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Cosmetics — Analytical methods — Nitrosamines: Detection and determination of N-nitrosodiethanolamine (NDELA) in cosmetics by HPLC-MS-MS

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National foreword

This British Standard is the UK implementation of ISO 15819:2014. It supersedes BS ISO 15819:2008 which is withdrawn.

The UK participation in its preparation was entrusted to Technical Committee CW/217, Cosmetics.

A list of organizations represented on this committee can be obtained on request to its secretary.

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ISBN 978 0 580 82029 8

ICS 71.100.70

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This British Standard was published under the authority of the Standards Policy and Strategy Committee on 30 November 2014.

Amendments issued since publication

Date	Text affected
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INTERNATIONAL STANDARD

ISO 15819

Second edition
2014-10-15

Cosmetics — Analytical methods — Nitrosamines: Detection and determination of N-nitrosodiethanolamine (NDELA) in cosmetics by HPLC-MS-MS

*Cosmétiques — Méthodes analytiques — Nitrosamines: Recherche
et dosage de la N-nitrosodiéthanolamine (NDELA) dans les produits
cosmétiques par CLHP-SM-SM*



Reference number
ISO 15819:2014(E)

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Published in Switzerland

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

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For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT) see the following URL: [Foreword - Supplementary information](#)

The committee responsible for this document is ISO/TC 217, *Cosmetics*.

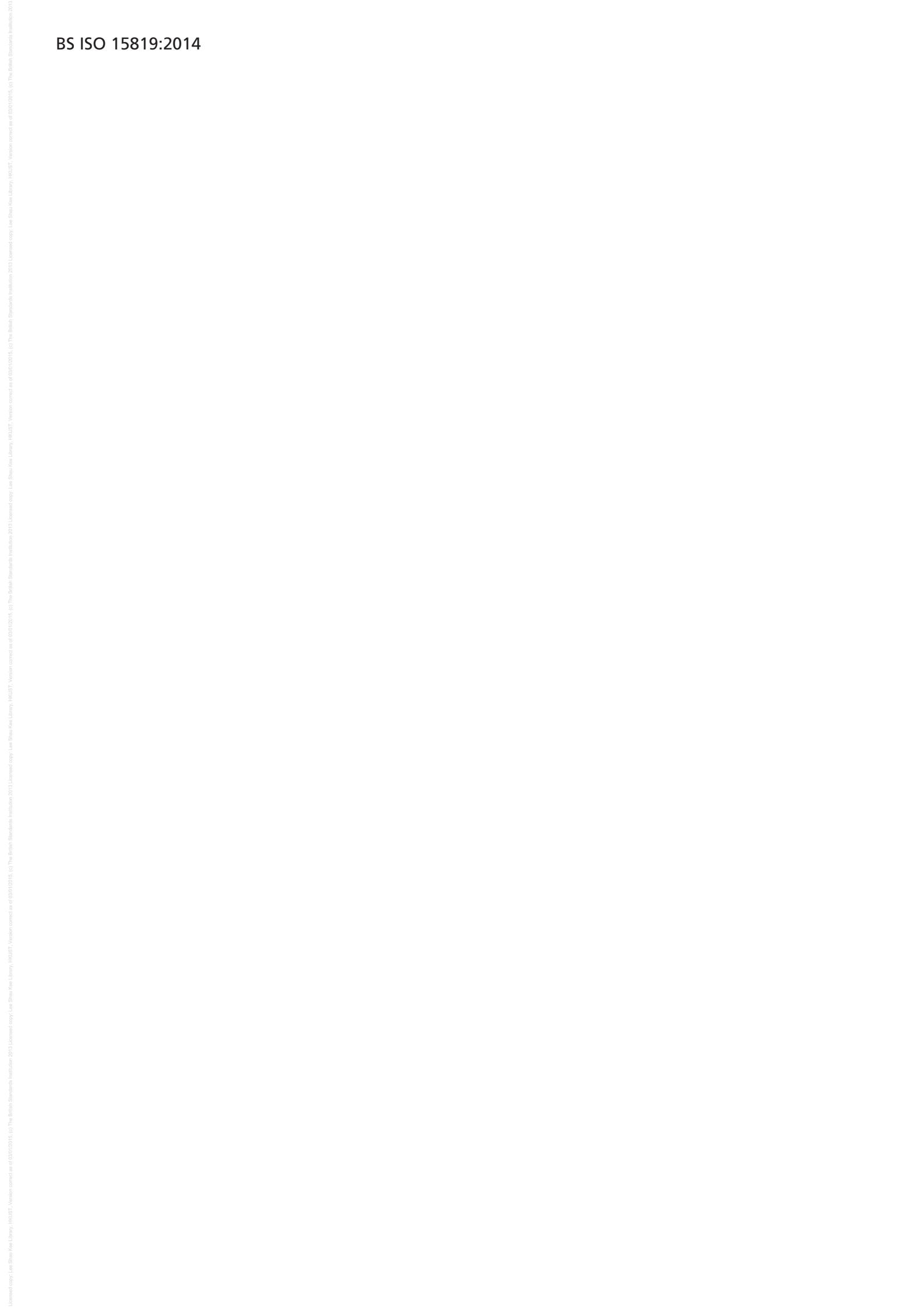
This second edition cancels and replaces the first edition (ISO 15819:2008), which has been technically revised.

