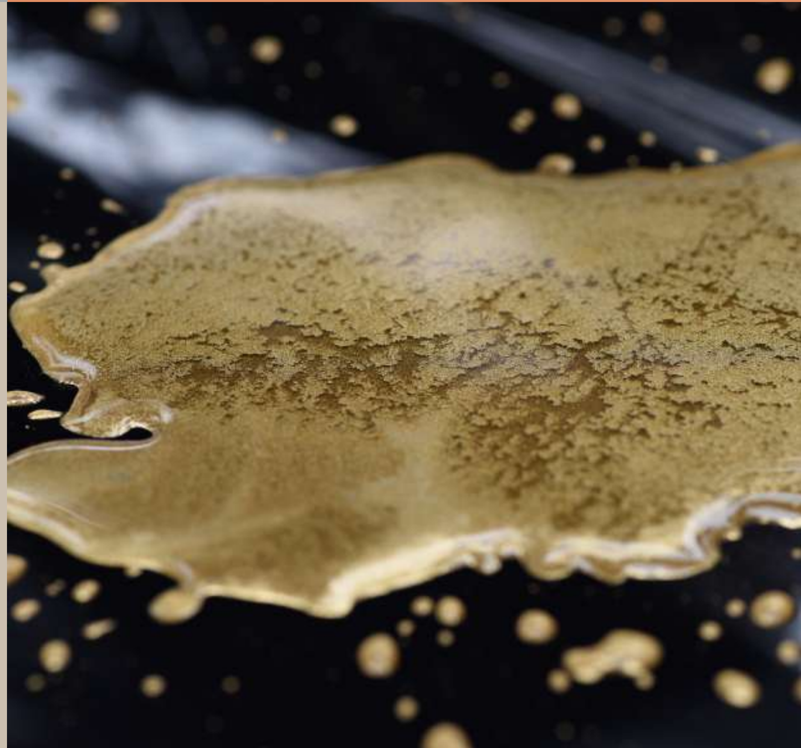
DETERMINATION OF POLAR *N*-NITROSAMINES IN COSMETIC PRODUCTS

using liquid chromatography coupled to mass spectrometry



"Safety is defined and measured more by its absence than its presence."

James Reason

European Network of Official Cosmetics Control Laboratories (OCCLs)

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European Directorate for the Quality of Medicines & HealthCare

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Determination of polar *N*-nitrosamines in cosmetic products using liquid chromatography coupled to mass spectrometry

Users should verify the performance of the method in their laboratory for each different matrix.

1. Scope

This procedure describes a method for the determination of polar *N*-nitrosamines in cosmetic products and tattoo inks at concentrations ranging from 10 to 1 000 µg/kg.

The method is suitable for the determination of *N*-nitrosodiethanolamine (NDELA), *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA), *N*-nitrosomorpholine (NMOR), *N*-nitrosomethylethylamine (NMEA), *N*-nitrosopyrrolidine (NPYR) and *N*-nitrosopiperidine (NPIP) in cosmetics and tattoo inks, in the concentration range 10–1 000 µg/kg.

Some non-polar nitrosamines, such as *N*-nitrosodiisobutylamine (NDIBA), *N*-nitrosodibutylamine (NDBA), *N*-nitrosodiphenylamine (NDPhA), *N*-nitrosomethylbenzylamine (NMBzA), and *N*-nitrosodibenzylamine (NDBzA), can be detected using this method. An alternative method should be used for their quantitative determination.

2. Principle

N-nitrosamines are extracted using a 1 % solution of formic acid in a water/methanol mixture (95/5 *V*/*V*).

In the case of non-compliant samples, or where acidic extraction is suspected to generate nitrosamines, an additional aqueous extraction is performed for confirmation. The pH of a 1 % solution of formic acid (in water) is about 2.2. According to the literature, nitrosation is optimal at pH 3–4, with a steep decrease on both sides of this interval [1, 2]. To date, no nitrosamine formation has been observed with the present method using acidic extraction.

Following extraction, the extracts are centrifuged, filtered and analysed by LC/MS. Quantitative determination may be performed by MS/MS or by high-resolution MS [HRMS(/MS)] experiments, depending on the technical capabilities of the equipment. It is recommended to run a full scan in parallel. This allows for the detection of interfering substances and precursors.

NDELA is quantified using a deuterated internal standard. Other nitrosamines are usually determined using an external standard, but the use of internal standards may improve the performance of the method for critical samples and for certain *N*-nitrosamines that are not completely extracted. In this latter case, a correction factor may be applied to take the recovery rate into account.

