	Title	Revision n°	date	Document N°
	IDENTIFICATION AND DETERMINATION OF HYDROQUINONE IN COSMETIC PRODUCTS BY TLC AND HPLC	0	31/08/05	ACM INO 03

A. IDENTIFICATION

1. SCOPE AND FIELD OF APPLICATION

The method describes the identification of hydroquinone in cosmetic products.

2. PRINCIPLE

Hydroquinone 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. Acetone
3.4. Toluene
3.5. Glacial acetic acid
3.6. phosphomolybdic acid
3.7. Silver nitrate
3.8. Ammonium hydroxide 25% (w/w)
3.9. Developing solvents:
  3.9.1. System n°1:
    n-Hexane/Acetone, 3:2
  3.9.2. System n°2:
    Toluene/Glacial acetic acid, 8:2
3.10. Reference substance (RS): Hydroquinone

3.11. Spray reagents
  3.11.1. To a 5 % (w/v) aqueous solution of silver nitrate, add ammonium hydroxide until the precipitate which forms is dissolved.

Warning:
the solution becomes explosively unstable on standing and should be discarded after use.

  3.11.2. 5 % (w/v) solution of phosphomolybdic acid in ethanol

4. APPARATUS

Normal laboratory equipment, and:
4.1 TLC plates, ready for use: silica gel GF/ 254 nm 20 cm x 20 cm . Layer thickness 0.25 mm
4.2 Ultrasonic bath
4.3 UV lamp, 254 nm

5. PROCEDURE

5.1 Preparation of the sample

5.1.1 Weigh accurately about 1.5 g of sample into a 25 mL beaker
5.1.2 Add gradually 15 ml of ethanol 96 % (v/v), and mix
5.1.3 Transfer into a 25 ml volumetric flask
5.1.4 Homogenize in an ultrasonic bath for 10 minutes, and then cool the flask to room temperature

5.1.5 Add ethanol 96 % (v/v) to volume, and mix
5.1.6 Put in an ice bath until separation of fats occurs (indication time: 10 minutes)
5.1.7 Filter through a paper filter

5.2 Preparation of the Reference Solution :

Warning:
This solution should be freshly prepared, and is stable for less than one day at room temperature.

5.2.1 Weigh accurately about 0.05 g of *hydroquinone* (R S) into a 25 mL volumetric flask
5.2.2 Add 5 ml of ethanol 96 % (v/v), and shake to dissolve it
5.2.3 Add ethanol 96 % (v/v) to volume, and mix

5.3 Thin Layer Chromatography (TLC)

5.3.1 Activate the plates for 10 minutes at 100 °C
5.3.2 Saturate two chromatographic tanks with both developing solvents
5.3.3 Deposit on both plates 20 µl of
- sample solution
- reference solution

notes:
- *spots may be duplicated,*
- *spiked sample solution (prepared by mixing one ml of reference solution with one ml of sample solution) may be spotted in parallel*

5.3.4 Develop in the dark at ambient temperature until the solvent front has migrated 15 cm from the start.
5.3.5 Remove the plates and allow to dry at room temperature.
5.3.6 Detection
5.3.6.1 Observe the plate under UV light at 254 nm, and mark the position of the spots.
5.3.6.2 Spray the plate with:
- silver nitrate reagent or
- phosphomolybdic acid reagent , and heat the plate to approximately 100°C for about 10 minutes

6. IDENTIFICATION

6.1 Calculate the Rf value for each spot :
- their Rf values,
- the colour of the spots under UV radiation,
- and the colours of the spots after visualization with the spray reagent.

6.2 Perform the HPLC described in the following section (B), and compare the retention times obtained for the sample peak with that for the standard solution.

6.3 Combine the results from TLC and HPLC to identify the presence of hydroquinone.

7. REMARKS

Under the conditions described above, the following Rf values were observed:
Developing system n°1: 0.5
Developing system n°2: 0.2 to 0.3

B. DETERMINATION

1. SCOPE AND FIELD OF APPLICATION

This method specifies a procedure for the determination of hydroquinone in cosmetic products

2. PRINCIPLE

The sample is extracted with a water/methanol mixture under gentle heating to melt any lipid material. Determination of the hydroquinone in the resulting solution is performed 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 Methanol (HPLC grade)
3.2 Mobile phase: water/methanol mixture 45:55 (V/V). Mix 55 volumes of methanol and 45 volumes of water.
3.3 Reference material: Hydroquinone

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 150 mm, internal diameter 4.6 mm, packed with ODS, particle size 10 µm, or equivalent.
4.4 Filter paper, diameter 90 mm, Schleicher and Schull, Weisshand No 5892, or equivalent (Whatman n° 1)
4.5 Vortex mixer
4.6 0.45 µm membrane filter (HVLP or equivalent)
4.7 laboratory centrifuge

5. PROCEDURE

5.1 Standard solution preparation
5.1.1 Weigh accurately about 0.05 g (w_{ref}) of *hydroquinone* into a 50 ml volumetric flask
5.1.2 Add 25 ml of mobile phase and shake to dissolve it
5.1.3 Add mobile phase to volume, and mix
5.1.4 Pipette 5 ml this solution into a 50 ml volumetric flask
5.1.5 Dilute and add mobile phase to volume, then mix

Note:

- dilution may be adapted to sample concentration
- these solutions must be freshly prepared. Amber (low actinic) glass may be used.

