

**Cosmetics —
Analytical methods
— Nitrosamines:
Detection and
determination of *N*-
nitrosodiethanolamine
(NDELA) in cosmetics
by HPLC, post-column
photolysis and
derivatization**

ICS 71.100.70

National foreword

This British Standard is the UK implementation of ISO 10130:2009.

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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This British Standard was published under the authority of the Standards Policy and Strategy Committee on 30 June 2010

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ISBN 978 0 580 61108 7

Amendments/corrigenda issued since publication

Date	Comments

INTERNATIONAL STANDARD

ISO
10130

First edition
2009-12-15

Cosmetics — Analytical methods — Nitrosamines: Detection and determination of *N*-nitrosodiethanolamine (NDELA) in cosmetics by HPLC, post- column photolysis and derivatization

Cosmétiques — Méthodes analytiques — Nitrosamines: Recherche et dosage de la N-nitrosodiéthanolamine (NDELA) dans les cosmétiques par CLHP, photolyse et dérivation post-colonne



Reference number
ISO 10130:2009(E)

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Published in Switzerland

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Foreword

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The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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ISO 10130 was prepared by Technical Committee ISO/TC 217, *Cosmetics*.

Introduction

Human exposure to *N*-nitrosamines can occur through diverse sources such as the 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 the presence of NDELA in cosmetics. Examples of these methods are gas chromatography/thermal energy analysis, and high performance liquid chromatography coupled either with a mass spectrometry determination or with photolysis and colorimetric quantification. The latter method uses specific technology to ensure 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 (HPLC) coupled with post-column photolysis and derivatization, in order to separate and detect trace levels of NDELA from a cosmetic ingredient or product matrix with specificity for NDELA.

This International Standard refers to a collaborative study (Reference [2]) involving seven laboratories and published in 2006. Validation criteria are given in Reference [2].

Cosmetics — Analytical methods — Nitrosamines: Detection and determination of *N*-nitrosodiethanolamine (NDELA) in cosmetics by HPLC, post-column photolysis and derivatization

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 by high performance liquid chromatography (HPLC) coupled with post-column photolysis and derivatization.

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 the possibility of either NDELA contamination from the ingredients or NDELA formation by the composition of ingredients, the method will be applied for the testing of cosmetic products and is an alternative to ISO 15819.

This method is not applicable to matrices containing oxidation dyes.

2 Principle

Extraction of the nitrosamine NDELA from cosmetic samples is carried out with water. Clean-up is performed either using solid phase extraction (SPE clean-up, see 5.3.2) with a C18 cartridge or dichloromethane (DCM clean-up, see 5.3.3) when the samples are not dispersible in water. The extracts are analysed by HPLC, post-column photolysis and derivatization. NDELA is separated from the cosmetic matrix using reversed-phase liquid chromatography. The *N*-nitroso bond is cleaved by UV photolysis with the formation of nitrite ion. According to the Griess reaction, the nitrite functional group is diazotized with sulfanilamide in an acid medium and is then coupled with *N*-(1-naphthyl)ethylenediamine dihydrochloride (NED) to form a purple-coloured azo dye that is quantitatively determined spectrophotometrically at a maximum wavelength, λ_{\max} , of 540 nm (see Annex B).

The presence of NDELA can be confirmed by repeating the analysis without photolysis (no nitrite ion is then produced because the *N*-nitroso bond is not cleaved). The absence of a chromatographic peak at the retention time of NDELA in the chromatogram confirms that the peak observed in the first analysis corresponds to NDELA.

3 Reagents

3.1 Methanol, HPLC grade.

3.2 Water, HPLC grade.

3.3 Dichloromethane, HPLC grade.

3.4 *N*-Nitrosodiethanolamine, with known purity greater than 95 %. CAS No. [116-57-7].

3.5 Orthophosphoric acid, 85 %, analytical grade.

3.6 Ammonium acetate, analytical grade.

3.7 Ammonium acetate solution, 1 mol/l.

Dissolve 77,08 g of ammonium acetate (3.6) in up to 1,0 l of water (3.2).

3.8 Ammonium acetate solution, 0,02 mol/l.

Take 20 ml of 1 mol/l ammonium acetate solution (3.7) and dilute to 1 l with water (3.2).

