL of ammonium hydroxide with 70% ethanol to 100 mL.

2.1.2.4. 1% acetic acid in 70% ethanol

Dilute 1 mL of acetic acid with 70% ethanol to 100 mL.

2.1.2.5. 10% ammonium hydroxide

Dilute 40 mL of ammonium hydroxide with deionized water to 100 mL.

2.1.2.6. 25% sodium chloride solution

Dissolve 25 g of sodium chloride with deionized water to 100 mL.

2.1.2.7. 6% acetic acid

Dilute 6 mL of acetic acid with deionized water to 100 mL.

2.1.3. Sample solution preparation

2.1.3.1. Liquid sample (alcoholic beverages, soft dinks, liquid seasoning, etc.): Transfer 20 ~ 200 mL of sample depending on the color intensity, dilute with appropriate amount of deionized water as the sample solution. For

alcohol product, neutralize first and then evaporate ethanol by hot water bath. Add deionized water to the original volume as the sample solution.

2.1.3.2. Candy, cookie and agricultural products

Transfer 20 ~ 200 g of sample depending on the color intensity, grind or fine cut. Follow the procedure described below to obtain the sample solution.

2.1.3.2.1. Candy

Dissolve sample with about 5 times the amount of hot deionized water as the sample solution. If the sample is only colored on the surface, take the colored portion for preparation of sample solution.

2.1.3.2.2. Pickled fruits and vegetables

Add about 4 ~ 5 times the amount of 80% ethanol to the sample and shake to mix. Stand for 2 - 3 hr, then collect the leaching solution. Leach the sample with 1% ammonium hydroxide in 70% ethanol once or several times. Neutralize the combined leaching solution with 6% acetic acid. Evaporate most of the ethanol by water bath, and then add deionized water to the original volume as the sample solution. If the sample is still colored, leach with 1% acetic acid in 70% ethanol once or several times, neutralize the combined leaching solution with 10% ammonium hydroxide. Evaporate ethanol by water bath, and then add deionized water to the original volume as the sample solution.

2.1.3.2.3. Jelly, jam, miso, and stuffing food

Add about 3 ~ 5 times the amount of hot deionized water to the sample, mix and stand for a while. Filter with glass wool or asbestos and collect the filtrate as the sample solution. If the color cannot be extracted by this method, follow the procedure described in section 2.1.3.2.2. to obtain the sample solution.

2.1.3.2.4. Chocolate, cocoa, and cream

Place the sample on a large filter paper or in a beaker. Degrease by diethyl ether for several times. Remove the residual diethyl ether by a filter paper or air drying. Follow the procedure described in section 2.1.3.2.2. to obtain the sample solution.

2.1.3.2.5. Cereal products

Add about 5 times the amount of 80% ethanol to the sample, stand for 24 hr, and shake frequently. Stand again. Collect the leaching solution, and concentrate to 1/5 the original volume by water bath. Add about 1/4 volume of 25% sodium chloride solution and alkalinize by 10% ammonium hydroxide. Transfer the solution to a separatory funnel, degrease by adding the same amount of petroleum ether and shake several times. Neutralize the lower layer by adding 6% acetic acid as the sample solution. If the petroleum ether layer is colored, add 6% acetic acid and shake to extract the colorants once again. Combine the acetic acid layer with other extracts as the sample solution.

2.1.3.2.6. Chewing gum

Add about 5 times the amount of deionized water to the sample and heat to boiling. Filter with a membrane filter after cooling down and collect the colored filtrate as sample solution. If the sample is still colored or the filtrate is not colored, add 10% ammonium hydroxide to neutralize or slightly alkalinize the solution. Heat to boiling. Filter with a membrane filter after cooling down. Combine the filtrate as sample solution.

2.1.3.3. Aquatic and livestock products

Transfer 20 ~ 200 g of sample depending on the color intensity. Follow the procedure described in section 2.1.3.2.4. to obtain the sample solution.