3. Limitation of the method

Only polar nitrosamines can be quantitatively extracted using aqueous/methanolic formic acid. The recovery rates for non-polar nitrosamines vary according to the matrix. Screening is only possible for nitrosamines with side chains up to dibenzyl at the cost of higher detection limits than for polar nitrosamines.

4. Terms, definitions and abbreviations

APCI: atmospheric pressure chemical ionisation

ESI: electrospray ionisation

FWHM: full width at half maximum

MRM: multiple reaction monitoring

PRM: parallel reaction monitoring

5. Health, safety and environment

It is the user's responsibility to use safe and proper techniques in handling materials for the methods of analysis specified in this document.

- Consult manufacturers for specific details such as material safety data sheets and other recommendations.
- Wear protective goggles, gloves and coats in all laboratory areas.
- Take great care with substances which are toxic and/or human carcinogens.
- Use a fume cupboard during the preparation of organic solvent solutions.
- Dispose of solvents in accordance with environmental requirements.

6. Apparatus, reagents and solutions

6.1. Apparatus

HPLC or UHPLC system with a pumping system capable of gradient elution	Optional: external hardware (divert valve)
High-resolution or tandem (MS/MS) mass spectrometer • ion source: APCI • polarity: positive • minimum mass resolution: 0.7 D for tandem mass spectrometers; 20 000 FWHM for high-resolution mass spectrometers • minimum mass accuracy: 5 ppm for high-resolution mass spectrometers	APCI has proven the method of choice for the determination of nitrosamines. ESI vaporisation can be used, as long as the necessary sensitivity is guaranteed.
Ultrasonic bath (possibly thermostated) and/or	
Ultrasonic homogeniser	e.g. Bandelin Sonopuls HD 3100
Vortex mixer	
Centrifuge capable of 4 000 g or greater	
Pipetting device for organic solvents	

6.2. Accessories

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

Chromatographic column: Nucleodur Sphinx RP, 3 µm, 200 × 3 mm	Macherey-Nagel (760814.30) with or without precolumn
Polyethylene syringes 1 mL	
Syringe filters for HPLC	e.g. 0.2 µm, PVDF e.g. target syringe filter 13 mm (PVDF), pore size 0.2 µm. CAUTION: syringe filters can be contaminated with NDELA.
HPLC vials, amber glass	CAUTION: vials can be contaminated with NDELA (from the packaging). Running of a blank is mandatory and it is recommended that the HPLC vials are first washed with water. The washed vials must not be stored in their original plastic packs, to avoid possible recontamination by NDELA.

6.3. Reagents

Reagents of at least analytical grade are used. Water for chromatography is to be used.

Internal standards

N-nitrosodiethanolamine-d8 (NDELA-d8)

Supplier e.g. CDN Isotopes, Art. D-5997, CAS [1173019-53-8]

N-nitrosodimethylamine-d6 (NDMA-d6) (if internal standard for NDMA is used)

Supplier e.g. CDN Isotopes, Art. D-2937, CAS [17829-05-9]

N-nitrosomorpholine-d8 (NMOR-d8) (if internal standard for NMOR is used)

Supplier e.g. Cambridge Isotopes Lab., DLM-8254, CAS [1219805-76-1]

N-nitrosodiethylamine-d10 (NDEA-d10) (if internal standard for NDEA is used)

Supplier e.g. Cambridge Isotopes Lab., DLM-7982-S, CAS [1219794-54-3]

Analytes

N-nitrosodiethanolamine (NDELA)

Supplier e.g. Sigma *N*-7632, CAS [1116-54-7]

N-nitrosodiisopropanolamine (NDIPLA)

Supplier e.g. Neochema 16000-0040, CAS [53609-64-6]

Nitrosamine mix (NMIX)

2 mg/mL solution in methanol of

- *N*-nitrosodimethylamine (NDMA), CAS [62-75-9]
- *N*-nitrosodiethylamine (NDEA), CAS [55-18-5]
- *N*-nitrosomethylethylamine (NMEA), CAS [10595-95-6]
- *N*-nitrosomorpholine (NMOR), CAS [59-89-2]
- *N*-nitrosopiperidine (NPIP), CAS [100-75-4]
- *N*-nitrosopyrrolidine (NPYR), CAS [930-55-2]

and possibly:

- *N*-nitrosodipropylamine (NDPA), CAS [621-64-7]
- *N*-nitrosodibutylamine (NDBA), CAS [924-16-3]
- *N*-nitrosodiphenylamine (NDPhA), CAS [86-30-6]

Supplier e.g. EPA 8270/Appendix IX Nitrosamines Mix, Supelco Art. 502138

Solvents for sample preparation

Methanol

Supplier e.g. J.T. Baker 8402, CAS [67-56-1]

Ethanol

Supplier e.g. J.T. Baker 8462, CAS [64-17-5]

Acetonitrile

Supplier e.g. J.T. Baker 9017, CAS [75-05-8]

Dichloromethane

Supplier e.g. Merck 6054, CAS [75-09-2]

Formic acid

Supplier e.g. Sigma-Aldrich 33015, CAS [64-18-6]

Solvents for chromatography

Methanol for LC/MS

Supplier e.g. Biosolve 136841, CAS [67-56-1]

Formic acid for LC/MS

Supplier e.g. Biosolve 069141, CAS [64-18-6]

6.4. Solutions

According to the literature, nitrosamines are light sensitive. Therefore, all reference and test solutions must be protected from light. However, sample preparation is quick and may be carried out in plastic or clear glass vessels.

6.4.1. *Stock solutions*

Stock solutions are preferably stored in a deep-freezer at a temperature ≤ −18 °C.

Internal standard NDELA-d8 stock solution (1 mg/mL)

Dissolve 50 mg of NDELA-d8 in acetonitrile and dilute to 50 mL with the same solvent.

Since the response factor for NDELA may show a strong drift, the use of NDELA-d8 as an internal standard is necessary for quantitative measurements.

NDELA stock solution (500 ng/µL)

Weigh accurately 25 mg of NDELA and dissolve in a sufficient amount of ethanol (about 50 mL) to give a solution with an exact concentration of 500 ng/µL.

NDELA stock solution may be stored for at least 4 years when stored below −18 °C. The degradation rate during this period is < 2 %.

NMIX stock solution (100 ng/µL)

Prepare a 0.1 mg/mL NMIX stock solution by appropriate dilution of Nitrosamine mix.

e.g.: Dilute 1 mL of a 2 mg/mL Nitrosamine mix to 20 mL with ethanol (100 ng/µL).

NMIX stock solution may be stored for at least 5 years when stored below −18 °C. The degradation rate of all components except *N*-nitrosodiphenylamine during this period is < 1 %. The degradation rate of *N*-nitrosodiphenylamine during this period is < 4 %.

Other nitrosamine stock solutions

Other nitrosamines available as pure substances are treated as NDELA (stock solution 500 ng/µL).

6.4.2. *Intermediate solutions*

Intermediate solutions must be stored in a deep-freezer at a temperature ≤ −18 °C.

Internal standard NDELA-d8 intermediate solution (5 ng/µL)

Dilute 100 µL of internal standard NDELA-d8 stock solution (1 mg/mL) to 20 mL with methanol.

NMIX intermediate solution (1 ng/µL) (NMIX interm. sol.)

Dilute 20 µL of NDELA stock solution (500 ng/µL), 20 µL of other nitrosamine stock solutions if available, 100 µL of NMIX stock solution (100 ng/µL) and 50 µL of internal standard NDELA-d8 intermediate solution (5 ng/µL) to 10 mL with ethanol.

6.4.3. *Extraction solutions*

Extraction solvent (1 % solution of formic acid in a 95/5 V/V water/methanol mixture)

To a mixture of 950 mL of water and 50 mL of methanol, add 10 mL of formic acid.

Extraction solvent with internal standard (25 pg/µL) (IS-Extraction solvent)

Dilute 500 µL of internal standard NDELA-d8 intermediate solution (5 ng/µL) to 100 mL with the extraction solvent.

This solution is used in the dilution of the calibration solutions and for extraction.

6.4.4. *Calibration solutions*

Prepare fresh dilutions daily. The dilutions given in Table 1 are examples and may be adapted to specific needs.

6.4.5. *Mobile phases*

0.1 % HCOOH in water

Dilute 1 mL of formic acid for LC/MS to 1 000 mL with water for chromatography.

0.1 % HCOOH in methanol

Dilute 1 mL of formic acid for LC/MS to 1 000 mL with methanol for LC/MS.

7. Procedure

7.1. Sample preparation

Protect all test solutions from light immediately after preparation.

- In a centrifuge tube, weigh about 250 mg of the sample to be examined to the nearest 0.1 mg.