3.9 *N*-(1-Naphthyl) ethylenediamine dihydrochloride, with known purity greater than 98 %. CAS No. [1465-25-4].

This reagent should be sealed and stored in the absence of light.

3.10 Sulfanilamide, with known purity greater than 99 %. CAS No. [63-74-1].

3.11 Griess reagent.

Dissolve 0,25 g *N*-(1-naphthyl) ethylenediamine dihydrochloride (3.9) in water (3.2) and dilute to 250 ml in a volumetric flask. Dissolve 4,0 g of sulfanilamide (3.10) in 250 ml of a 5 % (mass/volume) aqueous solution of 85 % orthophosphoric acid (3.5). Mix the reagents together in an amber glass bottle and keep the mixture away from the light.

The mixture can be used for five days and shall, in any case, remain colourless when it is stored between 2 °C and 8 °C.

4 Apparatus

Use standard laboratory glassware and equipment, and the following.

4.1 Mechanical shaker or **Vortex® mixer** ¹⁾.

4.2 High performance glass vial, 20 ml with Poly-Cone® lined urea screw caps.

4.3 Microtube 1,5 ml, for centrifugation in polypropylene.

4.4 Low dead-volume mixing tee.

4.5 Sample processing station, in SPE application (such as Vacmaster® or Visiprep®¹⁾) sample processing station.

4.6 Centrifuge, capable of reaching not less than 20 000g with a temperature control.

4.7 Solid-phase extraction columns, e.g. Sep Pak®¹⁾ C18 cartridges Waters or Bakerbond®¹⁾ C18-6 ml, 500 mg reversed phase octadecylsilane bonded to silica gel 40 µm average particle diameter (APD), 60 Å.

4.8 High performance liquid chromatograph, equipped with a pulse damper, eluent reservoir, a pump, an injection system, data processing, e.g. an integrator, coupled with a UV/visible detector equipped with a tungsten lamp, of sensitivity 0,001 AUFS or 0,0005 AUFS (AUFS: absorbance units full scan).

¹⁾ Poly-Cone®, Vortex® mixer, Vacmaster®, Visiprep®, Waters or Bakerbond®, Sep Pak®, Spherisorb® and Beam Boost® are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of these products.

4.9 Griess reagent delivery pump: low-pressure pump without pulsation, or a second liquid-chromatography (LC) pump with the same characteristics as the first may be used.

4.10 Photolysis unit: photochemical reactor comprising a “high-pressure” knitted PTFE reactor coil, of 5 m or 6 m × 0,3 mm internal diameter, placed around a low-pressure UV lamp emitting at 254 nm (e.g. Beam Boost®¹⁾ Photoreactors).

4.11 Post-column module or a column oven, equipped with a 1 ml knitted PTFE reactor coil and a low dead-volume mixing tee. The coil is convoluted in a defined manner in order to prevent band broadening (Reference [3]).

4.12 Analytical reversed-phase HPLC separating column, C18, e.g. Spherisorb®¹⁾ octadecylsilane (ODS) II protected with a guard column.

Separating column

- length: 150 mm
- internal diameter: 4,6 mm
- size of spherical particles: 5 µm

Guard column

- length: 10 mm
- internal diameter: 4,6 mm
- size of spherical particles: 5 µm

4.13 Configuration of the post-column reactor system (see Annex C).

After the injection valve, the connecting tubes shall be as short as possible.

The column is connected directly to an injection system equipped with a 50 µl or 100 µl sample loop. A photochemical reactor (1) fitted with a knitted-open tubular reactor coil and a 254 nm ultraviolet lamp is connected to the column exit. The photochemical reactor exit is connected to one branch of a mixing tee (2). The second branch of the tee is connected to the pump with the derivatization reagent (3). The third branch is connected to the tubular reactor which consists of a knitted PTFE tube under a post-column module (4). The exit of the tubular reactor is connected to the UV/visible detector (5). The eluted peaks are monitored using a microcomputer equipped with a commercial integration software package.

5 Sample preparation and storage

5.1 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 shall take place in an adequately ventilated fume hood or glove box.

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

Thought should be given to safe disposal of any solution of material containing *N*-nitrosamines.

Ultraviolet light (UV) degrades *N*-nitrosamines, so *N*-nitrosodiethanolamine and all solutions (standards/extracts) shall be stored in the absence of light between 2 °C and 8 °C.