Introduction

Human exposure to N-nitrosamines can occur through diverse sources such as environment, food, or personal care products. As a result of their perceived carcinogenic potential on several animal species, minimization of exposure to N-nitrosamines is recognized as important to the preservation of human health. Among N-nitrosamines, N-nitrosodiethanolamine (NDELA) has been recognized as a potential contaminant of cosmetics.

In this context, several analytical methods have been developed to detect and determine its presence in cosmetics, such as gas chromatography/thermal energy analysis, high performance liquid chromatography (HPLC) coupled either with photolysis and colourimetric quantification or with mass spectrometry (MS) determination. This latter method uses advanced technology to ensure the maximum specificity towards NDELA, to minimize the risk of artefactual formation of the analyte of interest and to allow precise quantification.

This analytical method uses high performance liquid chromatography coupled with mass spectrometry to separate and detect trace levels of NDELA from a cosmetic ingredient or product matrix with maximum specificity for NDELA.



Cosmetics — Analytical methods — Nitrosamines: Detection and determination of N-nitrosodiethanolamine (NDELA) in cosmetics by HPLC-MS-MS

1 Scope

This International Standard describes a method for the detection and quantification of N-nitrosodiethanolamine (NDELA) in cosmetics and raw materials used in cosmetics.

This method is not applicable to the detection and/or quantification of nitrosamines other than NDELA nor to the detection and/or quantification of NDELA in products other than cosmetics or raw materials used in cosmetics.

If a product has a possibility of either NDELA contamination from ingredients or NDELA formation by the composition of ingredients, the method is intended to be applied for quantitative determination of NDELA. Accordingly, the method does not apply to routine testing of cosmetic products. Because of the large variety of cosmetic products within this field of application, this method might need to be adapted for certain matrices (refer to ISO 12787).

Therefore, International Standards dedicated to alternative methods for testing nitrosamines in cosmetic products are being developed separately. Other methods can be employed provided that they are verified as to their detection of NDELA and validated in terms of recovery and quantification of the analyte.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 3696:1987, *Water for analytical laboratory use — Specification and test methods*

ISO 12787:2011, *Cosmetics — Analytical methods — Validation criteria for analytical results using chromatographic techniques*

3 Principle

Extraction of NDELA in cosmetics samples is carried out with water in the presence of deuterated d8-NDELA used as internal standard (IS). Clean-up is performed either using solid phase extraction (SPE clean-up, see 6.3.1) with a C18 cartridge or liquid-liquid extraction using dichloromethane (DCM clean-up, see 6.3.2) when the samples are not dispersible in water. The extracts are analysed by HPLC-MS-MS (high performance liquid chromatography coupled with tandem mass spectrometric detection).

Identification of NDELA is carried out by using the molecular ion and two diagnostic ions. NDELA quantification is done by comparing the ratio of the major fragment ions of NDELA and d8-NDELA with the calibration curve.

In accordance with the ISO 12787, the absence of NDELA in the sample could be confirmed with a second analysis. A spiked preparation at a target value could be performed to evaluate the limit of detection of NDELA in the sample.

If matrix effect is observed with significant impact on the performance of the method (sensitivity, accuracy, etc.) for specific cosmetic product, standard addition calibration procedures could be utilized (refer to ISO 12787).

