	Title	Revision n°	date	Document No
	IDENTIFICATION OF TRETINOIN(RETINOIC ACID) IN COSMETIC PRODUCTS BY TLC AND HPLC	1	28/11/13	ACM 001

A.THIN LAYER CHROMATOGRAPHY

1. SCOPE AND FIELD OF APPLICATION

The method describes the identification of Tretinoin in cosmetic products.

2. PRINCIPLE

Tretinoin is identified by thin layer chromatography (TLC).

3. REAGENTS

All reagents must be of analytical grade.

3.1 Absolute ethanol
3.2 n-Hexane
3.3 Diethyl ether
3.4 Methanol
3.5 Cyclohexane
3.6 Acetone
3.7 Glacial acetic acid
3.8 Developing Solvent for TLC
System A: n-Hexane/ 0.33% acetic acid in absolute ethanol = 9/1 (v/v)
System B: n-Hexane/ acetone = 6 / 4 (v/v)
System C: Cyclohexane/ Ether/ Acetone/ Acetic acid = 54/40/4/2 (v/v/v/v)
3.9 Spray reagent - 5% phosphomolybdic acid in absolute ethanol, freshly prepared (yields a clear yellow solution)
3.10 Reference material: Tretinoin, secondary or primary standard. To be stored under nitrogen and protected from light at room temperature.

4. APPARATUS

Normal laboratory equipment, and:

4.1 Precoated silica gel 60 F_{254} TLC plate, 10 cm x 20 cm, layer thickness 0.25 mm (Merck Art 5749 or equivalent)
4.2 UV lamp, 254 nm
4.3 Spray apparatus
4.4 PTFE syringe filter 0.45 um or equivalent
4.5 Light –resistant apparatus
4.6 Centrifuge tubes, stoppered, 30 mL
4.7 Vortex mixer
4.8 Filter paper Whatman n^0 41, or equivalent
4.9 Hot air hair dryer

5. PROCEDURE

Important note:
The weighing and transfer of tretinoin reference material and samples should be done expeditiously and away from light to minimize the degradation of tretinoin.
If light-resistant glassware is not available, use aluminium foil to wrap tubes and flasks.

5.1 Standard Preparation (1mg/ml)

Accurately weigh 0.01 g of tretinoin into a 10 mL volumetric flask. Add 5m of methanol. Sonicate for 5min and make up to volume with methanol.

5.2 Sample Preparation

5.2.1 Cream products

Weigh about 3 g of sample into a 30 mL centrifuge tube wrapped in aluminium foil. Add 10 mL of methanol to the tube and vortex for 5 min. Cool the tube in ice for 15 min and filter the solution through filter paper Whatman n^0 41 or equivalent.

5.2.2 Water based products (solutions and gels)

Weigh about 10 g of sample into a separating funnel. For gel-based sample, add 5 mL of distilled water to dissolve it. Extract the solution with 50 mL of n-hexane and wash the n-hexane extract with 10 mL of distilled water. Blow the n-hexane layer to dryness with nitrogen at room temperature. Dissolve the residue in 1 mL of methanol and filter through 0.45 um PTFE syringe filter.

5.3 TLC Procedure

5.3.1 Saturate a chromatographic tank with the appropriate developing solvent.

For cream products, use system A. If tretinoin is detected, repeat the TLC procedure using system B.

For water-based products, use system C.

5.3.2 Prepare the TLC plate by drawing the base line and front line 15 cm apart.

5.3.3 Spot between 5-20μL of sample solution and 5μL of the standard solution on the baseline of the TLC plate.

5.3.4 Develop the plate until the solvent front has migrated to a distance of 15 cm from the baseline.

5.4 Detection

5.4.1 After drying the plate, examine the plate under UV light at 254 nm.

5.4.2 Spray the plate with spray reagent and let it dry.

5.3.3 Use hot air hair dryer on the plate and observe the colour of spots

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 for the sample solution with the standard solutions with respect to Rf values, spots under UV radiation and the colour of spots.

