SEVENTH COMMISSION DIRECTIVE 96/45/EC

of 2 July 1996

relating to methods of analysis necessary for checking the composition of cosmetic products

(Text with EEA relevance)

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Community,

Having regard to Council Directive 76/768/EEC of 27 July 1976 on the approximation of the laws of the Member States relating to cosmetic products [1], as last amended by Commission Directive 95/34/EC [2], and in particular Article 8 (1) thereof,

Whereas Directive 76/768/EEC provides for the official testing of cosmetic products with the aim of ensuring that the conditions laid down by Community provisions concerning the composition of cosmetic products are satisfied;

Whereas all the necessary methods of analysis should be laid down as quickly as possible; whereas certain methods have already been adopted in Commission Directives 80/1335/EEC [3], as amended by Directive 87/143/EEC [4], 82/434/EEC [5], as amended by Directive 90/207/EEC [6], and by Commission Directives 83/514/EEC [7], 85/490/EEC [8], 93/73/EEC [9] and 95/32/EC [10];

Whereas the identification and determination of 2-phenoxyethanol, 1-phenoxypropan-2-ol, methyl, ethyl, propyl, butyl and benzyl 4-hydroxybenzoate in cosmetic products constitute a seventh step;

Whereas the measures provided for in this Directive are in accordance with the opinion of the Committee on the adaptation of Directive 76/768/EEC to technical progress,

HAS ADOPTED THIS DECISION:

Article 1

Member States shall take all the necessary steps to ensure that during official testing of cosmetic products, the identification and determination of 2-phenoxyethanol, 1-phenoxypropan-2-ol, methyl, ethyl, propyl, butyl and benzyl 4-hydroxybenzoate shall be carried out in accordance with the method described in the Annex.

Article 2

1. Member States shall bring into force the laws, regulations and administrative provisions needed to comply with this Directive no later than 30 September 1997. They shall forthwith inform the Commission thereof.

2. When Member States adopt these provisions, these shall contain a reference to this Directive or shall be accompanied by such reference at the time of their official publication. The procedure for such reference shall be adopted by the Member States.

3. Member States shall communicate to the Commission the provisions of national law which they adopt in the field covered by this Directive.

Article 3

This Directive shall enter into force on the 20th day following its publication in the *Official Journal of the European Communities.*

Article 4

This Directive is addressed to the Member States.

Done at Brussels, 2 July 1996.

For the Commission

Emma BONINO

Member of the Commission

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(1) OJ No L 262, 27. 9. 1976, p. 169.
(2) OJ No L 167, 18. 7. 1995, p. 19.
(3) OJ No L 383, 31. 12. 1980, p. 27.
(4) OJ No L 57, 27. 2. 1987, p. 56.
(5) OJ No L 185, 30. 6. 1982, p. 1.
(6) OJ No L 108, 28. 4. 1990, p. 92.
(7) OJ No L 291, 24. 10. 1983, p. 9.
(8) OJ No L 295, 7. 11. 1985, p. 30.
(9) OJ No L 231, 14. 9. 1993, p. 34.
(10) OJ No L 178, 28. 7. 1995, p. 20.

ANNEX

IDENTIFICATION AND DETERMINATION OF 2-PHENOXYETHANOL, 1-PHENOXYPROPAN-2-OL, METHYL, ETHYL, PROPYL, BUTYL AND BENZYL 4-HYDROXYBENZOATE IN COSMETIC PRODUCTS

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, 1-phenoxypropan-2-ol, methyl 4-hydroxybenzoate, ethyl 4-hydroxybenzoate, propyl 4-hydroxybenzoate, butyl 4-hydroxybenzoate and benzyl 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 perservatives 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

3.1. General

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

3.2. Acetone

3.3. Diethylether

3.4. n-Pentane

3.5. Methanol

3.6. Acetic acid, glacial

3.7. Hydrochloric acid solution, c(HCl) = 4mol/l

3.8. Potassium hydroxide solution, c(KOH) = 4 mol/l

3.9. Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)

3.10. Detection reagent: Millon's reagent

Millon's reagent (Mercury (II) nitrate) is a ready-made solution which is commercially available (Fluka 69820).