4 Reagents

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and only distilled water or water of grade 1 in accordance with ISO 3696:1987. Solvents shall be of a suitable quality for HPLC-MS.

- 4.1 **Methanol (MeOH)**, HPLC-MS grade.
- 4.2 **Ethanol (EtOH)**, HPLC-MS grade.
- 4.3 **Dichloromethane (DCM)**, HPLC-MS grade.
- 4.4 **N-nitrosodiethanolamine**, with known purity greater than 95 %.
- 4.5 **d8-N-nitrosodiethanolamine**, with known purity greater than 95 %.
- 4.6 **Ammonium acetate (NH₄Ac)**, analytical grade (suitable for HPLC-MS).
- 4.7 **1 mol/l ammonium acetate solution**, for the preparation of 1,0 l, dissolve 77,08 g of NH₄Ac in 1,0 l of water.
- 4.8 **Eluent A: 2 mmol NH₄Ac in water**, for the preparation of 1,0 l, by taking 2 ml of 1 mol/l NH₄Ac (4.7) and making up to 1 l with water.
- 4.9 **Eluent B: 2 mmol NH₄Ac in 90 / 10 MeOH / water v/v**, formed, for the preparation of 1,0 l, by taking 2 ml of 1 mol/l NH₄Ac (4.7) and making up to 1 l with mixture of 90/10 MeOH / water v/v.

5 Apparatus

Use standard laboratory glassware and equipment, with the addition of:

- 5.1 **Vortex mixer.**
- 5.2 **Sample processing station**, in SPE application [such as Vacmaster^{®1}] sample processing station.
- 5.3 **High speed centrifuge (ideally 20 000 G).**

NOTE If centrifuge speed below 20 000 G is used, pay attention to possible clogging problem during the SPE clean-up process. If necessary, additional filtration step with 0,2 µm pore size membrane filter could be added.

- 5.4 **Solid phase extraction columns**, e.g. Bakerbond^{®1}) C18 — 6 ml, 500 mg reversed phase octadecylsilane bonded to silica gel, 40 APD (Average Particle Diameter), 60 Å.

5.5 HPLC-MS-MS equipment.

5.5.1 High performance liquid chromatography apparatus, consisting of an eluent reservoir, a pump, an injection system, a data processor, e.g. an integrator with plotter, coupled with tandem mass spectrometry using electrospray ionization.

1) Vacmaster[®] and Bakerbond[®] are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

5.5.2 Analytical reversed phase HPLC separating column, C18, e.g. Spherisorb^{®2)} ODS II protected with a guard column, the dimensions of which are:

- separating column
 - length: 100 mm;
 - internal diameter: 2,1 mm;
 - size of spherical particles: 5 µm;
- guard column
 - length: 10 mm;
 - internal diameter: 2,1 – 3,0 mm;
 - size of spherical particles: 5 µm.

The use of a guard column is optional. If used, its ID is preferably same as that of separating column. The condition should be adjusted based on the brand that is used for analytical reversed phase HPLC separating column.

6 Sample preparation and conservation

6.1 General

WARNING — Most N-nitrosamines are potent carcinogens and every possible precaution shall be taken to avoid human exposure.

All operations involving handling of N-nitrosamines or their solutions should take place in an adequately ventilated fume hood or glove box.

Rubber surgical gloves, which are frequently employed, do not provide complete protection. They should be removed and disposed of immediately after use and not worn for long periods.

Use safe disposal to discard any solution of material containing N-nitrosamines (such as, for example, tins or buckets for hazardous chemical waste).

N-Nitrosodiethanolamine shall be stored in the absence of light between 2 °C and 8 °C.

UV degrades N-nitrosamines, so all solutions (standards/extracts) shall be stored in such a way that deterioration and change in composition are prevented.

HPLC-MS-MS analysis should typically be carried out within 30 min of preparation of the sample extracts. If analysis is delayed, then stability should be verified.

6.2 Standard solutions preparation

6.2.1 Accurately prepare a stock solution (A) of NDELA containing approximately 1,0 mg/ml in ethanol and store in the absence of light at less than –18 °C. Record the exact concentration.

6.2.2 Accurately prepare a stock solution (d8A) of d8-NDELA containing approximately 1,0 mg/ml in ethanol and store in the absence of light at less than –18 °C. Record the exact concentration.

