	Title	Revision n°	date	Document No
	IDENTIFICATION OF PROHIBITED COLORANTS IN COSMETIC PRODUCTS BY TLC AND HPLC	1	28/11/13	ACM 002

A. IDENTIFICATION BY TLC

1. SCOPE AND FIELD OF APPLICATION

This method describes the identification of the following prohibited colorants in cosmetic products:

Names	C I number
Pigment Orange 5	12075
Metanil Yellow	13065
Rhodamine B	45170 and 45170:1

2. PRINCIPLE

Prohibited colorants in cosmetic products are extracted and identified by Thin Layer Chromatography (TLC).

3. REAGENTS

All reagents used mustl be of analytical grade, unless otherwise stated.

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3.1 Ammonia hydroxide Solution 28%
3.2 n-Butanol
3.3 Dichloromethane
3.4 Ethyl acetate
3.5 Ethanol-96%
3.6 Glacial acetic acid
3.7 Iso-butanol
3.8 Iso-propanol
3.9 Methanol
3.10 N,N-Dimethylformamide
3.11 Orthophosphoric acid 85%
3.12 Petroleum ether (range 40 to 60^{o}C or 60 to 80^{o}C)
3.13 n-Hexane
3.14 Solvent Mixture (SM)
This is prepared by mixing N,N-dimethylformamide with orthophosphoric acid in the ratio of 95:5 (v/v)
3.15 Developing solvents
The samples are analysed by TLC using the developing solvents listed below.

3.15.1 For oil-soluble colorants

DS1: Dichloromethane (Methylene chloride)

3.15.2 For water-soluble colorants

DS2: Ethyl acetate/ Methanol/ [(Ammonium hydroxide / Water) 3/7] = 15/3/3 (v/v/v). This must be freshly prepared prior to use.

DS3: Ethanol / Water / Iso-butanol / Ammonia hydroxide = 31/32/40/1 (v/v/v/v)

DS4: Isopropanol / Ammonia hydroxide = 100/ 25 (v/v)

DS5: n-Butanol / Ethanol / Water / Acetic acid = 60/10/20/0.5 (v/v/v/v)

DS6: Ethyl acetate / n-Butanol / Ammonium hydroxide = 20/55/25 (v/v/v)

4. APPARATUS

Normal laboratory equipment, and:

4.1 TLC plates, 20 cm x 20 cm, precoated with 0.25 mm silica gel, layer thickness 0.2 mm, plastic roll or equivalent
4.2 TLC Developing Tanks
4.3 Capillary tubes for spotting – Micropipette (1 to 5 ul)
4.4 Paper Chromatograph No. 1
4.5 PTFE syringe filter, 13 mm diameter, 0.45 um pore size or equivalent
4.6 Vortex mixer
4.7 Ultraviolet light at 254 nm and 366 nm
4.8 Water bath
4.9 Whatman filter paper, medium to fast speed

5. PROCEDURE

5.1 Standard Preparation

5.1.1 Pigment Orange 5 (0.1 mg/ml)

Accurately weigh 1 mg of standard into a 10ml volumetric flask. Add 5ml of dichloromethane (DCM) or solvent mixture (SM). Sonicate for 30min and make up to volume with the same solvent.

5.1.2 Metanil Yellow and Rhodamine B (0.1 mg/ml)

Accurately weigh 1 mg of standard into a 10ml volumetric flask each. Add 5ml of methanol or N,N- dimethylformamide or solvent mixture (SM). Sonicate for 5min and make up to volume with the same solvent.

5.2 Sample preparation

5.2.1 Colour cosmetic products

Weigh about 0.1 g to 0.3 g of sample and add 2 mL of solvent mixture (SM). Sonicate for 5 min. If necessary, heat it at 90°C for 30min or until the sample dissolves.

Note:
For sample suspected to contain Pigment Orange 5, extraction with DCM can also be performed.

For oily cosmetic samples, extract the fatty material with 2 x 5 mL hexane. Combine the hexane extracts. If coloured, back extract with a 2 mL portion of solvent mixture (SM). Discard the hexane layer
Filter the solvent mixture layer through a 0.45 µm membrane filter.
Use the filtrate for application on the TLC plates.

5.2.2 Toiletries and other water-based cosmetic products

Weigh about 1 to 5 g of sample (depending on color intensity of sample). Add 20 ml of DMF and warm the sample solution on water bath for 10 min. Leave it to cool to room temperature and filter through Whatman filter paper The organic colour will dissolve in the DMF solution.

Excess oil can be eliminated by extraction with 40 mL of petroleum ether. The DMF layer is evaporated to dryness on water bath. If colour is present in the petroleum ether layer which indicates the presence of oil soluble colour, keep this layer and evaporate it to dryness.