  Note: as only limited amounts are available for some products, such as mascara or tattoo inks, smaller quantities (down to 50 mg) may be used if the samples are sufficiently homogeneous; the amount of IS-Extraction solvent used must then be adjusted accordingly to keep the IS-Extraction solvent/sample ratio constant.
- Add 5 mL of IS-Extraction solvent.

Table 1 – *Calibration solutions*

Calibration solution	Prepared from	Diluted to (with IS-Extraction solvent)	Concentration (pg/µL)	Amount injected* (pg)	Amount in sample (0.25 g ad 5 mL, 10 µL)
Cal. 1	1 mL NMIX interm. sol.	20 mL	50	500	1 000 µg/kg
Cal. 2	5 mL Cal. 1	10 mL	25	250	500 µg/kg
Cal. 3	2 mL Cal. 1	10 mL	10	100	200 µg/kg
Cal. 4	1 mL Cal. 1	10 mL	5	50	100 µg/kg
Cal. 5	0.5 mL Cal. 1	10 mL	2.5	25	50 µg/kg
Cal. 6	0.2 mL Cal. 1	10 mL	1	10	20 µg/kg
Cal. 7	0.1 mL Cal. 1	10 mL	0.5	5	10 µg/kg

* The standard injection volume for the calibration solutions is 10 µL. Injection volumes may be adapted to specific needs.

- Mix using a vortex mixer to homogenise.
- Extract for 15 min using an ultrasonic bath or use an ultrasonic homogeniser for 1 minute.
  Note: Ultrasonic homogenisers have proved superior for homogenising difficult samples. If no ultrasonic homogeniser is available and the sample cannot be homogenised using an ultrasonic bath (e.g. samples with high fat content), use one of the following methods:
  – extract at 50 °C in an ultrasonic bath, or
  – first suspend the sample in methanol (0.25 mL) then add 25 µL of IS-NDELA-d8 intermediate solution and finally 4.73 mL of 1 % solution of formic acid in water, or
  – extract with dichloromethane/water according to EN ISO 15819/10130 [3, 4] – in this case, only the most polar nitrosamine, NDELA, remains quantitatively in the aqueous sample solution. Adjust the amount of internal standard as needed.
- Centrifuge at ≥ 3 200 g for 5 min.
- Filter through a 0.2 µm PVDF filter, discarding the first droplets.

7.2. Analysis

Inject 10 µL each of the calibration solutions (Cal. 1 to Cal. 7) and test solutions.

It is recommended to inject a control standard (CS) at the beginning and at the end of each series of tests, and at least after running six test solutions in a series of tests (e.g. Cal. 3).

Inject a blank sample preparation in each series of tests.

7.2.1. *LC conditions*

Use the LC system with a gradient elution (see Table 2) and the following conditions.

- Column temperature: 40 °C
- Injection volume: 10 µL (may be increased to achieve suitable sensitivity)
- Flow rate: 600 µL/min

Table 2 – *Standard gradient with Nucleodur Sphinx RP 200 × 3 mm, 3 µm*

Time (min)	Water/HCOOH (%)	Methanol/HCOOH (%)
0	95	5
3.0	95	5
8.0	0	100
10.0	0	100
11.0	95	5
16.0	95	5

7.2.2. *Chromatographic separation with column switch for NDELA*

Two columns coupled in series may be used to improve analysis in cases of interference, e.g. when the samples contain high amounts of triethanolamine (see Annex 1).

7.2.3. *Mass spectrometer – instrument parameters*

Instrument parameters have to be chosen according to the capabilities of the mass spectrometers used.

High-resolution mass spectrometers

The sensitivity and ruggedness of the method is improved when running Orbitrap high-resolution mass spectrometers in MS/MS or single ion mode after preselection of the mother ion at a mass resolution of < 1 Da.

The following settings have been found to be suitable for high-resolution MS/MS (e.g. Orbitrap) capable mass spectrometers:

- SIM: Isolation window = 0.4 m/z; Resolution = 70 000 FWHM
- MS/MS (PRM): Isolation window = 0.4 m/z; Resolution = 35 000 FWHM
- Full scan MS: Resolution = 70 000 FWHM

Tandem mass spectrometers

- Q1 and Q3 are operated at a resolution of ≤ 0.7 m/z

Notes

NDELA is highly temperature-sensitive. For the determination of other nitrosamines, the sensitivity of the method can be significantly improved using higher vaporising and drying temperatures.