6.2.3 Prepare working solutions (B, C, D, E, and F) by successive dilutions of the stock solution (A). All solutions shall be stored in the absence of light between 2 °C and 8 °C.

2) Spherisorb[®] is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

Working solutions	Stock or working solution volume	Water volume	Final concentration	Stability
Working solution B	100 µl of A	900 µl	100,0 µg/ml	1 d
Working solution C	100 µl of B	900 µl	10,0 µg/ml	1 d
Working solution D	100 µl of C	900 µl	1,0 µg/ml	1 d
Working solution E	100 µl of D	900 µl	100,0 ng/ml	1 d
Working solution F	100 µl of E	900 µl	10,0 ng/ml	1 d

NOTE Users can determine actual volume used for the preparation as far as final concentration is secured.

6.2.4 Prepare d8 working solutions (d8B and d8C) by sequential dilutions of the stock solution (d8A). All solutions shall be stored in the absence of light between 2 °C and 8 °C.

d8 working solutions	Stock or working solution volume	Water volume	Final concentration	Stability
Working solution d8B	20 µl of d8A	20 ml	1,0 µg/ml	1 d
Working solution d8C	200 µl of d8B	1 800 µl	100,0 ng/ml	1 d

NOTE Users can determine actual volume used for the preparation as far as final concentration is secured.

6.2.5 Prepare standard solutions by dilutions of the working solutions. A standard curve from 1,0 ng/ml to 80,0 ng/ml is made, using at least five of the seven standard solutions given in the table below. A standard solution corresponding to the required limit of quantitation (or lower) should be included. The internal standard d8-NDELA was at 20 ng/ml in each solution. All solutions shall be stored in the absence of light between 2 °C and 8 °C.

Standard solutions	Working solution volume	Working solution d8C	Water volume	Final concentration		Stability
				NDELA	d8-NDELA	
Standard solution 1	800 µl of E	200 µl	-	80,0 ng/ml	20 ng/ml	1 d
Standard solution 2	400 µl of E	200 µl	400 µl	40,0 ng/ml	20 ng/ml	1 d
Standard solution 3	200 µl of E	200 µl	600 µl	20,0 ng/ml	20 ng/ml	1 d
Standard solution 4	100 µl of E	200 µl	700 µl	10,0 ng/ml	20 ng/ml	1 d
Standard solution 5	500 µl of F	200 µl	300 µl	5,0 ng/ml	20 ng/ml	1 d
Standard solution 6	250 µl of F	200 µl	550 µl	2,5 ng/ml	20 ng/ml	1 d
Standard solution 7	100 µl of F	200 µl	700 µl	1,0 ng/ml	20 ng/ml	1 d

NOTE 1 Depending on the instrument sensitivity, the standard calibration curve and the sample preparation could be adapted in order to minimize possible matrix effect (see [6.3.3](#)).

NOTE 2 Users can determine actual volume used for the preparation of as far as final concentration is secured.

6.3 Sample preparation

6.3.1 SPE clean-up

Weigh accurately approximately 1,0 g of the sample (note the exact mass), add 400 µl of d8B working solution and adjust to 20,0 ml with water. Shake for 15 min. If necessary, use additionally, with appropriate

care, a sonication bath and/or centrifuge for 10 min. In order to avoid further nitrosamine generation, sonication should be minimized.

NOTE Provided HPLC-MS-MS sensitivity allows, a smaller sample mass and/or larger initial water volume can be utilized to adapt to certain matrices and to reduce the potential for ionization suppression issues due to coeluting matrix components. When changing the water volume, make the IS concentration consistent both in standard solution and in sample solution. Unless otherwise correct calculation cannot be performed.

Condition the solid phase extraction C18 column with 3,0 ml of methanol followed by 3,0 ml of water with a flow-rate of approximately 3,0 ml/min. Do not allow the column to dry out.

Load about 5 ml of the sample extract preparation to the above solid phase extraction C18 column and discard the first about 3 ml of solution. Collect the following about 2 ml (with a flow-rate of approximately 3,0 ml/min) into a vial.

If needed, prepare the sample in triplicate.