3.11. 2-Phenoxyethanol

3.12. 1-Phenoxypropan-2-ol

3.13. Methyl 4-hydroxybenzoate (methylparaben)

3.14. Ethyl 4-hydroxybenzoate (ethylparaben)

3.15. n-Propyl 4-hydroxybenzoate (propylparaben)

3.16. n-Butyl 4-hydroxybenzoate (butylparaben)

3.17. Benzyl 4-hydroxybenzoate (benzylparaben)

3.18. Reference solutions

Prepare 0,1 % (m/V) solutions of each of the reference substances 3.11, 3.12, 3.13, 3.14, 3.15, 3.16 and 3.17 in methanol.

3.19. Development solvent

Mix 88 volumes of n-pentane (3.4) with 12 volumes of glacial acetic acid (3.6).

4. **Apparatus**

Normal laboratory equipment, and:

4.1. Waterbath, 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 × 20 cm, precoated with 0,25 mm silica gel $60F_{254}$, with concentrating zone (Merck No 11798, 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

4.9. Electric heating plate, with thermostatic temperature controller. Temperature setting: about 80 °C. The hot plate shall be covered with an aluminium plate of 20 cm × 20 cm and a thickness of about 6 mm, to obtain a uniform heat distribution.

5. **Procedure**

5.1. Sample preparation

Weigh approximately 1 g of sample in a 50-ml glass tube with screw cap (4.8). Add four drops of hydrochloric acid solution (3.7) and 40 ml of acetone.

For strongly basic cosmetic products, such as toilet-soap, 20 drops of hydrochloric acid solution shall be added. Close the tube, 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.

Measure the pH of the solution with pH indicator paper and adjust the pH of the solution $\leqslant 3$ with hydrochloric acid solution. Shake vigorously again for one minute.

Cool the solution to room temperature and filter through a filter paper into a conical flask. Transfer 20 ml of the filtrate into a 200-ml conical flask, add 60 ml water and mix. Adjust the pH of the mixture to approximately 10 with potassium hydroxide (3.8), using pH indicator paper.

Add 1 g calcium chloride dihydrate (3.9) and shake vigorously. Filter the solution through a filter paper into a 250-ml separating funnel containing 75 ml diethylether and shake vigorously for one minute. Allow the phases to separate and collect the aqueous layer in a 200-ml conical flask. Adjust the pH of the solution to approximately 2 with hydrochloric acid solution, using pH indicator paper. Subsequently, add 10 ml diethylether and shake vigorously for one minute. Allow the phases to separate and transfer approximately 2 ml of the diethylether layer into a 5-ml sample vial.

5.2. Thin-layer chromatography (TLC)

Place a TLC plate (4.4) on the heated aluminium plate (4.9). Apply 10 µl of each of the reference solutions (3.18) and 100 µl of the sample solution(s) (5.1) on a start line in the concentration zone of the TLC plate.

If desired, a stream of air can be used to facilitate evaporation of the solvent. Remove the TLC plate from the heating plate and allow to cool to room temperature. Transfer 100 ml of the development solvent (3.19) into a developing tank (4.2).

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. Remove the plate from the development tank and dry in a stream of hot air by means of a hot-air hair dryer.

Examine the plate under UV light (4.3) and mark the position of the spots. Heat the plate for 30 minutes in an oven (4.5) at 100 °C to remove excess acetic acid. Visualize the preservatives in the chromatogram with Millon's reagent (3.10), by dipping the paint roller (4.7) into the reagent and rolling over the TLC-plate until evenly wetted.

Note: Alternatively, the spots may be visualized by the careful application 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 and 1-phenoxypropan-2-ol 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**

Calculate the R_f-value for each spot. Compare the spots obtained from the sample solution with those of the reference solutions with respect to their R_f-values, their behaviour under UV radiation and the colour after visualization. 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. Combine the results from TLC and high-performance liquid chromatography (HPLC) to confirm the presence of the 2-phenoxyethanol, 1-phenoxypropan-2-ol and 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 R_f-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. Chomatography* 410, 395-411).

7.3. The R_f-values listed in the following table serve as an indication of the values that may be obtained:

Compound	hR_f	Colour
4-hydroxybenzoic acid	11	red
methylparaben	12	red
ethylparaben	17	red
propylparaben	21	red
butylparaben	26	red
benzylparaben	16	red
2-phenoxyethanol	29	yellow
1-phenoxypropan-2-ol	50	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, 1-phenoxypropan-2-ol, methyl 4-hydroxybenzoate, ethyl 4-hydroxybenzoate, propyl 4-hydroxybenzoate, butyl 4-hydroxybnenzoate and benzyl 4-hydroxybenzoate in cosmetic products.