2.2. Wool dyeing and separation method

2.2.1. Paper chromatography

2.2.1.1. Equipment

2.2.1.1.1. Developing tanks

2.2.1.1.2. UV lamp, 254 nm and 365 nm (or 375 nm).

2.2.1.2. Chemicals

Acetic acid, reagent grade;

Ammonium hydroxide (25%), reagent grade;

n-Butanol, reagent grade;

Ethanol, reagent grade;

Acetone, reagent grade;
iso-Pentanol, reagent grade;
Deionized water, resistivity ≥ 18 MΩ · cm (at 25 ℃);
New Coccine, Erythrosin, Tartrazine, Sunset Yellow FCF, Brilliant Blue FCF, Indigo Carmine, Fast Green FCF and Allura Red AC, reference standards.

2.2.1.3. Apparatus

2.2.1.3.1. Filter paper, chromatograph grade.

2.2.1.3.2. Capillary tube or micropipette.

2.2.1.3.3. Volumetric flask, 100 mL.

2.2.1.3.4. Separatory funnel.

2.2.1.3.5. Beaker.

2.2.1.3.6. Wool: Use degreased and uncolored pure wool that does not contain fluorescent substances, otherwise, follow the steps below.

(1) Degreasing: Use a Soxhlet extractor to fully degrease the wool with petroleum ether. Take out the wool and remove the petroleum ether at room temperature. Wash with deionized water. Gently squeeze the wool and air dry.

(2) Defluorescent: Transfer 10 g of degreasing wool in a beaker, add 1 ~ 4 mL of ammonium hydroxide, and add appropriate amount of deionized water to soak. Heat in a water bath at 45 ℃ for 30 ~ 60 minutes and stir frequently. Transfer the wool into diluted ammonium hydroxide, stir well. Take out the wool, wash with warm deionized water first, and then with cold deionized water. Gently squeeze the wool and air dry.

2.2.1.4. Reagents

2.2.1.4.1. Diluted ammonium hydroxide

Dilute 1 mL of ammonium hydroxide with deionized water to 100 mL.

2.2.1.4.2. 1N acetic acid

Dilute 6 g of acetic acid with deionized water to 100 mL.

2.2.1.4.3. 0.5N acetic acid

Dilute 1N of acetic acid with deionized water at the ratio of 1:1 (v/v).

2.2.1.4.4. 5% ammonium hydroxide

Dilute 20 mL of ammonium hydroxide with deionized water to 100 mL.

2.2.1.4.5. 1% ammonium hydroxide

Dilute 5% of ammonium hydroxide with deionized water at the ratio of 1:4 (v/v).

2.2.1.4.6. 10% acetic acid

Dilute 10 mL of acetic acid with deionized water to 100 mL.

2.2.1.4.7. 0.5N ammonium hydroxide

Dilute 7 mL of ammonium hydroxide with deionized water to 100 mL.

2.2.1.4.8. 25% ethanol

Dilute ethanol with deionized water at the ratio of 25: 75 (v/v).

2.2.1.5. Developing solvents

(1) *n*-Butanol: ethanol: 0.5N ammonium hydroxide (6: 2: 3, v/ v/ v).

(2) *n*-Butanol: ethanol: 0.5N acetic acid (6: 2: 3, v/ v/ v).

(3) Acetone: *iso*-pentanol: deionized water (6: 5: 5, v/ v/ v).

(4) 25% ethanol: 5% ammonium hydroxide (1: 1, v/ v).

2.2.1.6. Standard solution preparation

Transfer about 100 mg of reference standards accurately weighted to a 100-mL volumetric flask. Dissolve and dilute with deionized water to volume as standard solution.

2.2.1.7. Sample solution preparation

Transfer about 5 ~ 20 mL of the sample solution depending on the color intensity in a beaker. Acidify by 1 mL of 1 N acetic acid and put 0.1 g of wool in. After stirring and heating in a water bath for 30 min, take out the wool and wash sufficiently. Put the wool in another beaker and add 5 mL of 1% ammonium hydroxide. After heating in a water bath to dissolve the color, remove the wool. Acidify the solution by adding 2 mL of 10 % acetic acid. Then, add 0.1 g of new wool in the solution and heat in a water bath. When the wool is colored, there will be acidic colorants. Take out the colored wool and dissolve the color with the same procedure described above by adding 1% ammonium hydroxide. Concentrate the solution to about 0.5 mL as the sample solution.

