Title	Revision n°	date	Document No
IDENTIFICATION OF PROHIBITED COLORANTS IN COSMETIC PRODUCTS BY TLC AND HPLC	0	02/12/2005	ACM SIN 02

A. IDENTIFICATION BY TLC

1. SCOPE AND FIELD OF APPLICATION

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

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

2. PRINCIPLE

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

3. REAGENTS

All reagents used shall be of analytical purity. Water shall be distilled water or equal purity.

3.1 Ammonium hydroxide Solution 28%
3.2 n-Butanol
3.3 Dichloromethane (DCM)
3.4 Ethyl Acetate
3.5 Ethanol-96%
3.6 Glacial Acetic Acid
3.7 Iso-Butanol
3.8 Isopropanol
3.9 Methanol (MeOH)
3.10 N,N-Dimethylformamide (DMF)
3.11 Orthophosphoric acid 85%
3.12 Petroleum Ether (range 40 to 60 °C or 60 to 80 °C)
3.13 n-Hexane
3.14 Solvent Mixture (SM)

This is to be freshly prepared by mixing N,N-dimethylformamide with orthophosphoric acid in the ratio 95:5 (v/v),

3.15 Developing solvents

The samples are analysed by TLC using the developing solvents listed below. The TLC plate is removed and dried. The oil soluble dyes migrate with 3.15.1 and the water soluble dyes with the rest.

3.15.1 (DS1) Dichloromethane (Methylene chloride)
3.15.2 (DS 2) Ethyl Acetate / Methanol / (Ammonium hydroxide / Water 3/7) = 15/3/3 (v/v/v)
This must be freshly prepared prior to use.
3.15.3 (DS 3) Ethanol / Water / Iso-Butanol / Ammonium hydroxide = 31 / 32 / 40 / 1 (v/v/v/v)
3.15.4 (DS 4) Isopropanol / Ammonium hydroxide = 100 / 25 (v/v)
3.15.5 (DS 5) n-butanol / Ethanol / water / Acetic acid = 60 / 10 / 20 / 0.5
3.15.6 (DS6) Ethyl acetate / n-butanol / Ammonium hydroxide = 20/55/25

3.16 Reference Colorant solutions

Prepare the reference colorant solutions of CI 12075 (oil soluble colorant) in dichloromethane, or solvent mixture at a concentration of 0.1 mg / mL. Sonicate for half an hour or until dissolved.

Prepare the reference colorant solutions of CI 13065 and CI 45170 in methanol (or N,N-dimethylformamide - DMF) or solvent mixture at a concentration of 0.2 mg / mL.

Note: Concentrated colour solutions may give extra spots.

4. APPARATUS

Normal laboratory equipment, and:

- 4.1 Thin-layer plates, 20 cm x 20 cm, precoated with 0.25 mm silica gel 60 F_{254}, layer thickness 0.2 mm, plastic roll, or equivalent
- 4.2 TLC Tanks
- 4.3 Capillary tubes for spotting TLC - Micropipette (1 to 5 μl)
- 4.4 Paper Chromatograph No. 1
- 4.5 Whatman PTFE disposable syringe filter, 13 mm diameter, 0.45 μm pore size , or equivalent
- 4.6 Vortex mixer
- 4.7 Ultraviolet light source, 254 nm and 366 nm
- 4.8 Water bath

5. PROCEDURE

5.1 Sample preparation

5.1.1 Colour cosmetic products

Mix about 0.1 g to 0.3 g of the sample with 2 ml solvent mixture (SM).

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

If necessary, heat at 90°C for 1 hour or until the sample dissolves.
For oily cosmetic samples, extract the fatty material with 2 x 5 ml n-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 obtained above through 0.45 μm membrane filter.
Use the filtrate for application on the TLC plates.

5.1.2 Toiletries and other water-based cosmetic products

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

5.2 Thin-layer chromatography (TLC)

5.2.1 Colour cosmetic products

Line a TLC tank with chromatographic No. 1 paper.
Saturate the TLC tank with the appropriate developing solvent.
For each of the reference colorant solutions and sample solution, apply

- 1 to 5 μl for reference colorant solutions
- and about the same volume of sample solution depending on its colour concentration,

at equal distances on the start line of a TLC plate.
For all developing solvents, except DCM, develop the TLC plate at ambient temperature until the solvent front has migrated 15 cm from the start.
Note: for DCM, develop until the solvent front has migrated to about 11 cm.
Remove the TLC plate and dry at room temperature.

5.2.2 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 μm membrane filter. Spot the sample and reference colorant solutions on TLC plates. Develop in developing solvents. Allow to dry at room temperature.
Notes:

- For water soluble dyes (C. I. 13065 ,Metanil Yellow and C. I. 45170, Rhodamine B), developing solvent systems from DS2 to DS6 can be used.
- For oil soluble dye (C. I. 12075, Pigment Orange 5), use developing solvent DS1. Solvent DS2 may also be used for preliminary screening.

