SLOVENSKI STANDARD
oSIST prEN 16956:2016

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Kozmetika - Analizne metode - Metoda HPLC/UV za identifikacijo in določevanje hidrokinona, etrov hidrokinona in kortikosteroidov v kozmetičnih izdelkih za beljenje kože

Cosmetics - Analytical methods - HPLC/UV method for the identification and assay of hydroquinone, ethers of hydroquinone and corticosteroids in skin whitening cosmetic products

Kosmetische Mittel - Untersuchungsverfahren - HPLC/UV Verfahren für die Identifizierung und Bestimmung von Hydrochinon, Hydrochinonethern und Kortikosteroide in hautaufhellenden kosmetischen Mitteln

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English Version

Cosmetics - Analytical methods - HPLC/UV method for the identification and assay of hydroquinone, ethers of hydroquinone and corticosteroids in skin whitening cosmetic products

Kosmetische Mittel - Untersuchungsverfahren - HPLC/UV Verfahren für die Identifizierung und Bestimmung von Hydrochinon, Hydrochinonethern und Kortikosteroide in hautaufhellenden kosmetischen Mitteln

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

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European foreword

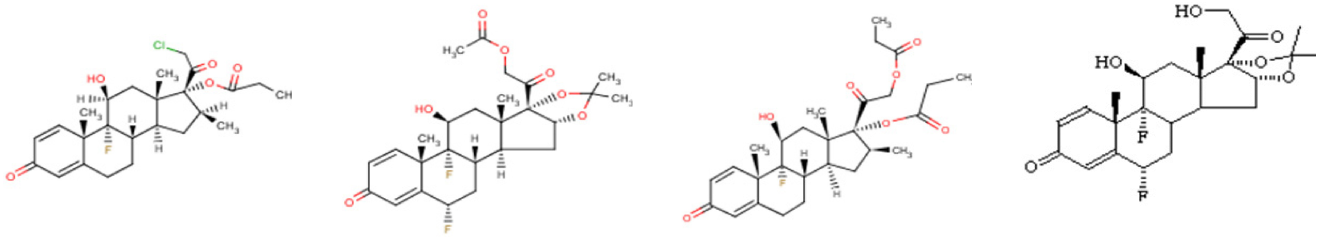
This document (prEN 16956:2016) has been prepared by Technical Committee CEN/TC 392 “Cosmetics”, the secretariat of which is held by AFNOR.

This document is currently submitted to the CEN Enquiry.

Introduction

Hydroquinone is no longer used in cosmetic products for skin whitening and depigmentation of dermal spots or imperfections. Due to its cytotoxic effects its use has been regulated. Hydroquinone and 3 of its ether (hydroquinone monomethylether (MME), hydroquinone monoethylether (MEE) and hydroquinone monobenzylether (MBE)) have been regulated since the 11 of July 2013 by the cosmetic regulation 1223/2009. Nowadays, its use is prohibited in skin whitening cosmetics.

Depigmentation is a side effect of topical steroids, in this way corticosteroids might be used as compounds in products illegally sold as cosmetics. Corticosteroids most commonly found in products illegally sold as cosmetics are clobetasol propionate, fluocinonide, betamethasone dipropionate, and fluocinolone acetonide (see Figure 1). Corticosteroids are registered in the list I of Poisons (6 Art.L.5132-CSP) and in the Regulation 1223/2009 Annex II "List of substances prohibited in cosmetic products" (reference number 300), and as such their use is prohibited in cosmetic products.



a) Clobetasol propionate — b) Fluocinonide — c) Betamethasone dipropionate — d) Fluocinolone Acetonide

Figure 1 — Corticosteroids most commonly found in illegal cosmetics

All these substances worked on the same principle as hydroquinone which mainly consists of inhibition of melanin synthesis.

The cosmetic directive 95/32/EC [2] gives an analytical method for the assay of hydroquinone and 3 of its ethers (hydroquinone monomethylether (MME), hydroquinone monoethylether (MEE) and hydroquinone monobenzylether (MBE)) in cosmetic products for lightening the skin. In order to update and extend this official method to the identification and assay of corticosteroids in cosmetic products, this standard describes HPLC/UV method for the identification and assay of hydroquinone, ethers of hydroquinone and corticosteroids in cosmetic products.

1 Scope

This European Standard specifies a HPLC/UV method for the assay of hydroquinone, 3 ethers of hydroquinone and 4 corticosteroids most frequently found in illegally sold skin whitening cosmetic products: clobetasol propionate, betamethasone dipropionate, fluocinonide and fluocinolone acetonide.

This standard also proposes HPLC/UV methods for the identification of 38 corticosteroids may be found in skin whitening products. Indeed, as corticosteroids could be deliberately introduced in skin whitening cosmetics, despite the fact that they are forbidden to use, an identification of the presence of one of this illicit compounds could be enough for a market survey control.