6.3.2 Alternative sample preparation for samples non-dispersible in water (DCM clean-up)

Weigh accurately approximately 0,2 g of the sample (note the exact mass) into a centrifuge tube, add 800 µl of d8C working solution and shake for 1 min. Add 4 ml of dichloromethane and shake for 1 min. Add 3,2 ml of water and shake for 5 min.

NOTE Provided HPLC-MS-MS sensitivity allows, a smaller sample mass and/or larger initial water volume can be utilized to adapt to certain matrices and to reduce the potential for ionization suppression issues due to coeluting matrix components. When changing the water volume, make the IS concentration consistent both in standard solution and in sample solution. Unless otherwise correct calculation cannot be performed.

Centrifuge at the highest possible speed (ideally 20 000 G) for 5 min. A portion of the upper aqueous layer is used for chromatographic analysis.

If necessary, filter the collected solution through an appropriate filter.

If needed, prepare the sample in triplicate.

6.3.3 Further dilution of prepared sample and standard solutions (Optional)

Additional dilution of sample extracts and (correspondingly) standard solutions is advised, to minimize the potential impact of coeluting matrix components that might suppress NDELA ionization, limiting method sensitivity. Dilution of sample extracts and standard solutions in water is suggested, up to the sensitivity capabilities on the specific HPLC-MS-MS system employed.

6.3.4 Sample storage

If necessary, sample could be stored below 8 °C in absence of light.

7 Procedure

7.1 General

With both types of sample preparation, the final extract is analysed for NDELA by HPLC-MS-MS.

7.2 Chromatographic conditions

Mobile phase: Eluent A — 2 mmol NH₄Ac in water (4.8)

Eluent B — 2 mmol NH₄Ac in 90 / 10 MeOH / water (4.9)

Gradient:

Interval min	% A	% B
0 – 1,5	97	3
1,5 – 1,6	97→0	3→100
1,6 – 4,0	0	100
4,0 – 4,1	0→97	100→3
4,1 – 6,0	97	3

Flow: 0,4 ml/min
 Injection volume: 20 µl
 Temperature column oven: 30 °C
 Sample storage: ≤8 °C in absence of light (recommended)
 Run injection: Every run consists of at least five calibration points, and at least one control sample (a standard solution) per each 15 sample extracts (for a run of less than 15 sample extracts, a minimum of one control sample is included). The allowable run size will include at least up to 20 sample extracts. Larger run sizes might be possible, provided sufficient on-tray stability is verified.

Chromatographic conditions might need to be adapted for certain matrices provided adequate verification (see ISO 12787).

7.3 HPLC-MS-MS conditions

Electrospray, positive mode.

Example of suitable parameters for the triple quadrupole mass spectrometer [such as AB Sciex 5500^{®3}].

Source settings

— Ion spray voltage (ISV)	2 800 V
— Declustering potential (DP)	26 V
— Source temperature (TEM)	650 °C
— Nebulizer gas (GS1)	70 units (nitrogen)
— Turbo gas (GS2)	70 units (nitrogen)
— Curtain gas (CUR)	30 units (nitrogen)

Analyser settings

— Scan mode	Multiple reaction monitoring (MRM) [two selected reaction monitoring (SRM) for NDELA and one SRM for IS]
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3) AB Sciex 5500 Triple Quad[®] is an example of a suitable product available commercially and suitable parameters might depend on the HPLC-MS-MS system. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

- Q1 resolution unit
- Q3 resolution unit
- Collision activated dissociation (CAD) gas 12 units (nitrogen)

Characteristic ions 135 (M+H), 104, and 74 (fragment ions) identify the presence of NDELA in the sample.

NDELA quantification is made by using the ratio of the intensity of the two major fragment ions 104 (from NDELA) and 111 [originating from the 143 (M+H) ion of d8-NDELA].

8 Calculation of results

8.1 Determination of the *R* value

For each injection (calibration point, sample extract, and control sample) an *R* value is determined, being defined as the intensity ratio between the intensity from ion 104 resulting from the fragmentation of the NDELA (M+H) ion and ion 111 resulting from the fragmentation of the d8-NDELA (M+H) ion (see, for example, [Figure A.2](#)).

These *R* values are used for the determination of the calibration curve and of concentration, *c*, as indicated in [8.4](#).

8.2 Calibration curve

Prepare a calibration curve by plotting the concentration of NDELA in standard solutions (see [6.2.5](#)) against their *R* value.