6. IDENTIFICATION

6.1 Detection / Calculation

Calculate the Rf value for each spot. Compare the spots obtained for the sample solution with those of the reference solutions with respect to their Rf values and the colour in daylight. Examine their characteristics under UV light. C. I. 45170 is a xanthene dye which shows brightness under UV light. The Rf values listed in the following table serve as an indication of the values that may be obtained:

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

Draw preliminary conclusions about the identity of the colorants. If the prohibited colorants appear to be present, the HPLC procedure described in the following section may be performed.

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 colorant(s) from the silica gel of each band with methanol, filter and evaporate the filtrate to dryness.

7. REMARKS

Llimit of detection (LOD)

Colorant/ LOD	Standard (µg)	Colour cosmetics sample (µg/g)	Toiletries sample (µg/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. IDENTIFICATION by HPLC

1 SCOPE AND FIELD OF APPLICATION

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

2 C I number	3 Other names
4 12075	5 Pigment Orange 5
6 13065	7 Metanil Yellow
8 45170 and 45170:1	9 Rhodamine B

2 PRINCIPLE

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

3 REAGENTS

All reagents used shall be of analytical purity or HPLC grade, where appropriate.

3.1 Dichloromethane (DCM)
3.2 Methanol (MeOH)
3.3 N,N-Dimethylformamide (DMF)
3.4 n-Hexane
3.5 Water, 18 mega 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 (SM)

This is to be freshly prepared by mixing N,N-dimethylformamide with orthophosphoric acid in the ratio 95:5 (v/v).

3.10 Reference Colorant solutions

Prepare the reference colorant solution for CI 12075 (oil soluble colorant) in dichloromethane or solvent mixture at a concentration of 0.1 mg / mL. Sonicate for half an hour or until dissolved.
Prepare the reference colorant solutions for CI 13065 and CI 45170 in methanol or N,N-dimethylformamide (DMF) or solvent mixture (SM) at a concentration of 0.2 mg / mL.

4 APPARATUS

Normal laboratory equipment and

4.1 High Pressure Liquid Chromatograph 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, 47mm 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

5 PROCEDURE

5.1 Sample preparation

Refer to the steps given in 5.1 under IDENTIFICATION BY TLC

5.2 HPLC

Warning: All solutions, including mobile phase, need to be filtered using 0.45 µm membrane filter prior to injection.

5.2.1 Mobile phase:
0.005M tetrabutylammonium (TBA) solution: water =75:25v/v.

The 0.005M TBA solution is prepared as follows:

- Add 65 ml of 20% TBA hydroxide solution (Merck)
- Make up to 100 ml with water.
- Add 2.8 g potassium hydroxide to 10 ml water.
- Adjust to pH 7 with orthophosphoric acid. This is a 0.5M TBA solution containing 0.385 mole TBA and potassium hydroxide each.
- Dilute 10 ml of 0.5M TBA solution to 1 litre with methanol. The solution becomes turbid. Let the precipitate settle for a few hours. Filter through 0.45 um membrane filter.

5.2.2 HPLC Conditions

Column oven temperature:	30 °C
Column:	Hypersil ODS (C18), 5 μm, 200 x 4.6 mm or equivalent
Flow:	1 ml/min
Detection Wavelength:	435 nm and 535 nm
Diode Array Sprectral Range:	275 to 760 nm
Injection Volume:	10 μl
Run time	35 min

5.2.3 Inject 10 µl of the sample solution into the HPLC. If the absorbance of the peak is too low or too high, increase or decrease the injection volume or dilute with solvent mixture to get a peak absorbance of 0.05 to 0.5 Au. Record the chromatogram and spectrum.

6 IDENTIFICATION

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

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

Colorants	Wavelength of Detection	Retention Time (min)
Pigment Orange 5	535nm	13
Metanil Yellow	435 nm	3
Rhodamine B	535 nm	6

Note:

- *The spectrum and retention time should give a match factor of over 900.*
- *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

Limit of detection (LOD)

Colorants/ LOD	Standard (µg / mL)	Colour cosmetics Sample (µg/g)	Toiletries Sample (µg/g)
Pigment Orange 5	16	153	32
Metanil Yellow	3	70	6
Rhodamine B	40	800	80

8 CONCLUSION

The results from the TLC and HPLC chromatograms are used to obtain a conclusion on the identity and presence of the prohibited colorant in the cosmetic product.

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 workshop in Kuala-Lumpur, on September 13th to 17th, 2004,**
- **Modified after Singapore training, Oct 11th to 16th, 2004**
- **Modified and approved after the Brunei workshop, Aug 30th to 31st, 2005**
- **Modified and approved after the final review in Singapore, Nov 30th to Dec 2nd, 2005**