2. **Definition**

The amounts of the preservatives determined by this method are expressed as percentage by mass.

3. **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 as the internal standard.

4. **Reagents**

4.1. 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.

4.2. Ethanol, absolute

4.3. 2-Phenoxyethanol

4.4. 1-Phenoxypropan-2-ol

4.5. Methyl 4-hydroxybenzoate (methylparaben)

4.6. Ethyl 4-hydroxybenzoate (ethylparaben)

4.7. n-Propyl 4-hydroxybenzoate (propylparaben)

4.8. Isopropyl 4-hydroxybenzoate (isopropylparaben)

4.9. n-Butyl 4-hydroxybenzoate (butylparaben)

4.10. Benzyl 4-hydroxybenzoate (benzylparaben)

4.11. Tetrahydrofuran

4.12. Methanol

4.13. Acetonitrile

4.14. Sulfuric acid solution $c(H_2SO_4) = 2 mol/l$

4.15. Ethanol/water mixture

Mix nine volumes of ethanol (4.2) and one volume of water.

4.16. Internal standard solution

Accurately weigh approximately 0,25 g isopropylparaben (4.8), transfer to a 500-ml volumetric flask, dissolve and make up to volume with ethanol/water mixture (4.15).

4.17. 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.

4.18. Preservative stock solution

Accurately weigh approximately 0,2 g 2-phenoxyethanol, 0,2 g 1-phenoxypropan-2-ol, 0,05 g methylparaben, 0,05 g ethylparaben, 0,05 g propylparaben, 0,05 g butylparaben and 0,025 g benzylparaben in a 100-ml volumetric flask, dissolve and make up to volume with ethanol/water mixture.

Kept in a refrigerator the solution is stable for one week.

4.19. Standard preservative solutions

From the stock solution (4.18) transfer respectively 20,00 ml, 10,00 ml, 5,00 ml, 2,00 ml and 1,00 ml into 50-ml volumetric flasks. To each flask, add 10,00 ml internal standard solution (4.16) and 1,0 ml sulfuric acid solution (4.14) and make up to volume with ethanol/water mixture. These solutions should be freshly prepared.

5. Apparatus

Normal laboratory equipment, and:

5.1. Waterbath, capable of maintaining a temperature of 60 °C ± 1 °C.

5.2. High performance liquid chromatograph with a UV-detector, wavelength 280 nm

5.3. Analytical column:

Stainless steel, 25 cm × 4,6 mm i.d. (or 12,5 cm × 4,6 mm i.d.) packed with Nucleosil 5C18, or equivalent (see 10.1)

5.4. 100-ml glass tubes with screw cap

5.5. Boiling chips, carborundum, size 2 to 4 mm, or equivalent

6. Procedure

6.1. Sample preparation

6.1.1. Sample preparation without addition of the internal standard

Weigh approximately 1,0 g of sample in a 100-ml glass tube with screw cap. Pipette 1,0 ml sulfuric acid solution (4.14) and 50,0 ml ethanol/water mixture (4.15) into the tubes. Add approximately 1 g of boiling chips (5.5), close the tube and shake vigorously until a homogeneous suspension is obtained. Shake for at least one minute. Place the tube for five minutes in a waterbath (5.1) kept at 60 °C ± 1 °C to facilitate the extraction of the preservatives into the ethanol phase.

Immediately cool the tube in a stream of cold water and store the extract in the refrigerator for one hour. Filter the extract using a filter paper. Transfer approximately 2 ml of the filtrate into a 5-ml sample vial. Store the extracts in the refrigerator and perform the HPLC determination within 24 hours.

6.1.2. Sample preparation including addition of internal standard

Weigh to three decimal places 1,0 g ± 0,1 g of sample in a 100-ml glass tube with screw cap.

Pipette 1,0 ml sulfuric acid solution and 40,0 ml ethanol/water mixture into the tube. Add approximately 1 g of boiling chips (5.5) and exactly 10,00 ml internal standard solution. Close the tube and shake vigorously until a homogeneous suspension is obtained. Shake for at least one minute. Place the tube for five 5 minutes in a waterbath kept at 60 °C ± 1 °C to facilitate extraction of the preservatives into the ethanol phase.

Immediately cool the tube in a stream of cold tap water and store the extract in the refrigerator for one hour. Filter the extract using a filter paper.