This standard is not dedicated to artificial nail products or soaps.

2 Principles

2.1 Identification and quantification of hydroquinone, 3 ethers of hydroquinone and 4 most frequently found corticosteroids

The considered corticosteroids are clobetasol propionate, betamethasone dipropionate, fluocinonide and fluocinolone acetonide.

The sample is extracted by a mixture of water/methanol then gently warmed in order to extract compounds presents in the product. The obtained mixture is filtered. The quantitation of present compounds in solution is made by reversed phase HPLC with DAD detection.

2.2 Screening methods for the identification of hydroquinone, 3 ethers of hydroquinone and 38 corticosteroids

As corticosteroids are not to be used in cosmetics and shall not be present as contaminants or impurities, an identification of one of these illicit compounds deliberately introduced in cosmetics could be enough for market survey control. Once illicit corticosteroids are identified, if necessary, an accurate quantification of these compounds could be then performed using dedicated validated methods.

The sample is extracted by a mixture of water/methanol then gently warmed in order to extract compounds presents in the product. The obtained mixture is filtered. The identification of present compounds in solution is made by reversed phase HPLC with DAD detection.

Two elution gradients are needed to separate all compounds. The main solvent gradient allows the separation of 39 compounds among the 43 compounds considered in 45 min. For compounds not separated a complementary gradient elution using the same solvents is proposed.

3 Reagents

If not otherwise specified, analytical-grade chemicals shall be used; the water shall be distilled or of a corresponding purity. "Solution" shall be understood as an aqueous solution unless otherwise specified.

3.1 Methanol, HPLC grade.

3.2 Water, HPLC grade.

3.3 Extraction solution, methanol/water (1/1).

Mix 500 ml of methanol (3.1) and 500 ml of water (3.2) in a 1 000 ml conical flask.

3.4 Compounds considered, see Table 1.

Table 1 — Compounds considered

Compound	CAS	Manufacturer[a]	Purity %	Used method – clause
Alclometasone dipropionate (ACD)	66734-13-2	USP	99,2	2.2
Amcinonide (AMC)	51022-69-6	Sigma	97,9	2.2
Beclomethasone dipropionate (BCD)	5593-20-4	Sigma	99	2.2
Betamethasone acetate (BMA)	987-24-6	Sigma	98,6	2.2
Betamethasone (BM)	378-44-9	Sigma	98,4	2.2
Betamethasone dipropionate (BMD)	5593-20-4	Sigma	98,6	2.1/2.2
Betamethasone valerate (BMV)	2152-44-5	Sigma	98,1	2.2
Budesonide (BUD)	51333-22-3	Ph. Eur.	99,7	2.2
Clobetasol propionate (CP)	25122-46-7	Sigma	98,8	2.1/2.2
Clocortolone pivalate (CLP)	34097-16-0	USP	98,9	2.2
Cortisone (CS)	53-06-5	Sigma	98,3	2.2
Desonide (DSN)	638-94-8	Cil	98	2.2
Desoximetasone (DXM)	382-67-2	Sigma	99	2.2
Dexamethasone phosphate (DMPS)	2392-39-4	Sigma	100,2	2.2
Dexamethasone acetate (DMA)	1177-87-3	Sigma	99	2.2
Diflorasone diacetate (DFD)	33564-31-7	USP	99,8	2.2
Diflucortolone valerate (DFCV)	59198-70-8	Schering	/	2.2
Difluprednate (DFP)	23674-86-4	Sigma	99,5	2.2
Flumethasone pivalate (FMP)	2002-29-1	Farmabios	100,4	2.2
Fluocinolone acetonide (FCA)	67-73-2	Sigma	99,6	2.1/2.2
Fluocinonide (FCAA)	356-12-7	Sigma	99	2.1/2.2
Fluocortolone –hexanoate (FCH)	303-40-2	Schering	/	2.2
Fluocortolone pivalate (FCP)	29205-06-9	Ph. Eur.	/	2.2
Flurandrenolide (FDL)	1524-88-5	USP	/	2.2
Halcinonide (HAL)	3093-35-4	Sigma	99	2.2
Hydrocortisone (HC)	3093-25-4	Sigma	98	2.2
Hydrocortisone aceponate (HCAP)	74050-20-7	Toronto Research Chemicals	98	2.2
Hydrocortisone butyrate (HCB)	13609-67-1	Sigma	99,1	2.2
Hydrocortisone valerate (HCV)	57524-89-7	Sigma	99	2.2
Methylprednisolone acetate (MPLA)	53-36-1	USP	99,8	2.2
Mometasone furoate (MMF)	83919-23-7	USP	99,8	2.2
Prednicarbate (PCN)	73771-04-7	Ph. Eur.	/	2.2