Note: For further purification, the coloured solution may be streaked onto the TLC plate as much as applicable. Develop the plate in system DS1 to eliminate the oil. The same plate may be further developed in system DS2, which will separate the water soluble colours.
Remove each band of colour and keep separately in a flask.
Extract the colourant(s) from the silica gel of each band with methanol, filter and evaporate the filtrate to dryness.

5.3 TLC Procedure

5.3.1 Colour cosmetic products

Line a TLC tank with a chromatographic No. 1 paper. Saturate the tank with an appropriate developing solvent. Apply on the baseline of a TLC plate, the following

- 1 to 5 µL of standard solutions
- 1 to 5 µL of sample solution depending on its colour intensity

For all developing solvents, except DCM, develop the TLC plate at room temperature until the solvent front has migrated about 15 cm from the baseline.

Note: For DCM, develop the plate until the solvent front has migrated to about 11 cm from baseline. Remove the TLC plate and dry at room temperature.

5.3.2 Toiletries and other water-based cosmetic products (gels and solutions)

Dissolve the residue with 0.5 to 1 mL of methanol. Filter through 0.45 um membrane filter. Spot the standard and sample solution on TLC plates. Develop the plate in an appropriate developing solvent. Allow it to dry at room temperature.

Notes:

- For water soluble colorants (CI 13065, Metanil Yellow and CI 45170, Rhodamine B), developing solvent DS2 to DS6 can be used.

- For oil soluble colorants (CI 12075, Pigment Orange 5), use developing solvent DS1. Solvent DS2 may also be used for preliminary screening.

6. INTERPRETATION

6.1 Calculate the Rf value for each spot using the following formula.

$$\text{Rf value} = \frac{\text{Distance between the spot and the baseline}}{\text{Distance between the baseline and the solvent front}}$$

6.2 Compare the spots from sample solution with those of the standard solutions, with respect to their Rf values and the colour spots under normal daylight and UV light.

CI number	Name	Colour of spot/ban	Estimated Rf				
			DS1	DS2	DS3	DS4	DS5
CI 12075	Pigment Orange 5	Orange	0.4				
CI 13065	Metanil Yellow	Yellow		0.4	0.9	0.7	0.6
CI 45170	Rhodamine B	Bright pink		0.8	0.8	0.7	0.4

Further confirmation test shall be carried out by HPLC described in the following section (B).

7. REMARKS

7.1 Limit of detection (LOD)

Colourant / LOD	Standard (ug)	Colour cosmetics sample (ug/g)	Toiletries sample (ug/g)
Pigment Orange 5	0.02	133-400	0.4-4
Metanil Yellow	0.005	33-100	0.1-1
Rhodamine B	0.04	266-800	0.8-8

B. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

1. SCOPE AND FIELD OF APPLICATION

This method describes the identification of the following prohibited colorants in cosmetic products:

Names	CI number
Pigment Orange 5	12075
Metanil Yellow	13065
Rhodamine B	45170 and 45170:1

2. PRINCIPLE

Prohibited colorants in cosmetic products are extracted and identified by reverse phase High Pressure Liquid Chromatography (HPLC) and visible wavelength detection (UV).

3. REAGENTS

All reagents must be of analytical grade unless otherwise stated.

3.1 Dichloromethane
3.2 Methanol (HPLC)
3.3 N,N-Dimethylformamide
3.4 n-Hexane
3.5 Water (ultrapure,18 ohm)
3.6 Orthophosphoric acid 85%
3.7 Tetrabutylammonium hydroxide (TBA), 20% solution in water, Merck or equivalent
3.8 Potassium hydroxide
3.9 Solvent Mixture
This is freshly prepared by mixing N,N-dimethylformamide with orthophosphoric acid in the ratio 95:5 (v/v)
3.10 Mobile Phase (0.005M tetrabutylammonium hydroxide solution/water = 75/25 (v/v)

The 0.005 M TBA hydroxide solution is prepared as follows:

- Add 65 mL of 20% TBA hydroxide solution (Merck)
- Make up to 100 mL with water
- Transfer the solution to a 250 mL beaker
- Add 2.8 g potassium hydroxide to the above solution and make it dissolve
- Adjust to pH 7 with orthophosphoric acid. This is a 0.5 M TBA hydroxide solution
- Dilute 10 mL of 0.5 M TBA hydroxide solution to 1 litre with methanol
- The solution becomes turbid. Let the precipitate settle for few hours. Filter through 0.45 um membrane filter

4. APPARATUS

Normal laboratory equipment and

4.1 High Pressure Liquid Chromatography with a variable visible wavelength detector
4.2 Whatman PTFE disposable syringe filter, 13 mm diameter, 0.45 um pore size or equivalent
4.3 Nylon filter, 47 mm diameter, 0.45 um pore size or equivalent
4.4 Vortex mixer or ultrasonic bath
4.5 Water bath
4.6 Whatman filter paper, medium to fast speed

5. PROCEDURE

5.1 Standard Preparation

5.1.1 Pigment Orange 5 (0.1 mg/ml)

Accurately weigh 1 mg of standard into a 10ml volumetric flask. Add 5ml of dichloromethane (DCM) or solvent mixture (SM). Sonicate for 30min and make up to volume with the same solvent.

