	Title	Revision n°	date	Document N°
	IDENTIFICATION AND DETERMINATION OF 2-PHENOXY-ETHANOL, METHYL, ETHYL, PROPYL, AND BUTYL 4-HYDROXYBENZOATE in COSMETIC PRODUCTS BY TLC AND HPLC	0	2/12/05	ACM INO 04

A. IDENTIFICATION

1. SCOPE AND FIELD OF APPLICATION

This method specifies a TLC procedure that, in combination with the determination method described in Section B, allows the identification of 2-phenoxyethanol, methyl 4-hydroxybenzoate, ethyl 4-hydroxybenzoate, propyl 4-hydroxybenzoate and butyl 4-hydroxybenzoate in cosmetic products.

2. PRINCIPLE

The preservatives are extracted from the acidified cosmetic sample with acetone. After filtration, the acetone solution is mixed with water, and in an alkaline medium the fatty acids are precipitated as their calcium salts. The alkaline acetone/water mixture is extracted with diethylether to remove lipophilic substances. After acidification the preservatives are extracted with diethylether. An aliquot of the diethylether extract is spotted on a silica-gel coated thin-layer plate. After development of the plate, the chromatogram obtained is observed under UV light and visualized using Millon's reagent.

3. REAGENTS

General : all reagents used shall be of analytical purity.
Water shall be distilled water, or water of at least equal purity.

3.1 Acetone
3.2 Diethylether
3.3 n-Pentane
3.4 Ethanol
3.5 Acetic acid, glacial
3.6 Hydrochloric acid solution, 4 M
3.7 Potassium hydroxide solution, 4 M
3.8 Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)
3.9 Detection reagent: Millon's reagent. Millon's reagent (Mercury (II) nitrate) is a ready-made solution which is commercially available (Fluka 69820).
3.10 2-Phenoxyethanol
3.11 Methyl 4-hydroxybenzoate (methylparaben)
3.12 Ethyl 4-hydroxybenzoate (ethylparaben)
3.13 n-Propyl 4-hydroxybenzoate (propylparaben)
3.14 n-Butyl 4-hydroxybenzoate (butylparaben)
3.15 Standard solutions:
Prepare 0.1 % (w/v) solutions of each of the reference materials in ethanol….except for 2-Phenoxyethanol where a solution of 1% (w/v) is to be prepared.
3.16 Development solvent:
Mix 88 volumes of n-pentane with 12 volumes of glacial (concentrated) acetic acid

4. APPARATUS

Normal laboratory equipment, and:

4.1 Water bath, capable of maintaining a temperature of 60 °C
4.2 Developing tank (not lined with filter paper)

4.3 Ultraviolet light source, 254 nm
4.4 Thin-layer plates, 20 cm x 20 cm, precoated with 0,25 mm silica gel $60F_{254}$, with concentrating zone (Merck No 111798, Darmstadt, or equivalent)
4.5 Oven, capable of maintaining up to 105 °C
4.6 Hot-air hair dryer
4.7 Woollen paint roller, length approximately 10 cm, outside diameter approximately 3.5 cm. The thickness of the wool-layer shall be 2 to 3 mm. Trim the wool if necessary. See note under 5.2
4.8 50 mL glass tubes with screw cap, or equivalent

5. PROCEDURE

5.1 Sample preparation: Make a duplicate preparation.

5.1.1 Weigh accurately about 1 g of sample into a 125 mL Erlenmeyer flask
5.1.2 Add 4 drops of HCl 4 M, add 40 mL of acetone and mix (see note below)
5.1.3 Heat the mixture to about 60°C until complete extraction (for 10 minutes)
5.1.4 Cool and shake for one minute with Vortex
5.1.5 Adjust the pH of the solution at ≤ 3 using 4M H Cl. Measure with pH indicator paper
5.1.6 Vortex for one minute
5.1.7 Filter the solution through a paper filter into a 125 mL Erlenmeyer flask
5.1.8 Pipette 20 mL of the filtrate into a 250 mL Erlenmeyer flask, add 60 mL water, and mix
5.1.9 Adjust the pH of the solution to about 10 using 4M KOH. Measure with pH indicator paper
5.1.10 Add 1 g calcium chloride dihydrate, and shake
5.1.11 Filter the solution into a 250 mL separating funnel, containing 75 mL diethyl ether, and shake for 5 minutes
5.1.12 Allow the phases to separate
5.1.13 Discard the upper layer (diethyl ether phase)
5.1.14 Collect the aqueous phase (lower layer) in a 100 mL separating funnel
5.1.15 Adjust the pH to about 2 with HCL 4 M
5.1.16 Add 10 mL diethyl ether, and shake for 5 minutes
5.1.17 Allow the phases to separate
5.1.18 Discard the lower layer (aqueous phase)
5.1.19 Transfer 2 mL of diethyl ether phase (upper layer) into a 5 mL sample vial

