	Title	Revision n°	date	Document No
	IDENTIFICATION OF RETINOIC ACID (TRETINOIN) IN COSMETIC PRODUCTS BY TLC AND HPLC	0	02/12/05	ACM SIN 01

A. IDENTIFICATION by TLC

1. SCOPE AND FIELD OF APPLICATION

The method describes the identification of retinoic acid in cosmetic products.

2. PRINCIPLE

Retinoic acid 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 yellow clear solution)
3.10 Reference material: retinoic acid, 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 µm or equivalent
4.5. Light-resistant glassware
4.6. Centrifuge tubes, stoppered, 30 mL
4.7. Vortex mixer
4.8. Filter paper Whatman n°41, or equivalent

5. PROCEDURE

Important note:
The weighing and transfer of the retinoic acid reference material and samples should be done expeditiously and away from light to minimize the degradation of retinoic acid.
Both tubes and vessels, if light-resistant glassware is not available, should be wrapped in Aluminium foil.

5.1. Standard Preparation

Standard solution, 1 mg/mL
Accurately weigh 0.01 g of retinoic acid in 10 mL volumetric flask. Dissolve in methanol and dilute to volume with methanol.

5.2. Preparation of the sample

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 in ice for 15 min and filter through filter paper Whatman n° 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 50mL of n-hexane (3.2) 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 (3.4) and filter through 0.45 µm PTFE syringe filter.

5.3. TLC procedure

5.3.1.Cream products

5.3.1.1. Prepare the TLC plate by marking the base line and front line, eg. about 10 cm.

5.3.1.2. Spot about 5 to 20 µL of the sample solutions and 5 µL of the standard solution on the baseline of the TLC plate.

5.3.1.3. Develop the plate in developing system A.

5.3.1.4. After drying, examine under UV light at 254 nm. Spray with the spray reagent. The formation of a dark blue spot corresponding to the standard spot indicates the presence of retinoic acid.

5.3.1.5. Blow hot air on the plate. A bluish green spot for retinoic acid will appear.

5.3.1.6. If a positive result for retinoic acid is obtained, repeat steps “5.3.1.1” to “5.3.1.5” by using developing system B.

5.3.2.Water based products

5.3.2.1. Saturate the chromatographic tank lined with chromatographic paper with the developing system C.

5.3.2.2. Apply about 5 to 20 µL of the sample solution and 5 µL of the standard solution on the base line of a TLC plate. Allow to dry.

5.3.2.3. Develop the plate over a distance of 15 cm.

5.3.2.4. Dry the plate in air and observe the plate under ultra-violet light at 254 nm (4.2). Spray the plate with spray reagent (3.9). For details, refer to ”5.3.1.4” to “5.3.1.5”.

5.3.2.5. Compare the Rf value and colour of the spot obtained for the sample solution with that obtained for the standard solution.

6. IDENTIFICATION

Calculate the Rf value for each spot.

Rf value = distance of spot of substance / distance of solvent front

Compare the spot obtained for the sample solution with that for the standard solution with respect to their Rf values, the spots under UV radiation and the colour of the spots after visualization with the spray reagent.

	Estimated Rf values	Limit of Detection
Retinoic Acid	0.1 - 0.3 (system A) 0.5 (system B) 0.4 (system C)	0.l25 µg (1ul of 0.125 mg/mL)

To verify the presence and identity of retinoic acid, perform further screening by HPLC as described in the following section (B).

B. IDENTIFICATION BY HPLC

1. SCOPE AND FIELD OF APPLICATION

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

2. PRINCIPLE

Identification of retinoic acid is performed by reverse phase high pressure liquid chromatography (HPLC) with ultraviolet (UV) detection.

3. REAGENTS

All reagents must be of analytical quality or HPLC grade where applicable.

3.1 Water used must be Ultrapure water, 18 mega ohm
3.2 Methanol (HPLC grade)
3.3 Glacial acetic acid
3.4 Mobile phase: mixture of methanol, water and acetic acid = 85/15/0.5 (v/v/v)
3.5 Reference material: retinoic acid, 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, stoppered, 30 mL
4.3 Amber coloured autosampler vials
4.4 High Pressure Liquid Chromatograph, consisting of:
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 µL.
4.4.3 Analytical column: Hypersil ODS-C18, 5 µm, 200 x 4.6 mm, or equivalent
4.4.4 UV detector, with detection capability at 353 nm.
4.4.5 0.45 µm syringe membrane filter, PVDF or equivalent

5. PROCEDURE

The same precaution should be taken as described in section 5 for TLC procedure

5.1 Standard preparation

Standard solution: 0.05 mg/mL of retinoic acid in methanol for HPLC (3.2).
Accurately weigh 5.00 mg of retinoic acid in a 100 mL volumetric flask. Dissolve in 50 mL methanol, and if necessary, sonicate for 1 to 2 min. Make up to volume with methanol.

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

5.2 Sample preparation

5.2.1 For clear solutions, filter the solution through a 0.45 µm syringe filter into an amber-coloured autosampler vial, discarding the first few mL. Use the filtrate directly for HPLC injection.
5.2.2 For creams, weigh about 1 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 in ice for 15 min and filter an aliquot through a 0.45 µm syringe filter into an amber-coloured autosampler vial, discarding the first few mL. Use the filtrate for HPLC injection.

5.3 High performance liquid chromatography

5.3.1 Conditions:

Column temperature	: 30 ^{0}C
Flow rate	: 1.4 mL/min
UV detector	: 353 nm
Injection volume	: 20 µL
Instrument run time	: 30 min

5.3.2 Inject the standard solution and sample solutions into HPLC.

5.3.3 Compare the retention time and spectrum obtained for the sample chromatogram with that obtained for the retinoic acid standard. The retention time of retinoic acid is about 9 min and the maximum absorbance is at wavelength of 353 nm.

5.4 The limit of detection (LOD) is 0.002 mg/mL in the sample solution (5.3.2)

C. CONCLUSION

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

D. REMARKS

1. DETERMINATION BY HPLC

Use the areas of the retinoic acid peaks to calculate the concentration of the retinoic acid in the sample. Calculate the retinoic acid concentration in the sample, as a percentage by weight, (R) using the formula:

$$R\ \%\ (w/w) = \frac{area\text{–}of\text{–}sample\text{–}peak}{area\text{–}of\text{–}reference\text{–}material\text{–}peak} \times \frac{weight\text{–}of\text{–}reference\text{–}material(g)}{weight\text{–}of\text{–}sample(g)} \times \frac{volume\text{–}of\text{–}sample}{volme\text{–}of\text{–}reference\text{–}material} \times 100 \times P$$

where P is the purity (%) of the reference material.

A dilution factor is needed if the sample has been further diluted.

The sample and the standard should be of similar concentration for quantification. A stronger standard or a diluted sample should be prepared if necessary. The peak area of the sample and standard should not differ by more than 10%.

2. IDENTIFICATION OF ISORETINOIC ACID

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

3. SAFETY PRECAUTIONS

When handling the standards or the samples, all personnel, especially pregnant women or women considering pregnancy, should strictly follow proper safety policies concerning handling of retinoids.

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,**
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