	Title	Revision n°	Date	Document No
	IDENTIFICATION OF STEROIDS IN COSMETIC PRODUCTS BY TLC AND HPLC	1	28/11/13	ACM 007

A. THIN LAYER CHROMATOGRAPHY (TLC)

1. SCOPE AND FIELD OF APPLICATION

The method describes the identification of hydrocortisone acetate, dexamethasone, betamethasone, betamethasone 17-valerate and triamcinolone acetonide in cosmetic products.

2. PRINCIPLE

Liquid samples suspected of containing steroid compounds are neutralized and extracted with ethyl acetate. Cream samples suspected of containing steroid compounds are neutralized and extracted with methanol. The extracted solutions are evaporated to dryness. Residues are dissolved in methanol for identification by thin layer chromatography (TLC).

3. REAGENTS

All reagents must be of analytical grade.

3.1 Dichloromethane
3.2 Ethyl acetate
3.3 Water
3.4 Methanol
3.5 Anisaldehyde
3.6 Sulphuric Acid (Concentrated)
3.7 Hydrochloric Acid (0.5 M HCl)
3.8 Ammonium Hydroxide (0.5 M NH_4OH)
3.9 Glacial acetic acid (GAA)
3.10 Developing Solvent for TLC
Dichloromethane/ Methanol/ Water, 95/5/0.2 (v/v).
3.11 Reference materials:
3.11.1 Hydrocortisone acetate
3.11.2 Dexamethasone
3.11.3 Betamethasone
3.11.4 Betamethasone 17-valerate
3.11.5 Triamcinolone acetonide
3.12 Reference Standard Solutions
Weigh 10 mg of every reference material into separate 10 mL volumetric flask. Add 5 mL of methanol. Sonicate for 5 min, make up to volume with methanol.
3.13 Mixture of standard solutions
Weigh 10 mg of every reference material into a 10 mL volumetric flask. Add 5 mL of methanol. Sonicate for 5 min, make up to volume with methanol.
3.14 Spray Reagents (should be freshly prepared)
Add 50 mL GAA into a 100 mL volumetric flask. Then, add 0.5 mL anisaldehyde, and finally 11 mL of sulphuric acid. Shake gently.

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 or equivalent)
4.2 UV scanner, 254 nm and 366 nm
4.3 Spray apparatus
4.4 Oven
4.5 Water bath
4.6 Shaker
4.7 pH Meter
4.1 Filter paper
4.8 Centrifuge tubes
4.9 PVDF syringe filter ,0.45 µm or equivalent

5. PROCEDURE

5.1 Preparation of sample solutions

5.1.1 Liquid Sample

Take about 15 mL of sample. Neutralize to pH 7 with 0.5M HCl or 0.5 M NH_4OH, extract two times with 20 mL of ethyl acetate, and discard the aqueous layer. If necessary, filter the combined extract into an evaporating dish and let it evaporates to dryness using a water bath for about 30 min. Dissolve the residue in 5 mL of methanol and filter the solution using a syringe filter.

5.1.2 Cream Sample

Weigh about 5 g of sample in a centrifuge tube and add 20 mL of methanol. Warm the sample using a water bath for about 10 min. Shake vigorously for 5 min using a shaker. Centrifuge at 3000 rpm – 4000 rpm for 10 min and then leave it in a freezer for 10 min. Evaporate the clear supernatant solution to dryness using a water bath for about 1 hr. Dissolve the residue in 5 mL of methanol and filter the solution using a syringe filter.

5.2 TLC

5.2.1 Saturate a chromatographic tank with the developing solvent.
5.2.2 Prepare the TLC plate by making the base line and front line with a distance about 15 cm.
5.2.3 Spot about 20 µL of each of the following solutions on the baseline of the TLC plate.
5.2.3.1 Each of reference solutions.
5.2.3.2 The mixture of reference solution.
5.2.3.3 Sample solution.
5.2.4 Develop the plate until the solvent front has migrated at a distance of 15 cm from the base line.

5.3 Detection

5.3.1 After drying the plate, observe the plate under UV light at 254 nm.

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

5.3.3 Place the plate in an oven at 120°C for 10 min and observe the colour of spots obtained.

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.

