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अनुभाग 1 प्रत्यक्ष पद्धति

Methods of Chemical Testing of
Leather
Part 15 Determination of Ethoxylate
Alkylphenols (APEO)
Section 1 Direct Method

ICS 59.140.30

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NATIONAL FOREWORD

This Indian Standard (Part 15/Sec 1) which is identical to ISO 18218-1 : 2023 'Method of chemical testing of leather — Part 15: Determination of ethoxylate alkylphenols (EPEO), Section 1 Direct method' issued by the International Organization for Standardization (ISO) was adopted by the Bureau of Indian Standards on the recommendation of the Leather, Tanning Materials and Allied Products Sectional Committee and approval of the Chemical Division Council.

Nonylphenol ethoxylate belongs to the non-ionic surfactants. The biodegradation of nonylphenol ethoxylate releases the persistent pollutant, the branched nonylphenol. Nonylphenol is a hormonalacting substance that is toxic for waterborne organisms and many other organisms. For this reason, the release of nonylphenol ethoxylate into the environment should be avoided.

This standard is a method for determining ethoxylated alkylphenols (APEO) [nonylphenol ethoxylate (NPEO_n, where 2 ≤ n ≤ 16) and octylphenol ethoxylate (OPEO_n, where 2 ≤ n ≤ 16)] in leather. This direct method is especially suitable when a larger number of leather samples are to be checked for the presence of ethoxylated alkylphenols.

This method requires the use of liquid chromatography (LC) with a triple quadrupole mass spectrometer (MS/MS) to identify and quantify the ethoxylated alkylphenols.

The Committee further decided to publish the adopted/harmonized standards in the following manner:



- a) Wherever an existing test method prescribed in IS 582 is being replaced by the corresponding ISO/IULTCS test method, the relevant part will be published with the information in the national foreword about the method of IS 582 being superseded; and
- b) When a new test method is being incorporated in IS 582, the same will be published as a new standard and as subsequent part of IS 582.

This Indian Standard is published in several parts. The other parts of this standard are:

Part 1	Determination of volatile matter
Part 2	Determination of water-soluble matter, water soluble inorganic matter and water-soluble organic matter
Part 3	Determination of sulphate total ash and sulphated water-insoluble ash
Part 4	N-methyl-2-pyrrolidone (NMP) in leather
Part 5	Determination of certain azo colourants in dyed leather,
Sec 1	Determination of certain aromatic amines derived from azo colorants
Sec 2	Determination of 4-aminoazobenzene
Part 6	Determination of metal content,
Sec 1	Extractable metals
Sec 2	Total metal content
Part 7	Quantitative analysis of tanning agents by filter method
Part 8	Determination of the preservative (TCMTB, PCMC, OPP, OIT) content in leather by liquid chromatography,
Sec 1	Acetonitrile extraction method
Sec 2	Artificial perspiration extraction method
Part 9	Determination of pH and difference figure
Part 10	Determination of chromic oxide content,

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Introduction

Nonylphenol ethoxylate belongs to the non-ionic surfactants. The biodegradation of nonylphenol ethoxylate releases the persistent pollutant, the branched nonylphenol. Nonylphenol is a hormonal acting substance that is toxic for waterborne organisms and many other organisms. For this reason, the release of nonylphenol ethoxylate into the environment should be avoided.

In 2003 the European Directive 2003/53/EC^[4] restricted the sale and use of nonylphenol and nonylphenol ethoxylate in product preparations for industries with discharges to wastewater. Preparations containing concentrations equal to or higher than 0,1 % of nonylphenol ethoxylate or nonylphenol were forbidden. This Directive is included as part of the EU Regulation 1907/2006 (REACH).^[3]

No detailed composition of the chemical substance nonylphenol ethoxylate can be given; it is assigned the general structural formula:



where Ph = phenyl, $n \geq 1$.

To cover the group of ethoxylates of 4-nonylphenol, branched and linear, the European Chemical Agency (ECHA) has assigned the substance the following definition:

"4-nonylphenol, branched and linear, ethoxylated [substances with a linear and/or branched alkyl chain with a carbon number of 9 covalently bound in position 4 to phenol, ethoxylated covering UVCB and well-defined substances, polymers and homologues, which include any of the individual isomers and/or combinations thereof]."^[5]

In the leather industry, nonylphenol ethoxylate and octylphenol ethoxylate surfactants have been used. However, the water-insoluble substances nonylphenol and octylphenol have not been used. For this reason, two different analytical procedures have been prepared for analysing leather samples.

This document is a method that directly determines the ethoxylated alkylphenol. It is an efficient procedure for the analysis of a larger number of leather samples. This procedure requires liquid chromatography (LC) with triple quadrupole mass spectrometer (MS/MS) to identify the nonylphenol ethoxylate and octylphenol ethoxylate.

ISO 18218-2 specifies a procedure for analysing the alkylphenol component. The ethoxylated alkylphenol is cleaved to form the alkylphenol, which is identified using LC or gas chromatography-mass spectrometry (GC-MS) equipment. This method can also be used to indirectly determine the alkylphenol ethoxylate content in leather.