Compound	Estimated Rf values	Colour of spots ,after treatment with spray reagent
Tretinoin	0.1 – 0.3 (system A) 0.5 (system B) 0.4 (system C)	Bluish green

7. REMARKS

7.1 The limit of detection (LOD) in sample is 12.5 µg/g.

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

B. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

1. SCOPE AND FIELD OF APPLICATION

This method specifies a procedure for the identification of tretinoin in cosmetic products.

2. PRINCIPLE

Identification of tretinoin is performed by reversed phase High Pressure liquid chromatography (HPLC) with diode array detector (DAD) detection.

3. REAGENTS

All reagents must be of analytical grade, unless otherwise stated.

3.1 Water (Ultrapure,18 ohm)
3.2 Methanol (HPLC grade)
3.3 Glacial acetic acid
3.4 Mobile phase: methanol/water/acetic acid = 85/15/0.5 (v/v/v)
3.5 Reference material: Tretinoin, secondary or primary standard. To be stored under nitrogen and protected from light.

4. APPARATUS

Normal laboratory equipment and

4.1 Light-resistant glassware
4.2 Centrifuge tubes with stopper, 30mL
4.3 2ml amber autosampler vials
4.4 High Pressure Liquid Chromatography:
- 4.4.1 Constant flow solvent delivery system, capable of delivering a flow of 1.4 mL/min
- 4.4.2 Injection device, suitable for injection of a sample volume of 20 uL
- 4.4.3 Analytical column: Hypersil ODS-C18, 5um, 200 x 4.6mm, or equivalent
- 4.4.4 DAD detector, with detection capability at 353 nm
- 4.4.5 0.45 um syringe membrane filter, PVDF or equivalent

5. PROCEDURE

Important note:
The weighing and transfer of tretinoin reference material and samples should be done expeditiously and away from light to minimize the degradation of tretinoin.
If light-resistant glassware is not available, use aluminium foil to wrap tubes and flasks.

5.1 Standard preparation (0.05 mg/mL)

Accurately weigh 5.00 mg of Tretinoin into a 100 mL volumetric flask. Add 50 mL methanol and sonicate for 5 min. Make up to volume with methanol.

This solution must be freshly prepared and used within 24 hours.

5.2 Sample preparation

5.2.1 For solution-based products

Filter the solution through a 0.45 um syringe filter into an amber autosampler vial, discarding the first few mL. Use the filtrate for HPLC injection.

5.2.2 For cream products

Weigh 1g of sample into a 30 mL centrifuge tube wrapped in aluminium foil. Add 10 mL of methanol into the tube and vortex for 5 min. Cool the tube in ice for 15 min. Filter an aliquot through 0.45 um syringe filter into an amber autosampler vial, discarding the first few mL. Use the filtrate for HPLC injection.

5.3 HPLC Conditions:

Column oven temperature	: 30^0C
Flow rate	: 1.4 mL/min
Detection wavelength	: 353nm
DAD spectral range	: 200-500nm
Injection volume	: 20 µL
Run time	: 30 min

5.4 Inject the 20 µL of standard and sample solution into the HPLC. Record the chromatogram and spectrum.

6. INTERPRETATION

6.1 Compare the retention time (RT) and spectrum scan (200 - 500nm) of the sample solution with that of the standard solution.

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6.2 The RT of Tretinoin is about 9 min. Tretinoin in the sample solution is positively identified when the sample RT is within +/- 0.5min of standard RT. The spectrum scan of sample solution must match that of the standard solution with a match factor of at least 900.

7. REMARKS

7.1 The limit of detection (LOD) is 20 µg/g

7.2 This method may be used to identify Isotretinoin (13-cis form of retinoic acid)

7.3 Tretinoin is also known as Retinoic acid or Vitamin A acid. It is listed under Annex II – List of substances which must not form part of the composition of cosmetic products

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8. CONCLUSIONS

The results from TLC and HPLC are used to conclude the identity and presence of tretinoin in 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 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**