5.1.2 Metanil Yellow and Rhodamine B (0.1 mg/ml)

Accurately weigh 1 mg of standard into a 10ml volumetric flask each. Add 5ml of methanol or N,N- dimethylformamide or solvent mixture (SM). Sonicate for 5min and make up to volume with the same solvent.

5.2 Sample Preparation

5.2.1 Colour cosmetic products

Weigh about 0.1 g to 0.3 g of sample and add 2 mL of solvent mixture (SM). Sonicate for 5 min. If necessary, heat it at 90°C for 30min or until the sample dissolves.

<u>Note:</u>
For sample suspected to contain Pigment Orange 5, extraction with DCM can also be performed.

For oily cosmetic samples, extract the fatty material with 2 x 5 mL hexane. Combine the hexane extracts. If coloured, back extract with a 2 mL portion of solvent mixture (SM). Discard the hexane layer
Filter the solvent mixture layer through a 0.45 µm membrane filter.

5.2.2 Toiletries and other water-based cosmetic products

Weigh about 1 to 5 g of sample (depending on color intensity of sample).
Add 20 ml of DMF and warm the sample solution on water bath for 10 min.
Leave it to cool to room temperature and filter through a 0.45 µm membrane filter.

Excess oil can be eliminated by extraction with 40 mL of petroleum ether.
The DMF layer is evaporated to dryness on water bath. Dissolve the residue in 1-2mL of solvent mixture and filter through a 0.45 µm membrane filter.

Note:
If colour is present in the petroleum ether layer, this indicates the presence of oil soluble color. Keep this layer and evaporate it to dryness.

For further purification, the coloured solution may be streaked onto the TLC plate as much as applicable. Develop the plate in system DS1 to eliminate the oil.
The same plate may be further developed in system DS2, which will separate the water soluble colours.
Remove each band of colour and dissolve the residue in 1-2mL of solvent mixture.
Filter through a 0.45 µm membrane filter.

5.3 HPLC Conditions

Column oven temperature:	30^{o}C
Column:	Hypersil ODS (C18), 5 um 200 x 4.6 mm or equivalent
Flow:	1 ml/min
Detection Wavelength:	435 nm and 535 nm
DAD Spectral Range:	275 to 760 nm
Injection Volume:	10 uL
Run time	35min

6. INTERPRETATION

6.1 Compare the retention time and spectrum of sample chromatogram with that of the colorant standards.
The same retention time and spectra for sample and standard indicates the presence of the colorant of interest.

6.2 The optimum wavelength of detection and the retention time of the standard colorants are as follows:

CI number	Name	Wavelength of Detection	Estimated Retention Time (min)
12075	Pigment Orange 5	535 nm	13
13065	Metanil Yellow	435 nm	3
45170 and 45170:1	Rhodamine B	535 nm	6

Note:

- The spectrum and retention time should give a match factor of a least 950.
- If a colourant is suspected to be present, spike the colourant standard into the sample. One peak should be obtained for the suspected peak and the colourant standard peak.
- Further confirmation by Mass Spectrometry may be required.

7. REMARKS

7.1 Limit of detection (LOD)

Colourant	Standard	Colour cosmetics sample (ug/g)	Toiletries sample (ug/g)
Pigment Orange 5	16	153	32
Metanil Yellow	3	70	6
Rhodamine B	40	800	80

7.2 Pigment Orange 5, Metanil Yellow and Rhodamine B are listed under Annex II – List of substances which must not form part of the composition of cosmetic products.

8. CONCLUSION

The results from the TLC and HPLC chromatograms are used to conclude the identity and presence of the prohibited colourant in the cosmetic products.

Harmonised method:

- **Issued by the chemical analysis group at the harmonization workshop in Kuala Lumpur, on September 13th to 17th, 2004**
- **Approved by the harmonization workshop delegates in Kuala-Lumpur on September 13th to 17th, 2004**
- **Modified after the Singapore training on October 11th to 16th, 2004**
- **Modified and approved after the Brunei workshop on August 30th to 31st, 2005**
- **Modified and approved after the final review in Singapore on November 30th to December 2nd, 2005**