Indian Standard

**LEATHER — DETERMINATION OF ETHOXYLATED
ALKYLPHENOLS (APEO)**

PART 1 DIRECT METHOD

1 Scope

This document is a method for determining ethoxylated alkylphenols (APEO) [nonylphenol ethoxylate (NPEO_n, where $2 \leq n \leq 16$) and octylphenol ethoxylate (OPEO_n, where $2 \leq n \leq 16$)] in leather. This direct method is especially suitable when a larger number of leather samples are to be checked for the presence of ethoxylated alkylphenols.

This method requires the use of liquid chromatography (LC) with a triple quadrupole mass spectrometer (MS/MS) to identify and quantify the ethoxylated alkylphenols.

NOTE 1 In the leather industry, the most commonly used ethoxylated alkylphenol is the NPEO, with an average of 9 EO. It has an optimum cloud point in water for the typical leather processing temperatures of 40 °C to 55 °C.

NOTE 2 This document and ISO 18218-2 use different solvents for the extraction of the ethoxylated alkylphenols from leather. Consequently, the two analytical methods are expected to give similar trends but not necessarily the same absolute result for the ethoxylated alkylphenol content in leather.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 2418, *Leather — Chemical, physical, mechanical and fastness tests — Position and preparation of specimens for testing*

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

ISO 4044, *Leather — Chemical tests — Preparation of chemical test samples*

3 Terms and definitions

No terms and definitions are listed in this document.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at www.iso.org/obp
- IEC Electropedia: available at www.electropedia.org/

4 Principle

The leather sample is extracted with methanol using an ultrasonic bath. Subsequently, an aliquot of the solution can, after filtering, be directly analysed without further cleaning of the sample using LC with a MS/MS detector.

5 Apparatus

The usual laboratory apparatus and, in particular, the following shall be used:

- 5.1 **Ultrasonic bath**, with controllable heating capable of maintaining a temperature of (60 ± 5) °C.
- 5.2 **Glass vial with a screw cap**, for example, 22 ml is suitable.
- 5.3 **Polypropylene or polyethylene syringe**, 2 ml.
- 5.4 **Membrane filter**, for example, pore size 0,2 μm , for use with a syringe (5.3).
- 5.5 **Volumetric flasks**, 10 ml, 100 ml and 1 000 ml.
- 5.6 **Analytical balance**, weighing to 1 mg.
- 5.7 **Pipettes**, various sizes, 1 ml to 5 ml.
- 5.8 **LC vial with cap**.
- 5.9 **Instrumental equipment**, high-performance LC with MS/MS and electrospray ionization (ESI).

6 Reagents

Unless otherwise specified, all reagents shall be of a recognized analytical grade.

- 6.1 **Methanol**, LC-MS grade.
- 6.2 **Nonylphenol ethoxylate**, NPEO_n where $n = 9$ to 10, CAS Registry Number^{®1)} 68412-54-4, Sigma-Aldrich[®] Product No. 542334 (IGEPAL[®] CO-630)²⁾, technical grade.
- 6.3 **Octylphenol ethoxylate**, OPEO_n where $n = 9$ to 10, CAS Registry Number 9036-19-5, Sigma-Aldrich[®] Product No. 93443 (Triton[™] X-100)³⁾, technical grade.

NOTE In 6.2 and 6.3 the brand name is given to improve the comparability of test results among laboratories. The commercial nonylphenol or octylphenol ethoxylate contains groups of ethoxylates of nonylphenol or octylphenol with linear and branched structures, so use of another reference can lead to different results. Only technical grade references are currently available from laboratory chemical suppliers.

- 6.4 **Stock solution of nonylphenol ethoxylate**, $\beta = 250$ $\mu\text{g/ml}$

Weigh 25 mg of the nonylphenol ethoxylate (6.2) into a 100 ml volumetric flask (5.5), dissolve it in methanol (6.1) and fill up to the mark with methanol.

1) Chemical Abstracts Service (CAS) Registry Number[®] is a trademark of the American Chemical Society (ACS). This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

2) Sigma-Aldrich[®] Product No. 542334 (IGEPAL[®] CO-630) 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.

3) Sigma-Aldrich[®] Product No. 93443 (Triton[™] X-100) 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.

6.5 Stock solutions of octylphenol ethoxylate, $\beta = 250 \mu\text{g/ml}$

Weigh 25 mg of the octylphenol ethoxylate (6.3) into a 100 ml volumetric flask (5.5), dissolve it in methanol (6.1) and fill up to the mark with methanol.

6.6 Standard solution of nonylphenol ethoxylate, $\beta = 25 \mu\text{g/ml}$

Transfer 1,0 ml of the stock solution (6.4) to a 10 ml volumetric flask (5.5) and fill up to the mark with methanol (6.1).

6.7 Standard solution of octylphenol ethoxylate, $\beta = 25 \mu\text{g/ml}$

Transfer 1,0 ml of the stock solution (6.5) to a 10 ml volumetric flask (5.5) and fill up to the mark with methanol (6.1).