For quantification the calibration curve shall have a correlation coefficient better than or equal to 0,990 (see, for example, [Figure A.1](#)).

8.3 Validity check procedure and its criteria

The relative intensities of the two detected ions (104 and 74) from sample preparations, expressed as a percentage of the intensity of the most intense ion, shall correspond to those of the calibration standard solutions, at comparable concentrations, measured under the same conditions, within the tolerances listed in [Table 1](#).

Validity is checked for each sample preparation measurement which gives an NDELA concentration above the limit of detection (see Reference [\[1\]](#)).

Table 1 — Maximum permitted tolerances for relative ion intensities

Relative intensity (% of base ion intensity)	Relative range of the response
>50 %	±20 %
> 20 % to 50 %	±25 %
> 10 % to 20 %	±30 %
≤10 %	±50 %

8.4 Calculation of concentrations

Determine the concentrations, *c*, of the extracted samples in ng/ml by using their *R* values to read them directly from the calibration curve.

NDELA concentrations of unknown samples are calculated using Formula (1):

$$W = c \times V/m \quad (1)$$

where

- W* is the mass fraction of NDELA in the cosmetic sample, in nanograms per gram;
- c* is the concentration of NDELA determined in the extracts, in nanograms per millilitre;
- m* is the mass of cosmetic sample, in grams;
- V* is the volume of dilution of cosmetic product, in millilitres (ex: 20 ml for SPE clean-up, 4 ml for DCM clean-up).

NOTE The values of *V* would be adjusted in the event different volumes are used (as allowed, per note in [6.3.1](#) and [6.3.2](#)).

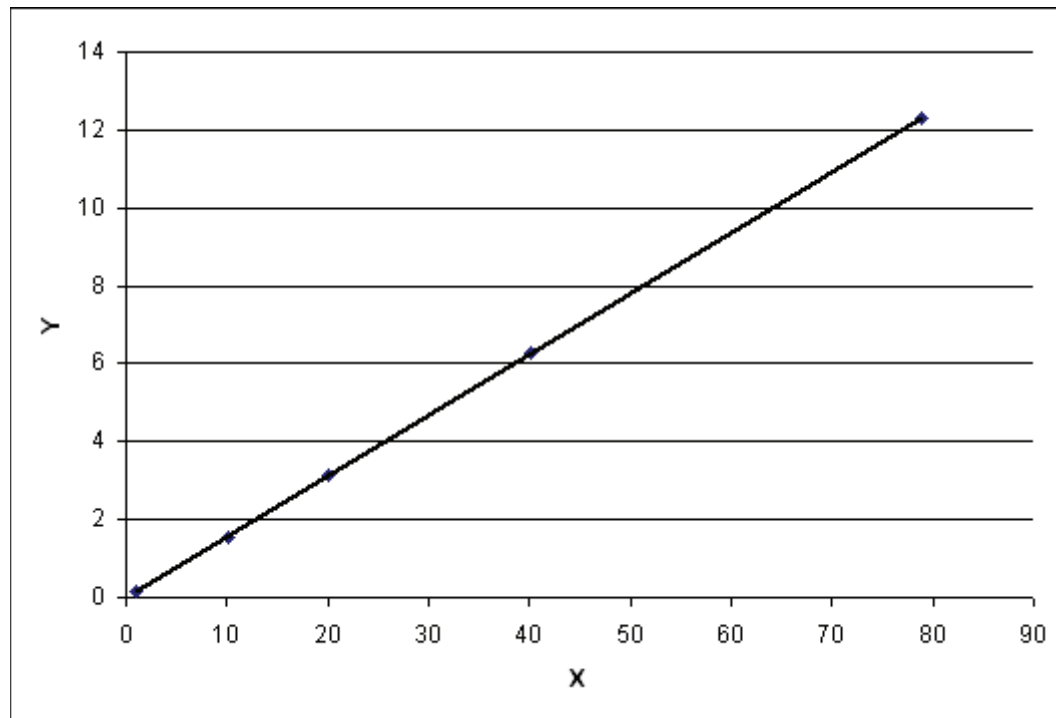
9 Test report

The test report shall contain at least the following information;

- a) all information necessary for the identification of the sample;
- b) all information necessary for the identification of the laboratory that performed the test;
- c) a reference to this International Standard (i.e. ISO 15819);
- d) date and type of sampling (if known);
- e) date of receipt of the laboratory sample;
- f) date of test;
- g) the results and the units in which the results have been expressed;
- h) the method of clean-up used;
- i) any particular points observed in the course of the test;
- j) any operations not specified in the method or regarded as optional which might have affected the results;
- k) the identification and signature of the person responsible for the report.

