A. IDENTIFICATION BY TLC

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, except for Hydroquinone RS

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 (v/v)
  3.9.2. System n°2:
    Toluene/Glacial acetic acid, 8:2 (v/v)
3.10. Reference Standard (RS) : Hydroquinone RS
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 60 F_{254} 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 Solution
  5.1.1 Weigh 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.

5.2.1 Weigh about 0.05 g of Hydroquinone RS 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.
- If the spot is too large, spotted be adjusted (reduced), i.e. 2 - 5 µL

5.3.4 Develop in a dark at room 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, the formation of black spot or
- phosphomolybdic acid reagent, and heat the plate to approximately 100°C for about 10 minutes, when necessary, the formation of blue spot.

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

Method validation information

No	Developing Solvents	Rf	LOD (µg/g)
1.	n-hexane/acetone, 3:2	0.32	100
2.	toluene/glacial acetic acid, 8:2	0.20	150

B. IDENTIFICATION AND DETERMINATION BY HPLC

1. SCOPE AND FIELD OF APPLICATION

This method specifies a procedure for the identification and 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 and suitable for HPLC where appropriate, except for Hydroquinone RS. 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 Standard (RS) : Hydroquinone RS

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 Photo Diode Array (PDA) 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-C18, particle size 5 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 Preparation of the Standard Solution

5.1.1 Weigh accurately 0.0125 g (w_{ref}) of Hydroquinone RS 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.

Notes:

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

5.2 Preparation of the Sample Solution

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 identification and determination of filtrate by HPLC within less than 24 hours.

5.3 High Performance Liquid Chromatography

5.3.1 Conditions

Column	:	ODS-C18, 5 µm, 250 x 4.6 mm or equivalent
Mobile phase	:	Water - Metanol (45:55)
Flow rate	:	1.0 mL/minute
PDA detector	:	292 nm
Inject volume	:	20 µL

5.3.2 Inject 20 µl of the standard and sample solutions obtained as described in section 5.1 - 5.2 and record the chromatograms. Measure the peak areas. Compare the chromatograms obtained for sample and standard solutions.

5.3.3 System suitability

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%].

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:

$$\text{Hydroquinone, \% w/w} = \frac{a_{sample}}{a_{std}} \times \frac{w_{std}\ (g)}{w_{sample}\ (g)} \times D \times 100$$

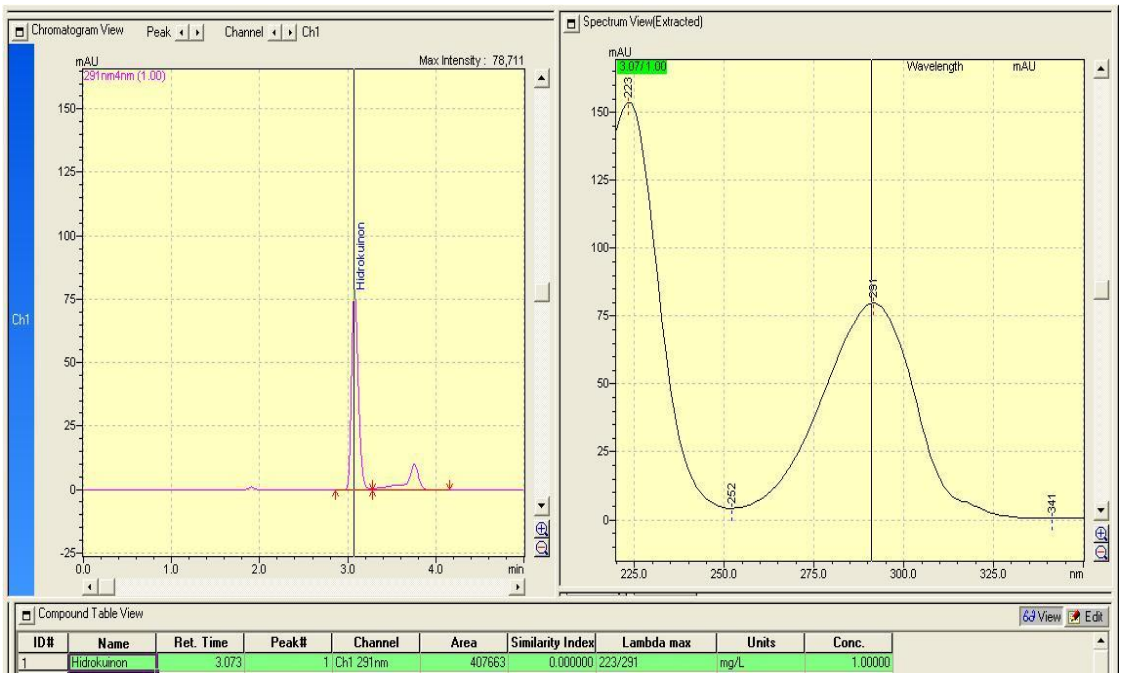
where:
a_{sample} = peak area of sample
a_{std} = peak area of standard
w_{sample} = weight of sample
w_{std} = weight of standard
D= dilution factor = 0.1 in the conditions above

7. REMARKS

7.1 Method validation information

No	Parameters of Validation	Value
1.	Retention time	3 minutes
2.	Precision	1,63 %
3.	Accuracy	(97.0 - 98.7)%
4.	LOD	7.5 µg/g
5.	LOQ	25 µg/g

7.2 Chromatogram of Hydroquinone



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
- Modified and approved after the 1st ACTLC Meeting in Solo (Indonesia), Nov 20nd, 2012