Steroid compounds	Estimated Rf values	Colour of after spray spots
Betamethasone	0.20	Greyish blue
Betamethasone 17-valerate	0.37	Dark purple
Dexamethasone	0.13	Grey
Hydrocortisone acetate	0.36	Dark brown
Triamcinolone acetonide	0.33	Yellow green

7. REMARKS

7.1 This method is also applicable to identify cortisone acetate, prednisolone, prednisone, flucinolone acetonide, Betamethasone 21-valerate and hydrocortisone.

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

B. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

1. SCOPE AND FIELD OF APPLICATION

This method specifies a procedure for the identification of the following steroids in cosmetic products:-

1.1 Prednisone
1.2 Prednisolone
1.3 Betamethasone
1.4 Dexamethasone
1.5 Cortisone
1.6 Hydrocortisone Acetate
1.7 Mometasone Furoate
1.8 Betamethasone 17-Valerate
1.9 Triamcinolone Acetonide

2. PRINCIPLE

Cosmetic samples suspected of containing steroid compounds are extracted with methanol. The extracted solutions are evaporated to dryness. Residues are dissolved in methanol for identification by reversed phase liquid chromatography (HPLC) with diode array detector (DAD) detection.

3. REAGENTS

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

3.1 Acetonitrile HPLC grade.
3.2 Methanol HPLC grade.
3.3 Water (Ultrapure water, 18 ohm)
3.4 Mobile phase in gradient system as shown below.

Time (minutes)	Ratio %	
	Acetonitrile	Water
Initial	20	80
2.00	25	75
14.00	30	70
18.00	40	60
20.00	50	50
22.00	70	30
30.00	20	80

3.5 Reference materials

3.5.1 Prednisone
3.5.2 Prednisolone
3.5.3 Betamethasone
3.5.4 Dexamethasone
3.5.5 Cortisone
3.5.6 Hydrocortisone Acetate

3.5.7 Mometasone Furoate
3.5.8 Betamethasone 17-Valerate
3.5.9 Triamcinolone Acetonide

3.6 Reference standard solutions (0.50 mg/mL)
Weigh 5 mg of every reference material into separate 10 mL volumetric flask. Add 5 mL of Methanol. Sonicate for 5 min and make up to volume with Methanol.

3.7 Mix Standard Solution (0.055 mg/mL).
Pipette 1mL of each reference standard solutions in part 3.6 and mix it in clean 10 mL vial. Filter the resulting solution with a 0.45 µm syringe membrane filter.

4. APPARATUS
4.1 Water bath with temperature monitoring
4.2 Ultrasonic bath
4.3 Shaker
4.4 Centrifuge tubes
4.5 High Performance Liquid Chromatography System with DAD or with detection capability at 240 nm.
4.6 Syringe membrane filter, 0.45 µm, PVDF or equivalent
4.7 Column Phenyl-Hexyl, 150 x 4.6 mm, 2.6 µm, or equivalent.

5. PROCEDURE
5.1 Preparation of Sample Solution
Weigh about 1g of cream sample into a centrifuge tube and add 5 mL of Methanol. Warm on a water bath (60°c) for 10 min and shake vigorously for 10 min using a shaker. Centrifuge the sample at 3000-4000 rpm and then place it in a freezer for 10 min. Collect the clear supernatant solution to and evaporate it to dryness using a water bath. Dissolve the residue in 5 mL of methanol and filter the solution using the 0.45 µm syringe membrane filter.

5.2 HPLC Conditions

Column temperature	: 40°C
Flow rate	: 0.7 mL/min
DAD detector	: 240 nm
Injection volume	: 10 µL
Instrument Run time	: 30 min

5.3 System Suitability Tests
5.3.1 Inject six (6) replicates of mix standard solution into the HPLC column and determine its percentage of relative standard deviation (RSD) of peak area or height. The value of RSD should not be less than 2% for all individual reference materials.
5.3.2 Resolution between adjacent peaks of reference materials should be more than 1.5.
5.3.3 The USP Tailing factor of peaks should be between 0.9 and 1.1.