Annex A (informative)

Examples of a calibration curve and of chromatograms



Key

X concentration of NDELA in standard solutions (ng/ml)

Y R-value

Figure A.1 — Example of a calibration curve for NDELA

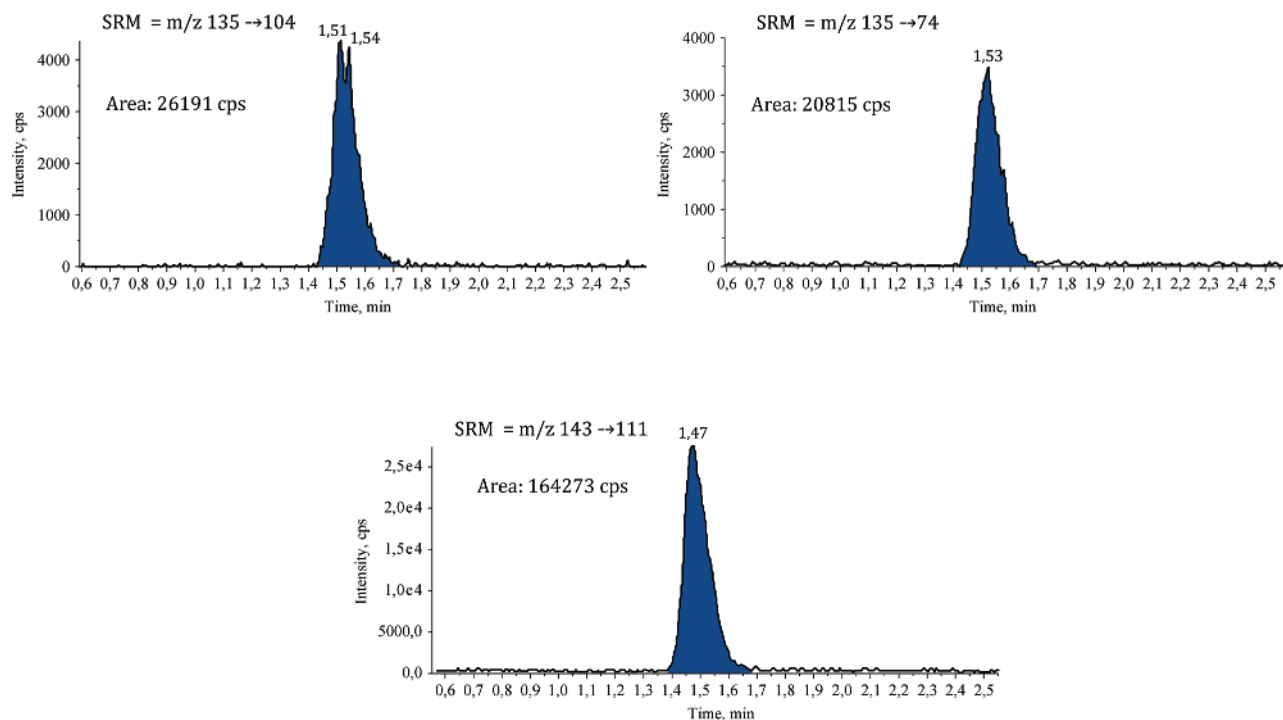


Figure A.2 — Examples HPLC-MS-MS chromatograms

From analysis of 1,0 ng/ml calibration standard solution, corresponding to 20 ng/g NDELA-equivalent in matrix. Data acquired using AB Sciex 5500 Triple Quad, with additional 4x dilution of the sample extract in water, prior to analysis. Top-left: NDELA (quantitation SRM); top-right: NDELA (secondary SRM); bottom: d8-NDELA (internal standard SRM).

Bibliography

- [1] 2002/657/EC: Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results [notified under document number C (2002) 3044]

BS ISO 15819:2014
ISO 15819:2014(E)

ICS 71.100.70

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