	Title	Revision n°	date	Document N°
	IDENTIFICATION OF HYDROCORTISONE ACETATE, DEXAMETHASONE, BETAMETHASONE, BETAMETHASONE 17-VALERATE AND TRIAMCINOLONE ACETONIDE IN COSMETIC PRODUCTS BY TLC AND HPLC	0	2/12/2005	ACM MAL 07

A. THIN LAYER CHROMATOGRAPHIC TECHNIQUE (TLC)

1. SCOPE

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 extracted with methanol. The extracted solutions are evaporated to dryness. Residues are dissolved in methanol for identification by thin layer chromatographic technique.

3. REAGENTS

All reagents must be of analytical grade.

3.1. Dichloromethane
3.2. Methyl acetate
3.3. Water
3.4. Methanol
3.5. Anisaldehyde
3.6. Sulfuric acid (concentrated)
3.7. Hydrochloric acid (0.5 M)
3.8. Ammonium hydroxide (0.5 M)
3.9. Glacial acetic acid
3.10. Tetrazolium blue
3.11. Sodium hydroxide (pellets)
3.12. Ethyl acetate
3.13. Developing solvent:
Dichloromethane/Methyl Acetate /Water, 100:50:50
Use the lower layer of the mixture.
3.14. Reference materials:
3.14.1. Hydrocortisone acetate
3.14.2. Dexamethasone
3.14.3. Betamethasone
3.14.4. Betamethasone 17-valerate
3.14.5. Triamcinolone acetonide
3.15. Standard solutions
3.15.1. 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.
3.15.2. Mixture of standard solutions: weigh 10 mg of every reference material into one 10 mL volumetric flask. Add 5 mL methanol. Sonicate for 5 min and make up to volume with methanol.

3.16. Spray reagents
3.16.1. Into a 100 mLvolumetric flask, add 50 mL glacial acetic acid. Then, add 0.5 mL anisaldehyde, and finally 11 mL sulfuric acid. Shake gently.

3.16.2. *Other spray reagent for information:*
Alkaline tetrazolium blue solution : mix 10 mL of a 0.2% w/v solution of tetrazolium blue in methanol with 30 mL of a 12% w/v solution of sodium hydroxide in methanol (this solution should be freshly prepared).

4. APPARATUS

Normal laboratory equipment, and:

4.1. TLC plates, ready for use: silica gel 60 F254 nm
4.2. TLC tank
4.3. Ultrasonic bath
4.4. UV lamp 254 nm
4.5. Oven
4.6. Filter paper, “1 PS” or equivalent
4.7. Water bath
4.8. Shaker
4.9. pH meter
4.10. 0.45 µm syringe membrane filter, PVDF or equivalent

5. PROCEDURE

5.1 Preparation of the sample

5.1.1 Liquid Sample
Take about 15 mL of sample. Neutralize to pH 7 with 0.5 M HCl or 0.5 M NH_4OH, extract 2 times with 20 mL ethyl acetate, discard aqueous layer. Filter – if necessary - the combined extract into evaporating dish and evaporate to dryness in a water bath (indication time: 30 min). Dissolve the residue in 5 mL of methanol and filter using the syringe membrane filter.

5.1.2 Cream Sample
Weigh about 5 g of sample in a centrifuge tube and dissolve by adding 20 mL of methanol. Warm on water bath for 10 minutes and shake vigorously for 5 minutes using a shaker. Centrifuge at 3000 – 4000 rpm for 10 minutes and leave it in the freezer for 10 minutes. Evaporate the clear supernatant solution to dryness in the water bath (indication time: 1h). Dissolve the residue in 5mL of methanol and filter the solution using the syringe membrane filter.

5.2 TLC

5.2.1 Saturate a chromatographic tank with the developing solvent.

5.2.2 On a plate, deposit:

- 20 µL of every reference solution,
- 20 µL of the mixture of reference solutions,
- 20 µL of the sample solution.

Develop until the solvent front has migrated 15 cm from the start.

5.2.3 Remove the plate and allow to dry at room temperature.

5.3 Detection

5.3.1 Observe the plate under UV light at 254 nm, and mark the position of the spots.
5.3.2 Spray the plate with reagent 3.16.1 . Let it dry and put the plate in the oven at 120°C for 10 min and observe the spots.

6. INTERPRETATION OF RESULT

Steroids	Colour of spot		Estimated Rf
	Spray reagent 3.16.1	Spray reagent 3.16.2	
hydrocortisone acetate	dark brown	violet blue	0.4
dexamethasone	grey	violet blue	0.2
betamethasone	greyish blue	violet blue	0.2
triamcinolone acetonide	yellow green	violet blue	0.3
betamethasone 17-valerate	dark purple	violet blue	0.35

7. REMARKS

7.1 This method is also applicable to identify the following steroids; cortisone acetate, prednisolone, prednisone, flucinolone acetonide, betamethasone 21-valerate, hydrocortisone.
7.2 Further screening by HPLC shall be carried out.
7.3 The limit of detection (LOD) shall be reported.