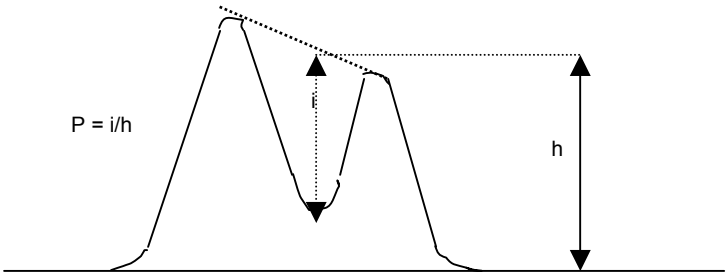
5.2 Sample preparation
5.2.1 Weigh accurately 1 ± 0.1 g of sample (w_{spl}) into a 25 mL beaker
5.2.2 Add gradually 25 mL of mobile phase, and mix until homogeneous
5.2.3 Transfer into a 50 mL volumetric flask

5.2.4 Vortex for about 1 minute
5.2.5 Place the flask in water bath at 60ºC for 15 minutes, then cool the flask to room temperature
5.2.6 Add mobile phase to volume, and mix
5.2.7 Filter the clear solution through a 0.45 µm membrane filter (when necessary, centrifuge first for 10 min)
5.2.8 Perform determination of filtrate by HPLC within less than 24 hours

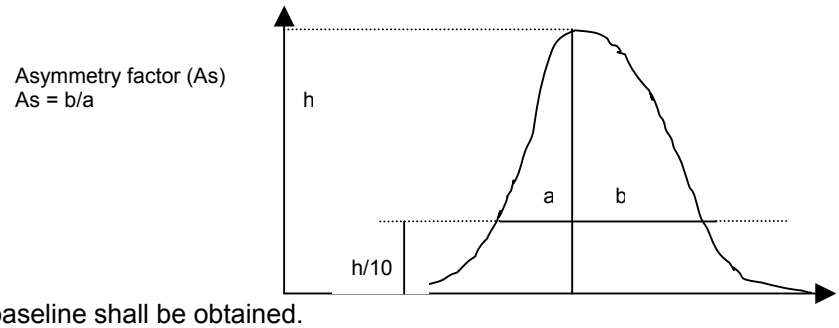
5.3 High performance liquid chromatography
5.3.1 Adjust the flow rate of the mobile phase to 1,0 mL/min and set the detector wavelength to 295 nm.
5.3.2 Inject 20 µl of the sample solution obtained as described in section 5.1, and record the chromatogram. Measure the peak areas. Perform a calibration as described under 5.2.3. Compare the chromatograms obtained for sample and standard solutions.

5.3.3 System suitability
5.3.3.1 Inject 20 µl of the reference solution and record the chromatogram. Inject 6 times to ensure that a constant peak area is obtained
[CV (= standard deviation/mean) x 100) should be less than 2%].
5.3.3.2 Ascertain whether the chromatograms obtained for a standard solution and the sample solution meet the following requirements:

— the peak separation of the worst separated pair shall be at least 0,90. For definition of peak separation, see Figure:



— The asymmetry factor As of all peaks obtained shall range between 0,9 to 1,5. To record the chromatogram for the determination of the asymmetry factor a chart speed of at least 2 cm/min is recommended.



— A steady baseline shall be obtained.

6. CALCULATION

Use the areas of the hydroquinone peaks to calculate the concentration of the hydroquinone in the sample. Calculate the hydroquinone concentration in the sample, as a percentage by mass, (xi) using the formula:

$xi\ \%\ (w/w) = bi/pi \times w_{ref}/w_{spl} \times d \times 100$

in which:

d (dilution factor)=0.1 in the conditions above

b_i = peak area of hydroquinone in the sample solution,

pi = peak area for hydroquinone in the reference solution,

w_{ref} and w_{spl} are the weight of hydroquinone (based on dry content) in the standard solution and in the sample solution respectively.

7. REMARKS

7.1 REPEATABILITY

For a hydroquinone content of 2,0 % the difference between the results of two determinations carried out in parallel on the same sample should not exceed an absolute value of 2 %.

7.2 REPRODUCIBILITY

For a hydroquinone content of 2,0 % the difference between the results of two determinations carried out on the same sample under different conditions (different laboratories, different operators, different apparatus and/or different time) should not exceed an absolute value of 2 %.

7.3 NOTE

When a hydroquinone content considerably higher than 2 % is found and an accurate estimate of the content is required, the sample extract (5.1) should be diluted to a similar concentration as would be obtained from a sample containing 2 % hydroquinone, and the determination repeated.

The sample and the standard should be of similar concentration. The peak area of the sample and of the standard should not differ by more than 10%.

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 Jakarta training, Nov 22nd to 26th, 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**