Note:
For strongly basic cosmetic products, such as toilet-soap, 20 drops of hydrochloric acid solution shall be added. Close the Erlenmeyer flask, gently heat the mixture to approximately 60 °C to facilitate the extraction of the preservatives into the acetone phase and shake vigorously for one minute.

5.2 Thin-layer chromatography (TLC)

5.2.1 Activate the plates at 100 °C for 10 minutes.
5.2.2 Apply 10 µL of each of the reference solutions and 100 µL of the sample solution(s) on a start line in the concentration zone of the TLC plate.
5.2.3 If desired, a stream of air can be used to facilitate evaporation of the solvent.
5.2.4 Transfer an adequate volume (100 mL, for instance) of the development solvent into a developing tank of suitable size.
5.2.5 Place the TLC plate immediately in the unsaturated chamber and develop at room temperature until the solvent front has run about 15 cm from the base line.

5.2.6 Remove the plate from the development tank and dry in a stream of hot air by means of a hot-air hair dryer.
5.2.7 Examine the plate under UV light and mark the position of the spots.
5.2.8 Heat the plate for 30 minutes in an oven at 100 °C to remove excess acetic acid.
5.2.9 Visualize the preservatives in the chromatogram with Millon's reagent, by dipping the paint roller into the reagent and rolling over the TLC-plate until evenly wetted.

Note:
Alternatively, the spots may be visualized by a careful application - using an HPLC syringe - of a drop of Millon's reagent on each of the spots marked under UV light.

Esters of 4-hydroxybenzoic acid appear as red spots, 2-phenoxyethanol as yellow spots. Note, however, that 4-hydroxybenzoic acid itself, which may be present in the samples as a preservative or decomposition product of the parabens, will also appear as a red spot. See 7.3 and 7.4.

6. IDENTIFICATION

6.1 Calculate the Rf value for each spot.

6.2 Compare the spots obtained from the sample solution with those of the standard solutions with respect to their Rf values, their behaviour under UV radiation and the colour after visualization.

6.3 Draw preliminary conclusions about the identity of the preservatives.
If parabens appear to be present, the HPLC procedure described in Section B should be performed.

6.4 Combine the results from TLC and high-performance liquid chromatography (HPLC) to confirm the presence of the 2-phenoxyethanol and of the parabens.

7. REMARKS

7.1 Because of the toxicity of Millon's reagent this reagent is best applied by one of the procedures described. Spraying is not recommended.

7.2 Other compounds containing hydroxyl groups may also give colours with Millon's reagent. A table of colours and Rf values obtained for a number of preservatives using this TLC procedure may be found in: N. de Kruijf, M. A. H. Rijk, L. A. Pranato-Soetardhi and A. Schouten (1987): Determination of preservatives in cosmetic products I: Thin-layer chromatographic procedure for the identification of preservatives in cosmetic products (J. Chromatography 410, 395-411).

7.3 The Rf value listed in the following table serve as an indication of the values that may be obtained:

Compound	Rf	Colour
methylparaben	0.12	Pink ++++
ethylparaben	0.17	Pink +++
propylparaben	0.21	Pink ++
butylparaben	0.26	Light Pink
2-phenoxyethanol	0.29	Lemon Yellow

7.4 No separation is obtained for 4-hydroxybenzoic acid and methylparaben, or for benzylparaben and ethylparaben. Identification of these compounds should be confirmed by performing the HPLC method described under Section B and comparing the retention times obtained from the sample with those of standards.

B. DETERMINATION

1. SCOPE AND FIELD OF APPLICATION

This method specifies a procedure for the determination of 2-phenoxyethanol, methyl 4-hydroxybenzoate, ethyl 4-hydroxybenzoate, propyl 4-hydroxybenzoate, and butyl 4-hydroxybenzoate in cosmetic products.