Depending on the mass spectrometer, measuring times may be too long when programming all experiments. Thus, the experiments may have to be segmented in order to get enough data points for each peak.

Whenever possible, a simultaneous data acquisition in full scan mode is recommended for the entire run. This allows for the detection of interfering and precursor substances.

When using a high-resolution mass spectrometer, full scan experiments allow for a less sensitive target screening for uncommon nitrosamines.

7.2.4. *Washing step and rinsing programme*

Optional – External hardware (divert valve)

If a divert valve is available, its use is recommended during the washing step of the method. During this period, the LC effluent may be directed to the waste bin. In addition, the mass spectrometer may be flushed with a second pump if available (see below).

Time (min)	Position
0.0 – 0.6	Waste
0.6 – 10.6	Detector
10.6 – 16.0	Waste

Optional – Rinsing programme to purge the mass spectrometer between runs

It is recommended to rinse the mass spectrometer using a second pump during the washing phase of the method. The eluent is water/methanol 9/1 (*V/V*) with 0.1 % formic acid.

Time (min)	Flow rate (µL/min)
0	200
0.6	200
0.7	20
10.5	20
10.6	200
16.0	200

7.3. Data evaluation

7.3.1. *Evaluation of the mass spectrometric experiments*

The evaluation of the data has to be tailored to the specific mass spectrometer:

- Useful MRM transitions and exact masses for data evaluation are given in Table 3.
- MRM transitions may vary between mass spectrometers.
  It is part of the in-house validation procedure to select the best-suited transitions for a sensitive and rugged determination.
- For high-resolution mass spectrometers, several data evaluation procedures are possible: single ion monitoring at high resolution (≥ 70 000 FWHM) or MS/MS experiments at high resolution (≥ 17 500 FWHM) are the method of choice and are recommended for the most frequently found nitrosamines (NDELA, NDELA-d8, NDMA, NMOR, NDEA).
  For Orbitraps, the Quadrupole should be operated at 0.4 D to avoid overfilling the Orbitrap.
  Extraction of the exact masses (< 5 ppm) out of full scan experiments (preferably at a mass resolution of ≥ 70 000 D) is also possible when the necessary sensitivity is guaranteed. Care has to be taken for possible signal suppression, especially when using Orbitrap mass spectrometers.
  Note: NDELA is generally determined using NDELA-d8 as internal standard; the other nitrosamines are generally determined using an external standard. Deuterated standards may improve precision and accuracy for other nitrosamines when needed.

Table 4 – *Retention times*

Substance	RT (min)
Nitrosodiethanolamine-d8 (NDELA-d8)	2.30
Nitrosodiethanolamine (NDELA)	2.38
Nitrosodimethylamine (NDMA)	3.56
Nitrosomorpholine (NMOR)	6.00
Nitrosodiisopropanolamine (NDIPLA)	6.48 (DP*)
Nitrosomethylethylamine (NMEA)	6.62 (DP*)
Nitrosopyrrolidine (NPYR)	6.82
Nitrosodiethylamine (NDEA)	7.85
Nitrosopiperidine (NPIP)	8.15

* DP: double peak

7.3.2. *Acceptance criteria*

Calibration

For quantitative applications, the deviation of a calibration point from the nominal value should be within a predefined acceptable range. Otherwise, recalibrate or adjust the calibration range if possible.

Routine tests

For quantitative applications, the deviation of the control standard from the nominal value should be checked. In case of significant deviation, the response factor should be calculated using the bracketing technique (for regular drift).

For each series of tests, a blank analysis should be performed. If there is a significant blank signal, this should be

Table 3 – *Masses and MRM transitions for high-resolution and tandem mass spectrometers*

Notation	Mass [m/z]	Molecular formula	Adduct	Daughter ions		
NDMA	75.05529	$C_2H_6N_2O$	+ H+	(75.05529)	58.05824	42.9
NMEA	89.07094	$C_3H_8N_2O$	+ H+	(89.07094)	61.03982	43.3
NPYR	101.07094	$C_4H_8N_2O$	+ H+	(101.07082)	55.05454	
NDEA	103.08659	$C_4H_{10}N_2O$	+ H+	75.05529	57.04500	
NPIP	115.08659	$C_5H_{10}N_2O$	+ H+	69.06988	53.00252	40.9
NMOR	117.06585	$C_4H_8N_2O_2$	+ H+	(117.06584)	87.06787	86.05992
NDPA	131.11789	$C_6H_{14}N_2O$	+ H+	89.07085	57.07013	(75.05522)
NDELA	135.07642	$C_4H_{10}N_2O_3$	+ H+	104.07064	74.06007	
NDELA D8	143.12663	$C_4H_2D_8N_2O_3$	+ H+	80.09824	111.11449	
NDIPLA	163.10772	$C_6H_{14}N_2O_3$	+ H+	88.07561	70.35	

investigated and measures should be taken to reduce the blank signal and/or the blank signal should be taken into account for calculation.