Compound	CAS	Manufacturer[a]	Purity %	Used method – clause
Prednisolone (PL)	50-24-8	Aventis Pharma	98	2.2
Prednisolone acetate (PLA)	52-21-1	Dr Ehrenstrofer	99,4	2.2
Prednisolone hexanoate (PLH)	69164-69-8	Schering	/	2.2
Prednisolone sulfobenzoate Na (PLSB)	630-67-1	Pharmaceutical drug	/	2.2
Triamcinolone (TRI)	124-94-7	Sigma	98,2	2.2
Triamcinolone acetonide (TRA)	76-25-5	Sigma	99,6	2.2
Hydroquinone (HQ)	123-31-9	Sigma	> 99,9	2.1/2.2
Hydroquinone monomethylether (MME)	150-76-5	Sigma	99,7	2.1/2.2
Hydroquinone monoethylether (MEE)	622-62-8	Sigma	99,9	2.1/2.2
Hydroquinone monobenzylether (MBE)	103-16-2	Sigma	99,5	2.1/2,2
a Examples of manufacturer				

3.5 Mobile phase for HPLC in 3.7.1, mobile phase A: methanol (3.1); mobile phase B: water (3.2).

3.6 Specific reagents for the screening methods, see 2.2.

3.6.1 Acetonitrile, HPLC grade.

3.6.2 Formic acid, purity > 98 %.

3.6.3 Ethanol, HPLC grade.

3.6.4 Mobile phase for HPLC in 3.7.2

Mobile phase A: Add 1,0 ml formic acid (3.6.2) to 500 ml acetonitrile (3.6.1) in a 1 l volumetric flask. Swirl to dissolve and dilute with acetonitrile (3.6.1). Final solution is 0,1 % formic acid in acetonitrile.

Mobile phase B: Add 1,0 ml formic acid (3.6.2) to 500 ml water (3.2) in a 1 l volumetric flask. Swirl to dissolve and dilute with water (3.2). Final solution is 0,1 % formic acid in aqueous solution.

3.7 Reference solutions

Methanol (3.1) is used as a solvent for the preparation of stock solutions. Standard solutions are prepared by dilution in a methanol / water solution (3.3).

3.7.1 Specific reference solutions for identification and quantification methods, see 2.1.

3.7.1.1 Standard Stock Solution of hydroquinone and its 3 ethers (between 1,2 mg/ml and 2,4 mg/ml).

Weigh approximately into a 25 ml volumetric flask:

— 0,03 g of hydroquinone;

— 0,04 g of monomethylether of hydroquinone;

— 0,05 g of monoethylether of hydroquinone;

— 0,06 g of monobenzylether of hydroquinone.

Firstly, dissolve in 15 ml of methanol (3.1), if necessary use a shaker and then fill up to the calibration mark with methanol. This solution shall be prepared daily.

3.7.1.2 Standard Stock Solutions (1 mg/ml) and Standard Working Solution of 4 most frequently found corticosteroids (10 µg/ml).

Weigh separately and approximately into a 10 ml volumetric flask:

— 10 mg of betamethasone dipropionate;

— 10 mg of clobetasol propionate;

— 10 mg of fluocinonide;

— 10 mg of fluocinolone acetonide.

Firstly, dissolve in 5 ml of methanol (3.1), if necessary use a shaker and then fill up to the calibration mark with methanol. Theses 4 solutions can be stored during 6 months at 4 °C.

Then prepare a daughter solution of 4 corticosteroids by introducing with a glass pipette 1 ml of each standard stock solution in a 100 ml volumetric flask. Fill up to the mark with mixture methanol/water (3.3).

3.7.1.3 Calibration Solutions of hydroquinone and its 3 ethers

The calibration solutions shall be freshly prepared (see Table 2). With a glass pipette, introduce the volume of standard stock solution (3.7.1.2) in a volumetric flask then fill up to the mark with the mixture methanol/water (3.3).

Table 2 — Calibration solutions

Number of calibration solution	Volume of Standard Stock Solution ml	Volume of flask ml	Concentration of calibration solutions µg/ml
1	0,5	100	6 – 8 – 10 – 12
2	1	50	24 – 32 – 40 – 48
3	5		120 – 160 – 200 – 240
4	10		240 – 320 – 400 – 480
5	20		480 – 640 – 800 - 960

3.7.1.4 Calibration Solutions of 4 most frequently found corticosteroids

The calibration solutions shall be freshly prepared (see Table 3). With a glass pipette, introduce the volume of standard daughter solution (3.7.1.3) in a volumetric flask then fill up to the mark with the mixture methanol/water (3.3).