5.4 Inject 20 uL of the following solutions into the HPLC column.
5.4.1Each of reference standard solutions
5.4.2Mix standard solution
5.4.3The sample solution

5.5 Compare the retention time (RT) obtained from sample solution and reference standard solutions.

6. INTERPRETATION

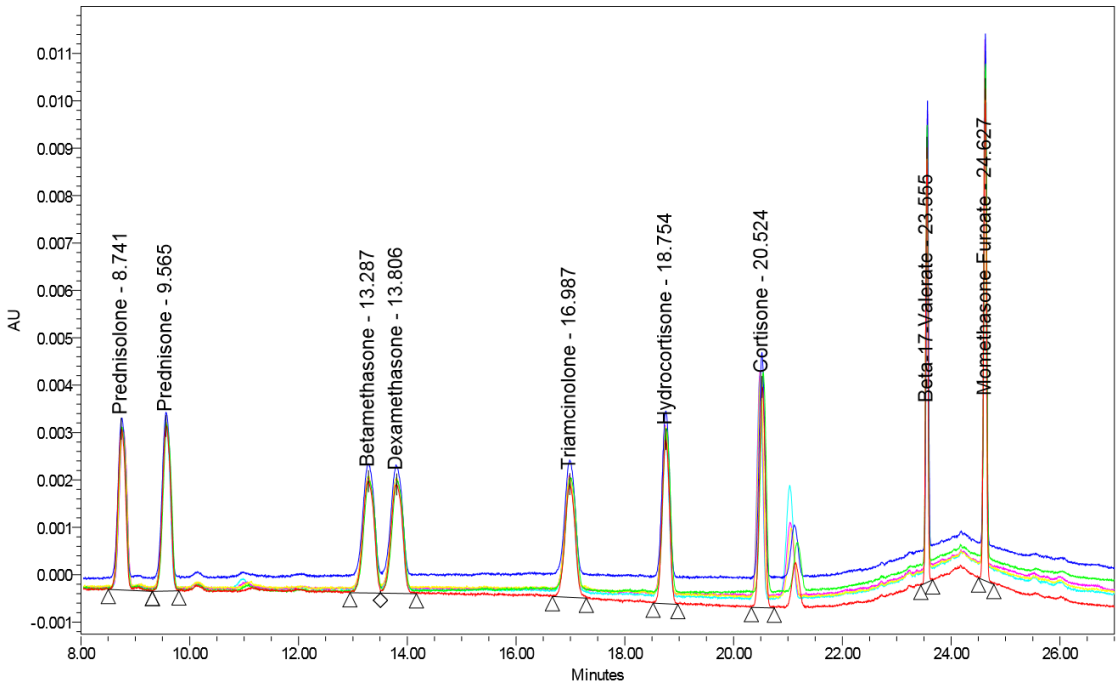
6.1 A steroid compound in the sample solution is positively identified when its RT should not differ by +/- 1% of RT obtained from reference standard solution. The example of chromatogram of mix standard solution and 4for each steroid compound, resolution and USP tailing factor are as shown Figure 1 while Figure 2 shows spectra for each steroid to support the result.

6.2 The peak should also comply with requirement under part 5.3.2 (when necessary) and 5.3.3.

6.3 Limit of Detection (LOD)

6.3.1LOD for all steroids specified in sample solution is 4.54µg/g and standard solution is at 1.0µg/mL.

6.3.2 A steroid compound in the sample solution is negatively identified when peak area or height of the compound is below than peak area or height of the LOD concentration spiked in the sample solution. Figure 1 represents an example of the chromatogram of LOD mix standards solution.



(a)

No	Sample Name	RT	Peak Area	Peak Height	s/n
1	Prednisolone	8.741	33939	3401	51
2	Prednisone	9.565	36851	3484	53
3	Betamethasone	13.287	53916	2380	36
4	Dexamethasone	13.806	33647	2380	34
5	Triamcinolone	16.987	31295	2390	36
6	Hydrocortisone	18.754	33437	3476	53
7	Cortisone	20.524	35084	4660	71
8	Beta-17-Valerate	23.555	27748	9241	134
9	Mometasone Furoate	24.677	38950	10387	157

(b)

Figure 1: (a) A chromatogram of LOD mix standards solution and (b) Its RT, peak area, peak height and s/n for each steroid compound.

7. HISTORY

7.1 Issued by the chemical analysis group at the harmonization workshop in Kuala Lumpur, on September 13th to 17th, 2004.

7.2 Approved by the harmonization workshop delegates in Kuala-Lumpur on September 13th to 17th, 2004.

7.3 Modified after the Kuala Lumpur training, Dec 6th to 10th, 2004.

7.4 Modified and approved after the Brunei workshop on August 30th to 31st, 2005.

7.5 Modified and approved after the final review in Singapore on November 30th to December 2nd, 2005.

7.6 Modified and approved after the final review in Malaysia on November 28th, 2013.