When an LC-MS/MS system is used, if the detected analytes do not comply with the maximum permitted tolerances for relative ion intensities in Table 5, it should be carefully checked (e.g. using optimised experiments) whether the signal comes from the analyte in question or whether it is a contaminant.

Table 5 – *Maximum permitted tolerances for relative ion intensities*

Relative intensity (% of base peak)	LC-MS/MS (relative)
≤ 10 %	± 50 %
> 10 to 20 %	± 30 %
> 20 to 50 %	± 25 %
> 50 %	± 20 %

Source: Table 4 in Annex to Commission Decision of 12 August 2002, 2002/657/EC.

If the measured values lie outside the calibration range, the analysis should be repeated. Several adjustments are possible:

- adaptation of the injection volume, depending on equipment and analytes,
- dilution of test solutions,
- adaptation of extraction solvent to sample ratio,
- adaptation of the calibration range, depending on the sensitivity and the analytical range of the assay.

7.3.3. *Out-of-specification results/controversial samples*

In the case of out-of-specification results or otherwise controversial samples, it is recommended to:

- perform at least a double determination;
- spike with the suspected substances at the expected concentration, in order to investigate co-elution and determine recovery;
- perform the extraction described in 7.1 with pure water instead of the extraction solvent to exclude possible formation of nitrosamines during the extraction step.

8. Calculation

From the nitrosamine content obtained, calculate the nitrosamine concentration in the sample:

$$\text{Concentration in µg/kg} \quad \frac{A \times V \times 1000 \times VF}{V_{inj} \times E}$$

A = mass of nitrosamine in the test solution (pg)

V = volume of extractant (mL)

VF = dilution factor

V_{inj} = injection volume (µl)

E = sample mass (mg)

1 000 conversion factor (mL to µL) for the volume of extraction solvent.

Nitrosamine contents are expressed in µg/kg.

9. Reporting

The test report should contain the following data:

- information necessary for the identification of the sample (type, origin and designation of the sample),
- the date of receipt and date of analysis,
- the test results (including the measurement uncertainty) and the units in which they have been expressed,
- justification of any deviation from the method,
- operations not specified in the method or regarded as optional, which might have affected the results.

10. Validation

The method was subjected to peer review. Inter-laboratory validation data (repeatability, reproducibility, recovery rates) are reported in Annex 2.

Linearity and detection limit data obtained by one of the laboratories are presented in Tables 6 and 7.

Table 6 – *Linearity data for a typical calibration on an Orbitrap mass spectrometer (deviation from nominal values in percent)*

Amount (pg)	NDELA	NDMA	NMOR	NMEA	NPYR	NDEA	NPIP
500	− 0.32	0.06	− 0.95	1.42	− 0.69	− 2.13	1.67
250	0.43	0.31	1.68	− 2.29	1.47	2.66	− 4.16
100	2.44	0.06	0.40	− 1.37	0.05	3.06	0.50
50	− 1.55	− 2.79	1.95	− 0.11	− 0.69	2.35	1.59
25	− 5.75	1.86	− 2.98	− 0.78	0.62	1.71	4.65
10	1.13	− 4.65	− 1.19	1.38	− 0.75	− 5.51	− 3.51
5	3.62	5.15	1.10	1.75	− 0.02	− 2.15	− 0.73
Average deviation	**3.06**	**3.15**	**1.79**	**1.57**	**0.82**	**3.28**	**3.09**
R^2	**0.9998**	**0.9999**	**0.9998**	**0.9997**	**0.9999**	**0.9993**	**0.9992**

Table 7 – *Limit of detection (LoD) observed on an Orbitrap Q-Exactive mass spectrometer*

Substance	Experiment	LoD (pg)	LoD (µg/kg)
NDELA	PRM	1.7	3
NDMA	PRM	2.5	5
NDEA	SIM	0.8	2
NMOR	PRM	1.8	4
NPYR	SIM	1.5	3
NPIP	SIM	0.8	2
NMEA	Full	17.3	35

PRM: parallel reaction monitoring (MS/MS);
SIM: single ion monitoring;
Full: full scan experiment.