2.2.1.8. Identification:

Draw a horizonal line on the bottom of a filter paper. Use capillary tubes or a micropipette to apply the sample and the standard solutions on the bottom line separately every 1.5 ~ 2 cm for a size about 0.5 cm dot. After air drying, place the filter paper into a developing tank. The paper must be vertical and not connect to the wall of the tank. Immerse about 1 cm of the bottom of the paper in the developing solvent. Cover the tank for developing to about 12 ~ 25 cm high of the paper. Take out the paper and air dry. Identify each colorant by comparing positions and colors of dots of the sample solution to the standard solution. Observation under a UV lamp may be applied when needed.

2.2.2. Thin layer chromatography

2.2.2.1. Equipment

2.2.2.1.1. Developing tank

2.2.2.1.2. UV lamp, same as section 2.2.1.1.2.

2.2.2.2. Chemicals

Acetic acid, reagent grade;
Ammonium hydroxide (25%), reagent grade;
Ethyl acetate, reagent grade;
Ethanol, reagent grade;
n-Pentanol, reagent grade;
Methyl ethyl ketone, reagent grade;
Ethylene glycol monomethyl ether, reagent grade;
Methanol, reagent grade;
iso-Pentanol, reagent grade;
Deionized water, resistivity ≥ 18 MΩ · cm (at 25 ℃) ;
New Coccine, Erythrosin, Tartrazine, Sunset Yellow FCF, Brilliant Blue FCF, Indigo Carmine, Fast Green FCF and Allura Red AC, reference standards.

2.2.2.3. Apparatus

2.2.2.3.1. Capillary tube or micropipette.

2.2.2.3.2. Separatory funnel.

2.2.2.3.3. Wool, same as section 2.2.1.3.6.

2.2.2.3.4. Thin layer chromatograph plate, silica, 0.2 mm, 20 x 20 cm.

2.2.2.4. Reagents

2.2.2.4.1. 1 N acetic acid: same as section 2.2.1.4.2.

2.2.2.4.2. 1% ammonium hydroxide: same as section 2.2.1.4.5.

2.2.2.4.3. 10% acetic acid: same as section 2.2.1.4.6.

2.2.2.5. Developing solvents:

(1) Ethyl acetate: methanol: ammonium hydroxide (4: 5: 1 or 3:1:1, v/ v/ v).

(2) *n*-Pentanol: ethanol: ammonium hydroxide (10: 10: 1, v/ v/ v).

(3) Methyl ethyl ketone: ethylene glycol monomethyl ether: ethanol: ammonium hydroxide (20: 15: 12: 1, v/ v/ v/ v).

(4) Methanol: ethanol: iso-pentanol: ammonium hydroxide (15: 10: 5: 3, v/ v/ v/ v).

2.2.2.6. Standard solution preparation: same as section 2.2.1.6.

2.2.2.7. Sample solution preparation: same as section 2.2.1.7.

2.2.2.8. Identification

Use capillary tubes or a micropipette to apply the sample and the standard solutions on the bottom line separately every 1 cm for a size about 0.3 cm dot. After air drying, place the TLC plate into a developing tank. Immerse about 0.5 ~ 1 cm of the bottom of the paper in the developing solvent. Identify each colorant by comparing positions and colors of dots of the sample solution to the standard solution. Observation under a UV lamp may be applied when needed.