2. PRINCIPLE

The sample is acidified by adding sulfuric acid and then suspended in a mixture of ethanol and water. After gently heating the mixture to melt the lipid phase to promote quantitative extraction, the mixture is filtered.
The preservatives in the filtrate are determined by reversed phase HPLC using isopropyl 4-hydroxybenzoate or ethylparaben as the internal standard.

3. REAGENTS

3.1 General
All reagents must be of analytical purity and suitable for HPLC where appropriate. Water shall be distilled water, or water of at least equal purity.

3.2 Ethanol, absolute
3.3 2-Phenoxyethanol
3.4 Methyl 4-hydroxybenzoate (methylparaben)
3.5 Ethyl 4-hydroxybenzoate (ethylparaben)
3.6 n-Propyl 4-hydroxybenzoate (propylparaben)
3.7 Isopropyl 4-hydroxybenzoate (isopropylparaben)
3.8 n-Butyl 4-hydroxybenzoate (butylparaben)
3.9 Tetrahydrofuran
3.10 Methanol
3.11 Acetonitrile
3.12 Sulfuric acid solution 2 M
3.13 Ethanol/water mixture: Mix nine volumes of ethanol and one volume of water.
3.14 Internal standard solution:
Accurately weigh approximately 0.125g isopropylparaben (or ethylparaben). Transfer to a 250 mL volumetric flask, dissolve and make up to volume with ethanol/water mixture.
3.15 Mobile phase: tetrahydrofuran/water/methanol/acetonitrile mixture
Mix 5 volumes of tetrahydrofuran, 60 volumes of water, 10 volumes of methanol and 25 volumes of acetonitrile.
3.16 Preservative stock solution
3.16.1 Weigh accurately about 0.05 g of *methyl, ethyl, propyl, butyl 4-hydroxybenzoate* and 0.2 g of *2-phenoxyethanol* and mix them into a 100 mL volumetric flask, respectively.
3.16.2 Add 50 mL of ethanol/water mixture 9 : 1 (v/v), shake to dissolve it.
3.16.3 Add ethanol/water mixture 9 : 1 (v/v) to volume, and mix.

Kept in a refrigerator, they are stable for one week.

3.17 Standard preservative solutions

3.17.1 Pipette 20.0 mL, 10.0 mL, 5.0 mL, 2.0 mL and 1.0 mL of preservative stock solution into each a 50 mL volumetric flask, respectively.
3.17.2 Add 10.0 mL of internal standard solution into every flask,
3.17.3 Add 1.0 mL of H_2SO_4 2 M and shake to homogenize it,
3.17.4 Add ethanol/water mixture 9 : 1 (v/v) to volume, and mix.
3.17.5 Filter through 0.45 µm membrane filter into HPLC injection vials

These solutions should be freshly prepared.

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 UV-detector, wavelength 280 nm
4.3 Analytical column:
Stainless steel, 25 cm x 4.6 mm i.d. (or 15 cm x 4.6 mm i.d.) packed with Nucleosil C18 (particle size: 5 µm), or equivalent (see 7.1)
4.4 100 mL glass tubes with screw cap or equivalent
4.5 Boiling chips, carborendum, size 2 to 4 mm, or equivalent
4.6 PVDF with glass filter or HVLP membrane filter pore size 0.45 µm, or equivalent

5. PROCEDURE

5.1 Sample preparation

5.1.1 Sample preparation without addition of the internal standard

5.1.1.1 Weigh accurately about 1 g of sample into a 125 mL Erlenmeyer flask (with a screw cap, when available)
5.1.1.2 Add 1.0 mL of H_2SO_4 2 M, add 50.0 mL of ethanol/water mixture 9 : 1 (v/v) and add 1 g of boiling chips.
5.1.1.3 Shake vigorously for 1 minute until homogeneous suspension
5.1.1.4 Place in water bath at (60 ± 1)°C for 5 minutes
5.1.1.5 Cool the Erlenmeyer flask in a stream of cold water and store in refrigerator for 1 hour
5.1.1.6 Filter the solution through a membrane filter (0,45 µm) – after centrifugation when necessary - into a 125 mL Erlenmeyer flask (with a screw cap when available).
5.1.1.7 Transfer approximately 2 mL filtrate into a 5 mL sample vials
5.1.1.8 Perform determination of the filtrate by HPLC within less than 24 hours