Injection for analysis shall be made within 24 h after preparation of the extract sample.

5.2 Standards preparation

5.2.1 Primary stock solution

Prepare a primary stock standard solution (S_0) of NDELA containing 1 mg/ml in water. This solution can be stored for a maximum of six months in the absence of light between 2 °C and 8 °C.

5.2.2 Secondary stock solution

From the primary stock solution (S_0), make a 1/100 dilution in water in order to obtain a 10 µg/ml secondary stock solution (S_1). A second 1/100 dilution of S_1 is performed in order to obtain a 100 ng/ml secondary stock solution (S_2). All of the solutions are stored for a maximum of one week in the absence of light between 2 °C and 8 °C.

5.2.3 Working solutions

Sequentially dilute secondary stock solution (S_2) with water, in order to obtain standards titrating at 1 ng/ml, 2 ng/ml, 5 ng/ml, 10 ng/ml and 20 ng/ml. (See Figure A.1.) These solutions are prepared every day and are kept in the absence of light (see Table 1).

The linearity of the standard calibration curve is checked over a 1 ng/ml to 100 ng/ml range (see Figure A.2).

Typical chromatograms are shown in Figures A.3 and A.4.

Table 1 — Preparation of working solutions

Working solutions	Secondary stock solution (S_2) volume	Final volume	Final concentration	Stability	Conditions
Working solution W_1	100 µl	10 ml	1 ng/ml	1 day	Stored in the absence of light
Working solution W_2	200 µl	10 ml	2 ng/ml	1 day	Stored in the absence of light
Working solution W_3	500 µl	10 ml	5 ng/ml	1 day	Stored in the absence of light
Working solution W_4	1 ml	10 ml	10 ng/ml	1 day	Stored in the absence of light
Working solution W_5	2 ml	10 ml	20 ng/ml	1 day	Stored in the absence of light

5.3 Sample preparation

5.3.1 General

Where a cosmetic product is dispersible (soluble) in water, the solid phase extraction (SPE) clean-up shall apply (see 5.3.2).

Where a cosmetic product is not dispersible in water, the dichloromethane (DCM) clean-up shall apply (see 5.3.3).

To summarize, for a cosmetic product, the criteria of dispersibility of the product shall be estimated in order to use a suitable clean-up for the product. SPE clean-up and DCM clean-up should not be carried out for the same product; the suitable clean-up shall be chosen according to the dispersibility. A product which is not dispersible in water shall be dealt with under the conditions of DCM clean-up.

5.3.2 SPE clean-up

Weigh about 2,0 g of the sample in a glass vial (4.2) and note the exact mass, disperse and adjust to 20 ml with water. Shake for 15 min using a mechanical shaker or for 1 min using a Vortex® mixer. Centrifuge for 10 min (SPE preparation).

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

Load 5 ml of the SPE preparation to the above solid phase extraction C18 cartridge and discard the first 3 ml of solution. Collect the following 2 ml (with a flow rate of approximately 3,0 ml/min) in a vial for chromatographic analysis.

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

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

Weigh about 0,4 g of the sample in a glass vial (4.2) and note the exact mass. Add 4,0 ml of dichloromethane and shake for 1 min. Add exactly 4 ml of water and shake for 5 min.

Put an aliquot part of the upper aqueous phase in a microtube (4.3).

Centrifuge at 20 000g for 10 min.

Inject the upper aqueous layer into the chromatographic system.

6 Procedure

6.1 General

With both types of sample preparation, the final extract is analysed for NDELA by HPLC with UV photolysis and the Griess reaction.

6.2 Chromatographic conditions

LC pump	Pump equipped with a pulse damper
Column type	See 4.12
Guard-column type	See 4.12
Injection volume	50 µl or 100 µl
Mobile phase	0,02 mol/l ammonium acetate solution (3.8)
Mobile-phase flow rate	0,5 ml/min
Griess-reagent flow rate	0,5 ml/min
Temperature of post-column reactor	50 °C

Temperature column

Oven	30 °C
Detection wavelength	540 nm
Photolysis unit	See 4.10
Griess reagent delivery	See 4.9
Post-column reactor	See 4.11
Detector	See 4.8

6.3 Set-up of the reaction system

The reaction system consists of four distinct parts:

- a) the chromatographic system,
- b) the post-column photolysis reactor,
- c) the chemical reactor, and
- d) the detector (see Annex C).