11. References

1. SCCS (Scientific Committee on Consumer Safety) 1458/11, Opinion on Nitrosamines and Secondary Amines in Cosmetic Products of 27 March 2012.
2. Mirvish, S.S. Kinetics of dimethylamine nitrosation in relation to nitrosamine carcinogenesis. *J. Natl. Cancer Inst.*, 1970. 44 (3): p. 633-639.
3. ISO 15819: Cosmetics – Analytical methods – Nitrosamines: Detection and determination of *N*-nitrosodiethanolamine (NDELA) in cosmetics by HPLC-MS-MS.
4. ISO 10130: Cosmetics – Analytical methods – Nitrosamines: Detection and determination of *N*-nitrosodiethanolamine (NDELA) in cosmetics by HPLC, post-column photolysis and derivatization.

Annex 1. Chromatographic separation with Column switch for NDELA

Cosmetics can contain high amounts of triethanolamine (TEA) which may interfere with NDELA quantification. When using a Reprosil 80 ODS-1 (150 × 3 mm, 5 µm) stationary phase, the elution order of TEA and NDELA is inversed compared with a Sphinx column. This is used in a 2D column setup: NDELA is first eluted from the Reprosil ODS-1 to a Sphinx column connected in series (Nucleodur Sphinx RP 100 × 3 mm, 1.8 µm), then the Reprosil ODS-1 is disconnected from the flow. The actual separation then takes place in the Sphinx column. This method may also provide additional selectivity and reliability for other interference problems. This 2D column setup is only to be used for NDELA. All other nitrosamines are directed to the waste.

Gradient program with 2D Setup (Reprosil ODS-1 plus Nucleodur Sphinx columns)

Gradient pump (Pump 1)			
Time (min)	Water/ HCOOH (%)	Methanol/ HCOOH (%)	Flow rate (µL/min)
0	95	5	450
4.5	95	5	450
5.5	0	100	450
10.0	0	100	450
10.5	95	5	450
16.0	95	5	450

Rinsing pump (Pump 2)		
Time (min)	Methanol/water 70/30 *V/V* (%)	Flow rate (µL/min)
0	100	10
2.5	100	10
3.3	100	350
4.0	100	1000
9.5	100	1000
10.0	100	450
10.5	100	10
16.0	100	10

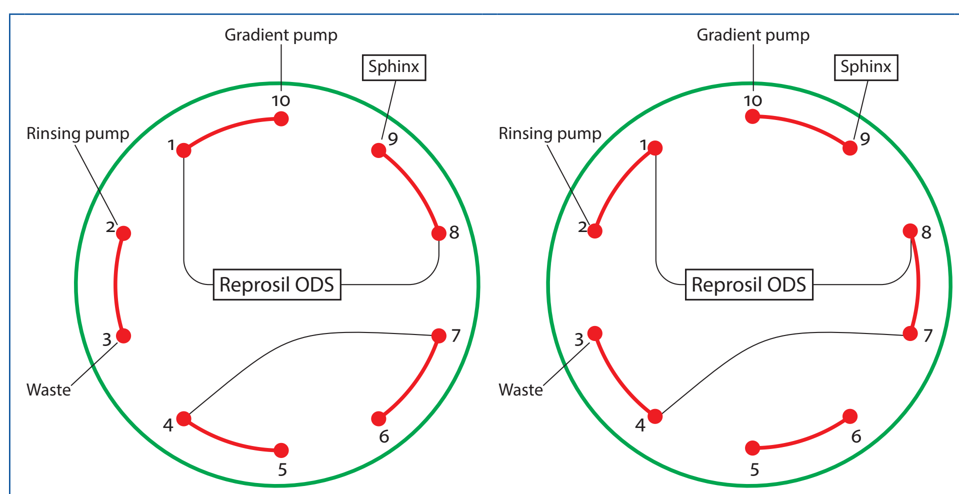
Divert Valve		
	Valve state	
Time (min)	Gradient pump	Rinsing pump
0.00	to precolumn + column	to waste
3.3	to column	to precolumn
10.0	to precolumn + column	to waste

HPLC conditions

Column thermostat
Column temperature: 40 °C

Autosampler
Injection volume: 10 µL

Figure 1 – *The setup for a 10-port valve is shown but a 6-port valve may also be used*



Annex 2. Inter-laboratory validation data (peer review)

Two nitrocellulose-based nail varnishes were tested by three independent laboratories during a peer review process. One laboratory used a high-resolution Orbitrap mass spectrometer; the other two laboratories used triple quadrupole mass spectrometers. The two products contained, respectively, four and three polar *N*-nitrosamines with a concentration range of 10 to 400 µg/kg.