5.1.2 Sample preparation including addition of internal standard. (Make duplicate solutions)

5.1.2.1 Weigh accurately about 1 g of sample into a 125 mL Erlenmeyer flask (with a screw cap when available)

5.1.2.2 Add 1.0 mL of H2SO4 2 M, 40.0 mL of ethanol/water mixture 9 : 1 (v/v), 1 g of boiling chips and 10.0 mL of internal standard solution containing 0.05 % ethyl 4-hydroxybenzoate
5.1.2.3 Shake vigorously for 1 minute until homogeneous
5.1.2.4 Place in water bath at (60 ± 1)°C for 5 minutes
5.1.2.5 Cool the Erlenmeyer flask under running cold water and store in refrigerator for 1 hour
5.1.2.6 Filter the solution through a membrane filter (0,45 µm) – after centrifugation when necessary - into a 125-mL Erlenmeyer flask (with a screw cap when available)
5.1.2.7 Transfer about 2 mL filtrate into a 5 mL sample vial
5.1.2.8 Perform determination of the filtrate by HPLC within less than 24 hours

5.2 High-performance liquid chromatography (HPLC)

5.2.1 Chromatographic conditions

5.2.1.1 Mobile phase: tetrahydrofuran /water/methanol/acetonitrile mixture (5/60/10/25)
5.2.1.2 Flow rate: 1.5 mL /minute
5.2.1.3 Detection wavelength: 280 nm
5.2.1.4 Oven temperature: 25 °C

5.2.2 Calibration

5.2.2.1 Inject 20 µL of each of the standard preservative solutions.
5.2.2.2 From the chromatograms obtained determine the ratios of the peak heights (or areas) of the standard preservative solutions to the peak height (or area) of the internal standard.
5.2.2.3 Plot a curve for each preservative relating these ratios to the concentrations of the standard solutions.
5.2.2.4 Perform linear calibration for each of the preservatives

5.2.3 System suitability
5.2.3.1 Inject 6 times 20 µL of the internal standard and / or standard solutions (tolerance: ≤ 2%)
5.2.3.2 Determine the asymmetric factor A_s = b/a, (see further for details).

5.2.4 Determination

5.2.4.1 Inject 20 µL of the sample solution without internal standard into the chromatograph and record the chromatogram.
5.2.4.2 Inject 20 µL of one of the standard preservative solutions and record the chromatogram. Compare the chromatograms obtained.
5.2.4.3 If, in the chromatogram of the sample extract, no peak is present having approximately the same retention time as isopropylparaben (recommended internal standard) or ethylparaben, continue by injecting 20 µL sample solution with internal standard.
5.2.4.4 Record the chromatogram and measure the peak heights (or area).

5.2.4.5 If an interfering peak is observed in the chromatogram of the sample solution having approximately the same retention time as isopropylparaben or ethylparaben, another internal standard should be selected.

5.2.4.6 If one of the preservatives under examination is absent in the chromatogram of the sample, this preservative can be used as an alternative internal standard.

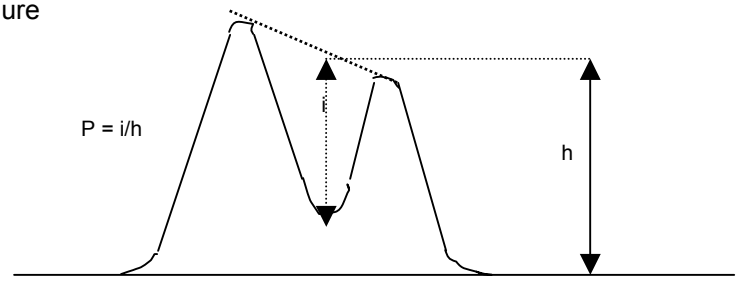
5.2.4.7 Calculate the ratios of the peak heights (or areas) of the investigated preservatives to the peak height (or areas) of the internal standard.

5.2.4.8 Ascertain that for the standard solutions used in the calibration procedure a linear response is obtained.

5.2.4.9 If the peak area of the sample is too low or too high, increase or decrease the amount of the sample so that the peak area falls within the calibration range.