B. HPLC TECHNIQUE

1. SCOPE

This method specifies a procedure for further 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 extracted with methanol. The extracted solutions are evaporated to dryness. Residues are dissolved in methanol for identification by reversed phase liquid chromatography with UV detection.

3. REAGENTS

All reagents must be of analytical quality. Water used must be distilled water, or water of at least equivalent purity

3.1 Acetonitrile, HPLC grade
3.2 Ammonium hydroxide (0.5 M)
3.3 Methanol, HPLC grade
3.4 Ethyl acetate
3.5 Hydrochloric acid (0.5 M)
3.6 Mobile phase : Acetonitrile : Water

Gradient :

Time (min)	Ratio (%)	
	Acetonitrile	**Water**
0.01 – 15.00	30	70
15.01 – 21.00	60	40
21.01 – 30.00	50	50
30.01 – 34.99	30	70
35.00	STOP	

Note: the above gradient conditions should be adjusted when necessary to the type of column in use.

3.7 Reference materials
- 3.7.1 Hydrocortisone acetate
- 3.7.2 Dexamethasone
- 3.7.3 Betamethasone
- 3.7.4 Betamethasone 17-valerate
- 3.7.5 Triamcinolone acetonide

3.8 Standard solutions

3.8.1 Weigh 10 mg of every reference material into separate 10 mL volumetric flask. Add 5 mL of methanol. Sonicate for 5 minutes, make up to volume with methanol. Filter using a syringe membrane filter.

3.8.2 Mixture of standard solutions: weigh 10 mg of every reference material into one 10 mL volumetric flask. Add 5 mL methanol. Sonicate for 5 minutes and make up to volume with methanol. Filter using a syringe membrane filter.

4. APPARATUS

Normal laboratory equipment and:

4.1 Water bath, capable of maintaining a temperature of 60 °C

4.2 High-performance liquid chromatograph with a variable wavelength UV detector and 20 µL injection loop

4.3 Analytical column
Stainless steel chromatographic column, length 250 mm, internal diameter 4,6 mm, (packed with ODS), particle size 5 µm, or equivalent.

4.4 Filter paper, 1 PS or equivalent

4.5 Ultrasonic bath

4.6 Shaker

4.7 pH meter

4.8 Syringe membrane filter (0.45 µm pore size)

5. PROCEDURE

5.1 Sample preparation

5.1.1 Liquid Sample
Take about 15 mL of sample. Neutralize to pH 7 with 0.5 M HCl or 0.5 M NH_4OH, extract 2 times with 20 mL ethyl acetate, discard aqueous layer. Filter – if necessary - the combined extract into evaporating dish and evaporate to dryness in a water bath (indication time: 30 min). Dissolve the residue in 5 mL of methanol and filter using the syringe membrane filter.

5.1.2 Cream Sample

Weigh about 5 g of sample in a centrifuge tube and dissolve by adding 20 mL of methanol. Warm on water bath for 10 minutes and shake vigorously for 5 min using a shaker. Centrifuge at 3000 – 4000 rpm for 10 min and leave it in the freezer for 10 min. Evaporate the clear supernatant solution to dryness in the water bath (indication time: 1h). Dissolve the residue in 5mL of methanol and filter the solution using the syringe membrane filter.

5.2 High performance liquid chromatography

5.2.1 Adjust the flow rate of the mobile phase to 1.2 mL/min and set the detector wavelength to 245 nm.

5.2.2 Inject

- 20 µL of every reference solution,
- 20 µL of the mixture of reference solutions,
- 20 µl of the sample solution.

5.2.3 System suitability

The relative standard deviation (RSD) of the retention time should be less than 1%, and the RSD of 6 replicate injections should be less than 2%.

5.2.4 Compare the retention time of the chromatograms obtained for sample and reference solutions.

6. INTERPRETATION

6.1 The retention time of reference and sample solution should not differ by more than ± 1 %.

6.2 The estimated retention (RT) for the steroids are as follows:

Steroids	Estimated retention time (RT) (min)
Betamethasone	15
Dexamethasone	16
Triamcinolone Acetonide	19
Hydrocortisone Acetate	20
Betamethasone 17-valerate	22

7. Limit of detection (LOD) and limit of quantitation (LOQ):

Steroids	LOD			LOQ		
	Standards (mg/mL)	Samples		Standards (mg/mL)	Samples	
		Liquid (µg/mL)	Cream (µg/g)		Liquid (µg/mL)	Cream (µg/g)
Hydrocortisone Acetate	0.02	7	20	0.07	23	70
Triamcinolone Acetonide	0.04	14	40	0.13	46	130
Betamethasone	0.05	17	50	0.15	58	150
Dexamethasone	0.05	17	50	0.16	62	160
Betamethasone 17-valerate	--	--	--	--	--	--

8. REMARKS

This method is also applicable to identify the following steroids; cortisone acetate, prednisolone, prednisone, flucinolone acetonide, betamethasone 21-valerate and hydrocortisone

C. CONCLUSION

Combine the results from TLC and HPLC to identify the presence of the steroids within the scope of the method.

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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 the Kuala-Lumpur training, Dec 6 th to Dec 10 th, 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**