2.2.3. High performance liquid chromatography

2.2.3.1. Equipment

2.2.3.1.1. High performance liquid chromatograph.

2.2.3.1.1.1. Detector: photodiode array detector.

2.2.3.1.1.2. Column: Atlantis T3, 3 μm, 2.1 mm × 10 cm, or an equivalent product.

2.2.3.2. Chemicals

Acetic acid, reagent grade;

Ammonium hydroxide (25%), reagent grade;

Phosphoric acid (85%), reagent grade;

Diammonium hydrogen phosphate, reagent grade;

Ammonium dihydrogen phosphate, reagent grade;

Methanol, HPLC grade;

Deionized water, resistivity ≥ 18 MΩ · cm (at 25 ℃);

New Coccine, Erythrosin, Tartrazine, Sunset Yellow FCF, Brilliant Blue FCF, Indigo Carmine, Fast Green FCF and Allura Red AC, reference standards.

2.2.3.3. Apparatus

2.2.3.3.1. Volumetric flask: 100 mL and 1000 mL.

2.2.3.3.2. Membrane filter: 0.45 μm, Nylon.

2.2.3.4. Reagents

2.2.3.4.1. 1N acetic acid: same as section 2.2.1.4.2.

2.2.3.4.2. 1% ammonium hydroxide: same as section 2.2.1.4.5.

2.2.3.4.3. 10% acetic acid: same as section 2.2.1.4.6.

2.2.3.4.4. 1M phosphoric acid:

Dilute 67.4 mL of phosphoric acid with deionized water to 1000 mL.

2.2.3.5. Mobile phase

2.2.3.5.1. Solvent A

Dissolve and dilute 1.15 g of diammonium hydrogen phosphate and 1.32 g of ammonium dihydrogen phosphate with deionized water to 1000 mL. Adjust pH of the solution to 6.0 by 1M phosphoric acid. Filter with a membrane filter.

2.2.3.5.2. Solvent B: Methanol.

2.2.3.6. Standard solution preparation

Transfer about 100 mg of each reference standard accurately weighted into a 100-mL volumetric flask, dissolve and dilute with deionized water to volume as standard stock solution. When to use, dilute with deionized water to 10 μg/mL as the standard solution.

2.2.3.7. Sample solution preparation

Follow the same procedure described in section 2.2.1.7. Filter with a membrane filter as the sample solution.

2.2.3.8. Identification

Accurately inject 10 μL of the sample solution and the standard solution into HPLC separately. Operate according to the conditions described below. Identify each colorant based on the retention time and the UV absorption spectrum.

HPLC operating conditions:

Photodiode array detector: 254 nm.

Column: Atlantis T3, 3 μm, 2.1 mm × 10 cm.

Column oven temperature: 30℃.

Mobile phase: gradient program of solvent A and solvent B is as follows

Time (min)	A (%)	B (%)
0 → 4	90 → 50	10 → 50
4 → 8	50 → 40	50 → 60
8 → 12	40 → 20	60 → 80
12 → 12.1	20 → 90	80 → 10
12.1 → 15	90 → 90	10 → 10

Flow rate: 0.7 mL/min.

Remark

1. Further validation should be performed when interfering compounds are found in the samples.
2. When confirming with LC-MS/MS, the multiple reaction monitoring (MRM) parameters are listed in the attached table.

Table. MRM parameters of 8 colorants (Allura red AC, etc.) by LC-MS/MS.

Analyte	Ionization mode	Quantitation ion pair			Qualitative ion pair		
		Precursor ion(*m/z*) >product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)	Precursor ion(*m/z*) >product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
New Coccine	ESI^-	268 > 206	-25	-18	206 > 80	-35	-40
Erythrosin	ESI^-	834 > 127	-80	-84	834 > 227	-80	-91
Tartrazine	ESI^-	244 > 80	-21	-62	244 > 198	-21	-20
Sunset Yellow FCF	ESI^-	407 > 207	-57	-41	407 > 80	-57	-108
Brilliant Blue FCF	ESI^-	373 > 170	-45	-42	373 > 80	-45	-92
Indigo Carmine	ESI^-	226 > 198	-42	-27	226 > 105	-42	-53
Fast Green FCF	ESI^-	381 > 170	-40	-38	381 > 341	-40	-25
Allura Red AC	ESI^-	225 > 136	-32	-34	225 > 80	-32	-59

Note: All the parameters can be adjusted depending on the instruments used if the above conditions are not applicable