For each sample, two sample preparations were performed on three different days. In addition, spiking was performed but at a different concentration on each of the three days. The mean concentration values, reproducibility and repeatability are shown in Table 8 and the recovery rates are shown in Table 9, below.

Table 8 – *Mean concentration values, reproducibility and repeatability*

	Nitrocellulose-based nail varnish – Sample A				Nitrocellulose-based nail varnish – Sample B		
Substance	**NDELA**	**NDMA**	**NDEA**	**NMOR**	**NDELA**	**NDMA**	**NMOR**
Mean concentration (µg/kg)	10.6	20.8	18.0	40.7	101.3	418.6	8.7
Inter-laboratory Reproducibility SD (µg/kg)	1.56	5.25	1.43	3.07	9.51	42.83	1.55
Intermediate Precision SD (µg/kg)	1.41	1.45	1.43	2.25	4.44	16.27	1.08
Intra-day Repeatability SD (µg/kg)	0.68	1.25	0.99	1.92	3.44	6.25	0.30
Relative Inter-laboratory Reproducibility	15 %	25 %	8 %	8 %	9 %	10 %	18 %
Relative Intermediate Precision	13 %	7 %	8 %	6 %	4 %	4 %	12 %
Intra-day Relative Repeatability	6 %	6 %	5 %	5 %	3 %	1 %	3 %

Table 9 – *Recovery rates*

Nitrocellulose-based nail varnish – Sample A

Substance	Statistic	Spike levels 50 µg/kg	Spike levels 100 µg/kg	Spike levels 300 µg/kg	ANOVA p-value	Mean Recovery	SD Recovery	95 % Confidence Limits
NDELA	**Mean**	100 %	102 %	103 %	0.86	102 %	2 %	97 % – 106 %
	SD	9 %	4 %	9 %				
NDMA	**Mean**	96 %	94 %	96 %	0.95	95 %	1 %	93 % – 98 %
	SD	8 %	9 %	4 %				
NDEA	**Mean**	79 %	78 %	84 %	0.37	80 %	3 %	74 % – 87 %
	SD	7 %	3 %	4 %				
NMOR	**Mean**	93 %	87 %	93 %	0.20	91 %	4 %	82 % – 99.7 %
	SD	6 %	3 %	5 %				
NPIP	**Mean**	79 %	78 %	85 %	0.84	81 %	3 %	72 % – 89 %
	SD	14 %	10 %	15 %				
NPYR	**Mean**	88 %	84 %	89 %	0.91	87 %	3 %	80 % – 94 %
	SD	19 %	13 %	10 %				
NMEA	**Mean**	91 %	90 %	96 %	0.77	92 %	3 %	85 % – 99.6 %
	SD	16 %	5 %	7 %				

Nitrocellulose-based nail varnish – Sample B

Substance	Statistic	Spike levels 50 µg/kg	100 µg/kg	300 µg/kg	ANOVA p-value	Mean Recovery	SD Recovery	95 % Confidence Limits
NDELA	Mean	n.e.	99 %	100 %	0.82	100 %	1 %	90 % – 109 %
	SD	n.e.	4 %	8 %				
NDMA	Mean	n.e.	n.e.	87 %	n.e.	n.e.	n.e.	n.e.
	SD	n.e.	n.e.	15 %				
NDEA	Mean	82 %	83 %	83 %	0.94	83 %	1 %	81 % – 85 %
	SD	7 %	7 %	3 %				
NMOR	Mean	94 %	94 %	91 %	0.64	93 %	2 %	89 % – 98 %
	SD	6 %	4 %	2 %				
NPIP	Mean	85 %	84 %	87 %	0.97	85 %	1 %	82 % – 88 %
	SD	13 %	9 %	13 %				
NPYR	Mean	94 %	93 %	90 %	0.95	92 %	2 %	88 % – 97 %
	SD	21 %	14 %	4 %				
NMEA	Mean	94 %	93 %	92 %	0.99	93 %	1 %	91 % – 95 %
	SD	14 %	8 %	6 %				

n.e.: not evaluated.

ENG

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