IMPORTANT — The run time should be extended at least 15 min after elution of the NDELA peak in order to allow the elution of potential matrix components.

The total chromatographic and post-column system shall be rinsed with water after use. In the case of non-utilization for longer than two days, this rinse shall be followed by a methanol rinse. Before restarting the system, it is necessary to rinse with water in order to avoid any recrystallization.

7 Calculation of results

7.1 Calibration curve

Prepare a calibration curve by plotting the concentration of NDELA standards (see 5.2.3) versus their peak area.

For the quantification with the calibration curve, the correlation coefficient shall be not less than 0,990.

7.2 Experimental conditions for the validity of the measurement

See Reference [2].

7.3 Calculation of concentrations

The external standard calibration method is used to quantify the concentration of NDELA present in the sample. A least-squares linear regression of peak areas versus concentration of the NDELA reference standard, expressed in nanograms per millilitre (ng/ml), is performed. The linear relationship of the increasing concentrations of the NDELA reference standard (expressed in nanograms per millilitre) versus their corresponding peak area is expressed as:

$$y = ax + b$$

where

- a is the slope of the regression line;
- b is the y -intercept.

From the chromatogram obtained with the sample extract under investigation, S_F is the peak area for NDELA.

The mass fraction, w , expressed in nanograms per gram (ng/g), of NDELA in the cosmetic product under investigation is calculated from the following equations:

$$\rho = (S_F - b)/a$$

$$w = \rho \times V/m$$

where

- ρ is the concentration of NDELA in the sample extract, expressed in nanograms per millilitre (ng/ml);
- S_F is the peak area of NDELA in the sample extract;
- b is the y -intercept of the calibration curve;
- a is the slope of the calibration curve;
- m is the mass of the cosmetic product under investigation, expressed in grams (g);
- V is
 - 20 ml for SPE (solid phase extraction),
 - 4 ml for DCM (dichloromethane);
- w is the mass fraction of NDELA in the cosmetic product under investigation, expressed in nanograms per gram (ng/g).

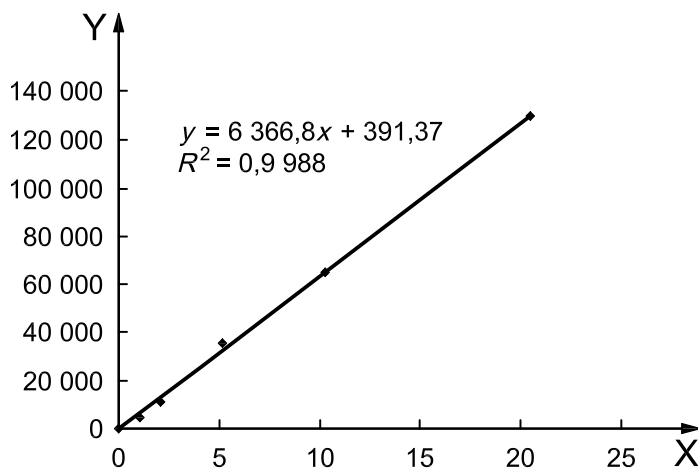
8 Test report

The test report shall contain at least the following information:

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

Annex A (informative)

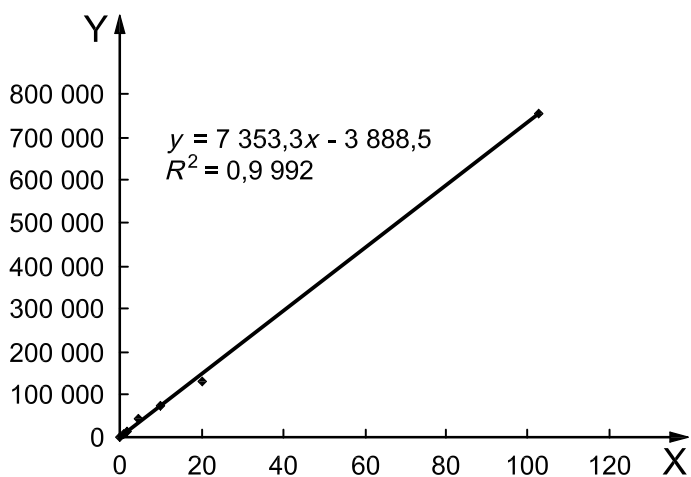
Examples of calibration curves and typical chromatograms