Transfer approximately 2 ml of the filtrate into a 5-ml sample vial (test solution). Store the extract in the refrigerator and perform the HPLC determinations within 24 hours.

6.2. High-performance liquid chromatography (HPLC)

6.2.1. Chromatographic conditions

— Mobile phase: tetrahydrofuran/water/methanol/acetonitrile mixture (4.17)

— Flow rate: 1,5 ml/minute

— Detection wavelength: 280 nm

6.2.2. Calibration

Inject 10µl of each of the standard preservative solutions (4.19). From the chromatograms obtained determine the ratios of the peak heights of the standard preservative solutions to the peak height of the internal standard. Plot a curve for each preservative relating these ratios to the concentrations of the standard solutions.

6.2.3. Determination

Inject 10 µl of the sample solution without internal standard (6.1.1) into the chromatograph and record the chromatogram.

Inject 10 µl of one of the standard preservative solutions (4.19) and record the chromatogram. Compare the chromatograms obtained.

If, in the chromatogram of the sample extract (6.1.1), no peak is present having approximately the same retention time as isopropylparaben (recommended internal standard), continue by injecting 10 µl sample solution with internal standard (6.1.2). Record the chromatogram and measure the peak heights.

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

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.

Calculate the ratios of the peak heights of the investigated preservatives to the peak height of the internal standard.

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

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 1).

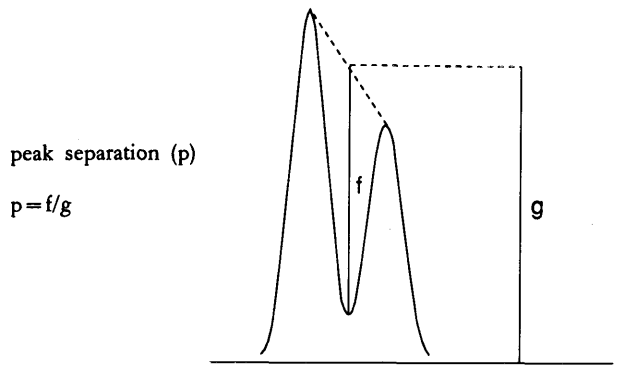

Figure 1: Peak separation

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 2.) To record the chromatogram for the determination of the asymmetry factor a chart speed of at least 2 cm/minute is recommended.

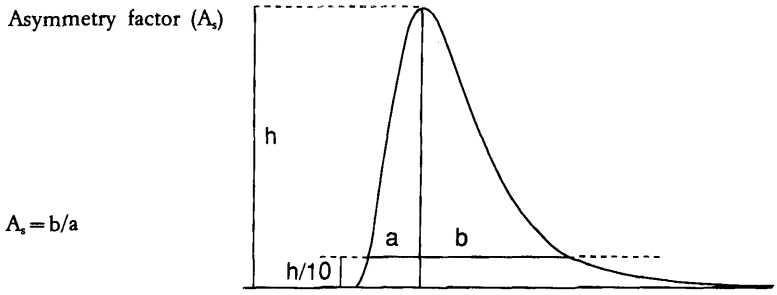

Figure 2: Peak asymmetry factor

— A steady baseline shall be obtained.

7. **Calculation**

Use the calibration curve (6.2.2) and the ratios of the peak heights of the investigated preservatives to the peak height of the internal standard to calculate the concentration of the preservatives in the sample solution. Calculate the 2-phenoxyethanol, 1-phenoxypropan-2-ol, methyl 4-hydroxybenzoate, ethyl 4-hydroxybenzoate, propyl 4-hydroxybenzoate, butyl 4-hydroxybenzoate and benzyl 4-hydroxybenzoate contents, w_i, as percentage by weight (% m/m), using the formula:

$$\% \, w_i \, (m/m) = \frac{b_i}{200 \times a}$$

in which:

b_i = the concentration (µg/ml) of preservative i in the test solution as read from the calibration curve; and

a = the mass (g) of the test postion.

8. **Repeatability** (1)

See remarks, 10.5.

9. **Reproducibility** (1)

See remarks, 10.5.

10. **Remarks**

10.1. 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 remarks 6.2.3). 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 optimized in order to obtain the required separation.

10.2. 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.

(1) ISO 5725.

10.3. 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).

10.4. To project the analytical column an appropriate guard column may be used.

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

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