Table 3 — Calibration solution

Number of calibration solution	Volume of Standard Stock Solution ml	Volume of flask ml	Concentration of calibration solutions µg/ml
6	5	100	0,5
7	5	50	1
8	10		2
9	20		4
10	25		5

3.7.2 Specific reference solutions for the screening methods, see 2.2.

3.7.2.1 Stock solution of the internal standard reference substance (1 000 µg/ml)

For the determination of the relative retention time (RRT) of the present substances prepare a stock solution of the internal standard reference substance propyl paraben by weighting approximately 10 mg into a 10 ml volumetric flask. Firstly dissolve in a small amount of methanol (3.1) and then fill up to the mark with methanol.

In this standard, hydroquinone, ethers of hydroquinone and corticosteroids are identified by comparing the relative retention time (RRT) calculated for substances present in the sample with those of standard solutions. If propyl paraben or an interfering compound is present in the sample the identification could be done directly using the retention time (instead of the RR). If needed a confirmation of the compound identity may also be carried out using spiked preparation or by using mass spectrometry detection (i.e. mobile phases used are compatible with LC-MS analysis).

3.7.2.2 Standard Stock solutions (1 000 µg/ml)

Prepare stock solutions of each standard substance according to Table 6 by weighting approximately 10,0 mg of each standard into a 10 ml volumetric flask. Firstly, dissolve in a small amount of methanol (3.1) and then fill up to the mark with methanol.

3.7.2.3 Qualitative analysis (identification)

3.7.2.3.1 Screening standard solutions

Prepare 4 standard solutions (Mixtures A, B, C and D), by mixing 250 µl of each selected stock solution into a 5 ml volumetric flask. Fill up to the mark with methanol.

3.7.2.3.2 Mixed standard solution A (50 µg/ml), hydroquinone (HQ), clobetasol propionate (CP), flucinonide (FCAA), monomethylether hydroquinone (MME), monobenzylether hydroquinone (MBE), monoethylether hydroquinone (MEE), betamethasone dipropionate (BMD), fluocinolone acetonide (FCA).

3.7.2.3.3 Mixed standard solution B (50 µg/ml), hydrocortisone (HC), prednisolone sulfobenzoate (PLSB), triamcinolone acetonide (TRA), prednisolone acetate (PLA), dexamethasone acetate (DMA) hydrocortisone valerate (HCV), difluprednate (DFP), amcinonide (AMC), difluocortolone valerate (DFCV), fluocortolone pivalate (FCP), fluocortolone hexanoate (FCH).

3.7.2.3.4 Mixed standard solution C (50 µg/ml), triamcinolone (TRI), prednisolone (PL), betamethasone (BM), desoximethasone (DXM), betamethasone acetate (BMA), hydrocortisone butyrate

(HCB), diflorasone diacetate (DFD), halcinonide (HAL), prednisolone hexanoate (PLH), prednicarbate (PCN), beclometasone dipropionate (BCD).

3.7.2.3.5 Mixed standard solution D (50 µg/ml), dexamethasone phosphate (DMPS), cortisone (CS), flurandrenolide (FDL), desonide (DSN), budesonide (BUD), betamethasone valerate (BMV), flumethasone pivalate (FMP), alclometasone dipropionate (ACD), mometasone furoate (MMF), clocortolone pivalate (CLP).

For identification purpose, an overall mixed standard preparation is prepared by mixing 1ml of each previous mixed standard solution (A, B, C and D) into a 5 ml volumetric flask. 100 µl of the ISTD stock solution (3.7.2.2) is added to this preparation. Fill up to the mark with methanol, and inject the preparation to identify the corticosteroids that may be present in sample analysed. The overall mixed standard solution has a 10 µg/mL concentration of each corticosteroids and 20 µg/mL of the ISTD.

Alternatively a single mix preparation of all standard can be prepared directly by mixing 100 µl of each stock solution (3.7.2.2) into a 10 ml volumetric flask. Add 200 µl of the ISTD stock solution (3.7.2.2). Fill up to the mark with methanol, and inject the preparation to identify the corticosteroid that may be present in the sample analysed.

4 Apparatus and equipment

In addition to the usual laboratory equipment, the following is required.

4.1 Analytical balance, with a precision of 0,1 mg.

4.2 Laboratory shaker.

4.3 Water-bath, allowing to heat at 60 °C.

4.4 High Performance Liquid Chromatography, consisting of:

— sampling device;

— pump system with gradient function;

— degasser;

— column oven;

— photodiode array detector;

— evaluation system.

4.5 Membrane filter, for sample filtration e. g. polypropylene, 0,45 µm pore size.

4.6 Specific apparatus and equipment for identification and quantification methods, see 2.1.

4.6.1 Analytical separation column, e.g. Thermo Fisher Scientific Hypurity Aquastar C18[1)] length = 0,25 m, internal diameter = 4,6 mm, diameter particle size = 5 µm.

1) This is an example of a suitable product available commercially. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of this product. Equivalent products may be used if they can be shown to lead to the same results.