Key

- X NDELA concentration, ng/ml
- Y NDELA area

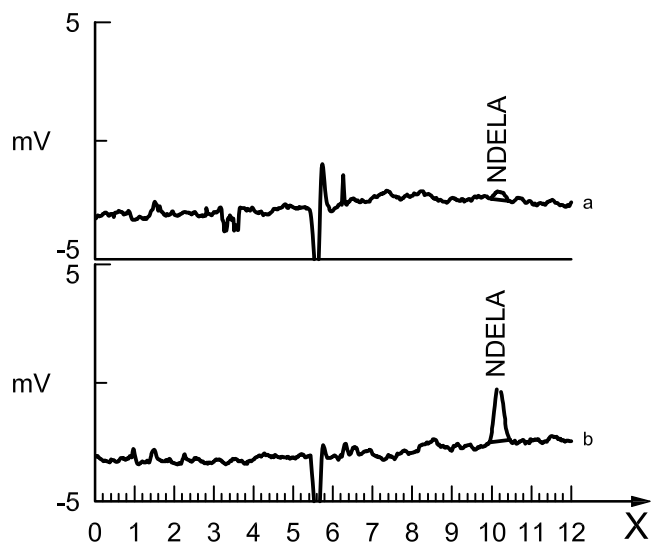
Figure A.1 — Calibration curve showing the linearity of response of NDELA over the concentration range 1 ng/ml to 20 ng/ml



Key

- X NDELA concentration, ng/ml
- Y NDELA area

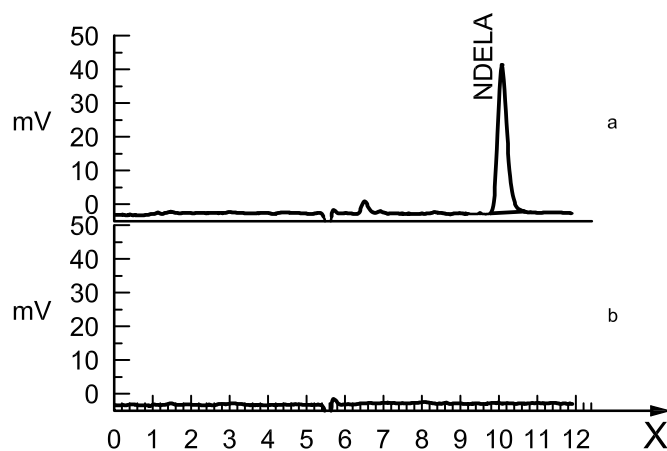
Figure A.2 — Calibration curve showing the linearity of response of NDELA over the concentration range 1 ng/ml to 100 ng/ml



Key

- X coefficient of determination, R^2 , in minutes
- a Chromatogram of a NDELA standard solution at 1 ng/ml.
- b Chromatogram of a NDELA standard solution at 5 ng/ml.

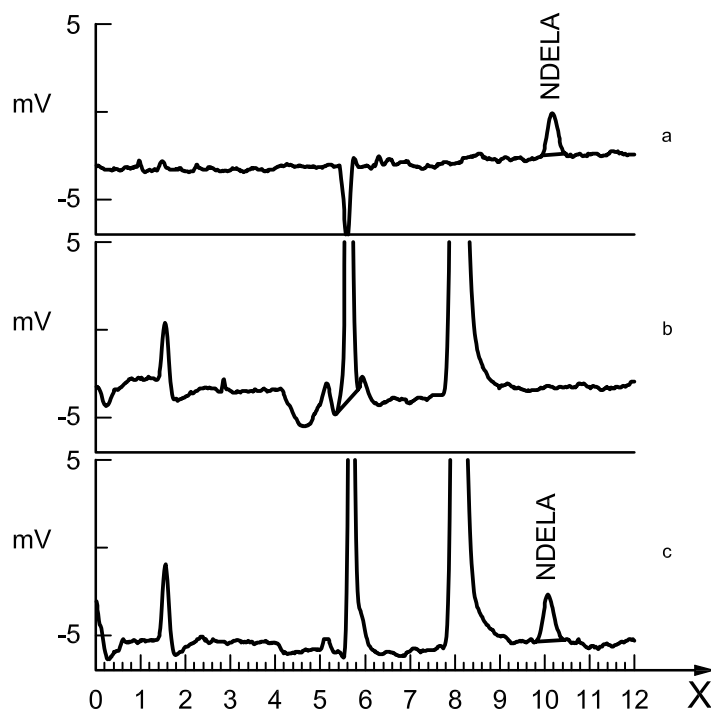
Figure A.3 — Examples of chromatograms of a NDELA standard solution at 1 ng/ml and 5 ng/ml