5.2.4.10 Ascertain whether the chromatograms obtained for a standard solution and the sample solution meet the following requirements:

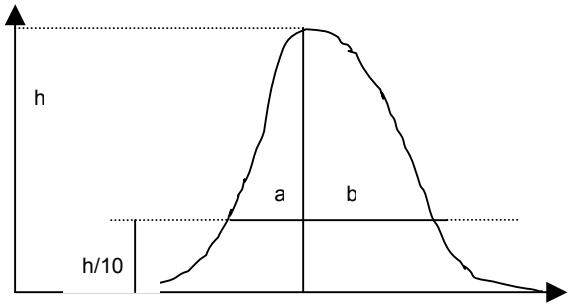
— the peak separation of the worst separated pair shall be at least 0,90. (For definition of peak separation, see Figure



If the required separation is not achieved, either a more efficient column should be used, or the mobile phase composition should be adjusted until the requirement is met.

— the asymmetry factor A_S of all peaks obtained shall range between 0,9 to 1,5.
(For definition of the peak asymmetry factor, see Figure . To record then chromatogram for the determination of the asymmetry factor a chart speed of at least 2 cm/minute is recommended.

Asymmetry factor (As)
As = b/a



— A steady baseline shall be obtained.

6. CALCULATION

Use the calibration curve and the ratios of the peak heights (or areas) of the investigated preservatives to the peak height (or area) of the internal standard to calculate the concentration of the preservatives in the sample solution.

Calculate the 2-phenoxyethanol, methyl 4-hydroxybenzoate, ethyl 4-hydroxybenzoate, propyl 4-hydroxybenzoate, butyl 4-hydroxybenzoate and w_i, as percentage by weight (% w/w), using the formula:

$$\% w_i (w/w) = b_i * 51/(10000 x a)$$

in which:
b_i = the concentration (µg/mL) of preservative i in the test solution as read from the calibration curve; and
a = the weight (g) of the test solution.

7. REMARKS

7.1 Stationary phase

The retention behaviour of the solutes in HPLC determinations is strongly dependent on the type, the brand and the history of the stationary phase. Whether a column can be used for the separation of the preservatives under examination, can be concluded from the results obtained for standard solutions (see 5.2.4). In addition to the proposed column packing material, Hypersil ODS and Zorbax ODS were also found to be suitable.

Alternatively, the recommended mobile phase composition can be optimised in order to obtain the required separation.

7.2 Detection wavelength

A ruggedness test on the described method has shown that a slight change in the detection wavelength can have a significant effect on the results of the determination. Therefore, this parameter must be controlled carefully during the analysis.

7.3 Interferences

Under the conditions described in this method many other compounds, such as preservatives and cosmetic additives, are eluted as well. Retention times of a large number of preservatives mentioned in Annex VI to the Council Directive regarding cosmetic products are listed in: N. de Kruijf, M.A.H. Rijk, L. A. Pranato-Soetardhi and A. Schouten, (1989). Determination of preservatives in cosmetic products II. High-performance liquid chromatographic identification (J. Chromatography 469, 317-398).

7.4 To protect the analytical column an appropriate guard column may be used.

7.5 The method has been investigated in a collaborative trial in which nine laboratories participated. Three samples were analysed. The following table lists, for each of the three samples, the means in % w/w (m), repeatabilities (r), reproducibilities (R) found for the analytes they contained:

Sample	2-Phenoxy-ethanol		1-Phenoxy-propan-2-ol	Methyl-paraben	Ethyl-paraben	Propyl-paraben	Butyl-paraben	Benzyl-paraben
Vitamin cream	m r R	1,124 0,016 0,176		0,250 0,018 0,030	0,0628 0,0035 0,0068	0,031 0,0028 0,0111	0,0906 0,0044 0,0034	
Vanishing cream	m r R	1,196 0,040 0,147		0,266 0,003 0,022	0,076 0,002 0,004			
Massage cream	m r R		0,806 0,067 0,112			0,180 0,034 0,078	0,148 0,013 0,012	0,152 0,015 0,016

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 & 31st, 2005**
- **Modified and approved after the final review in Singapore, Nov 30th to Dec 2nd, 2005**