Key

- X coefficient of determination, R^2 , in minutes
- a Chromatogram of a NDELA standard solution at 100 ng/ml.
- b Chromatogram of a NDELA standard solution at 100 ng/ml without photolysis.

Figure A.4 — Examples of chromatograms of a NDELA standard solution at 100 ng/ml



Key

X coefficient of determination, R^2 , in minutes

- a Chromatogram of a NDELA standard solution at 5 ng/ml.
- b Chromatogram of a cosmetic product (concentration of 2 g/20 ml).
- c Chromatogram of a cosmetic product spiked with NDELA at 50 ng/ml (concentration of 2 g/20 ml).

Figure A.5 — Three examples of typical chromatograms

Annex B (normative)

Photolysis and reaction of nitrite with Griess reagent to form the azo dye

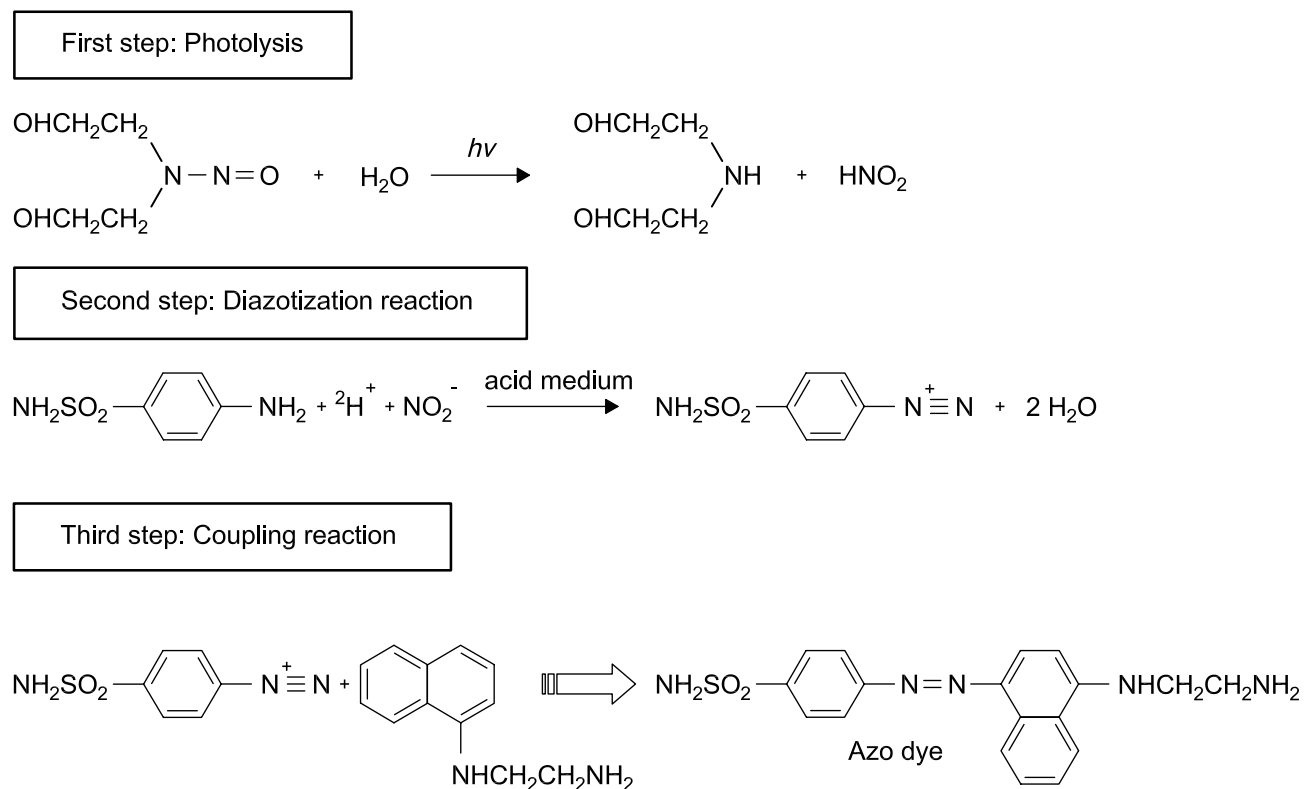
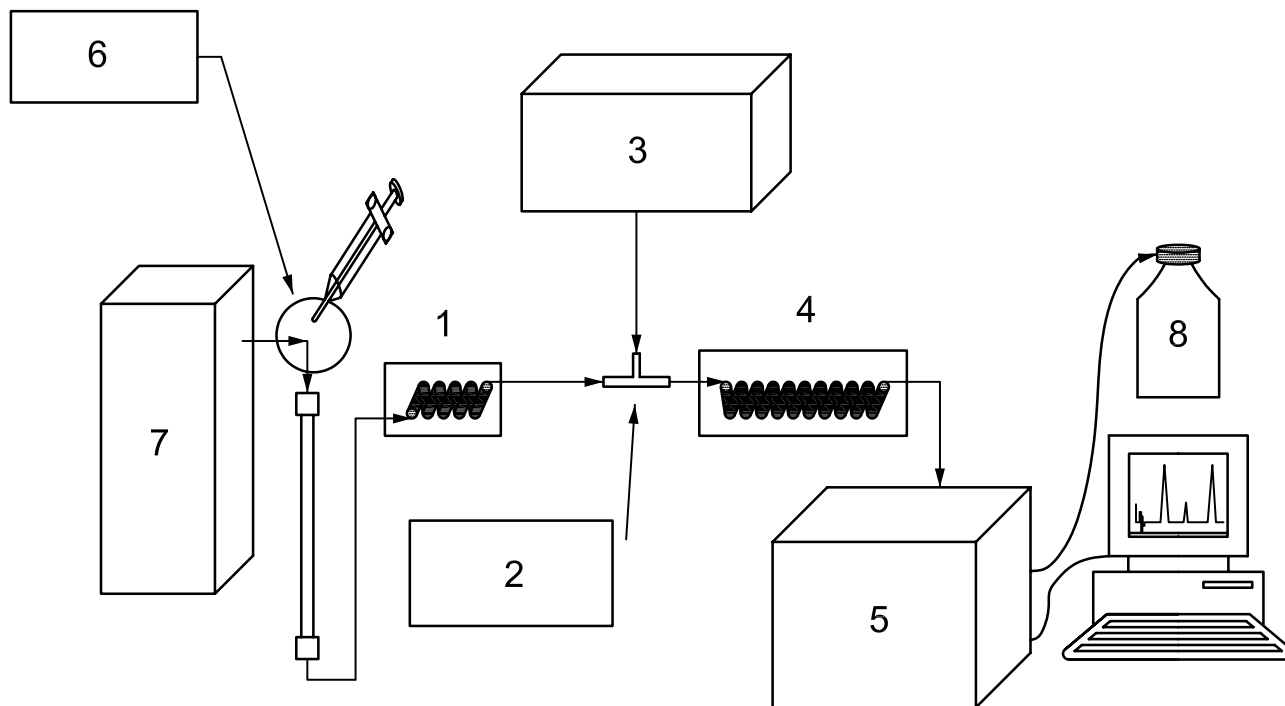


Figure B.1 — Photolysis and reaction

Annex C (normative)

Configuration of the post-column reactor system



Key

- 1 photolysis unit: 254 nm (see 4.10)
- 2 low dead-volume mixing tee (see 4.4)
- 3 Griess-reagent delivery pump (see 4.9)
- 4 post-column reactor module or column oven at 50 °C (see 4.11)
- 5 detector (tungsten lamp): 540 nm (see 4.8)
- 6 sampling valve
- 7 pump (HPLC)
- 8 waste

Figure C.1 — Configuration of the chromatographic and post-column reactor system for the determination of NDELA in cosmetic products

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- [1] ISO 15819, *Cosmetics — Analytical methods — Nitrosamines: Detection and determination of N-nitrosodiethanolamine (NDELA) in cosmetics by HPLC-MS-MS*
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