6.8 Calibration solutions of nonylphenol ethoxylate and octylphenol ethoxylate

Prepare at least four calibration solutions using the respective standard solutions (6.6 and 6.7). Table 1 gives an example of the calibration solutions prepared in a 10 ml volumetric flask.

Concentration ranges for the calibration standards are subject to change depending on the need of each laboratory and equipment used.

Table 1 — Example of calibration solutions

Concentration ($\mu\text{g/ml}$)	0,5	1,0	1,5	2,0	2,5
Volume methanol (ml)	9,8	9,6	9,4	9,2	9,0
Volume standard solutions (6.6 or 6.7) at 25 $\mu\text{g/ml}$ (ml)	0,2	0,4	0,6	0,8	1,0

6.9 Ammonium acetate

6.10 5 mM ammonium acetate

Dissolve 0,386 g ammonium acetate (6.9) in water (6.11) in a 1 000 ml volumetric flask (5.5). Fill the flask up to the mark with water (6.11).

6.11 Water, grade 3 according to ISO 3696.

7 Sampling

Cut a test specimen in accordance with ISO 2418. If cutting a test specimen according to ISO 2418 is not possible (e.g. in the case of leather from finished products such as shoes or clothing), details of the selection of the test specimen shall be given in the test report. Glue residuals shall be mechanically removed from leather pieces.

The leather test specimen shall be cut into small pieces or ground according to ISO 4044.

8 Sample preparation and analysis

8.1 Extraction

Weigh approximately 1 g of the leather pieces accurately to 10 mg in a glass vial (5.2). Add 20 ml methanol (6.1), close the vial and extract the sample at $(60 \pm 5) ^\circ\text{C}$ for (60 ± 5) min in an ultrasonic bath (5.1).

8.2 Analysis

After cooling down to room temperature, remove an aliquot of the extraction solution using a disposable syringe (5.3) and transfer into a LC vial (5.8) using a membrane filter (5.4). The aliquot is now ready for the LC analysis.

The detection of the alkylphenol ethoxylate is made using an LC with gradient elution and MS/MS (5.9). Congeners with 2 to 16 ethoxylate groups shall be used for quantification. Guidelines for suitable chromatographic conditions are given in Annex A.

8.3 Calibration

The calibration solutions (6.8) shall be transferred to an LC vial (5.8) and analysed along with each batch of test samples.

8.4 Calculation

8.4.1 Determination of the ratio of each APEO congener

Calculate the ratio of each APEO congener, R (%), according to Formula (1), using selected ion monitoring (SIM) data.

$$R = \frac{A_{(n)}}{A_{\text{sum}}} \times 100 \quad (1)$$

where

$A_{(n)}$ is the SIM area of each APEO congener;

A_{sum} is the sum of the SIM areas from all APEO congeners.

The calculation according to 8.4.1 shall be made for each new batch of the reference substances (6.2 and 6.3) and shall be done in the SIM mode. See Annex B, Tables B.1 to B.3, for the characteristic masses of the APEO congeners.

NOTE An accurate distribution of the APEO congeners cannot be received from multiple reaction monitoring (MRM) data because the APEO fragmentation efficiencies are highly dependent on the number of ethoxylate groups.

8.4.2 Determination of the real concentration of each APEO congener in the calibration standards

Calculate the real concentration of each APEO congener in the calibration standards (6.8) in $\mu\text{g/ml}$ according to Formula (2).

$$c_{\text{stdAPEO}(n)} = \frac{R \times c_{\text{std}}}{100} \quad (2)$$

where

R is the ratio of each APEO congener, in %;

c_{std} is the overall concentration of the respective calibration standard, in $\mu\text{g/ml}$.

8.4.3 Calibration graph

The calibration graphs are prepared with the help of the area of each APEO congener (y -axis) and the calculated real concentration of the respective calibration standard (x -axis) (8.4.2). For each

APEO congener, a separate calibration graph is necessary. The determination of the area of the daily calibration (6.8) and the area of the daily APEO samples shall be done in MRM mode.

8.4.4 Calculation of the APEO concentration

The NPEO content in the leather sample, s_{NPEO} , in mg/kg is calculated according to [Formula \(3\)](#).

$$s_{\text{NPEO}} = \frac{c \times V}{m} \times F_d \quad (3)$$

where

- c is the total sum of the concentrations of each NPEO congener in the sample ($\mu\text{g/ml}$) with $\sum_{n=2}^{16} c_{\text{NPEO}(n)}$;
- n is the ethoxylate chain length;
- $c_{\text{NPEO}(n)}$ is the concentration of the NPEO congener with n ethoxylate groups in the extract;
- V is the extraction volume according to [8.1](#), in ml;
- m is the mass of the leather sample, in g;
- F_d is the dilution factor (if used).

The OPEO content in the leather sample, s_{OPEO} , in mg/kg is calculated according to [Formula \(4\)](#).

$$s_{\text{OPEO}} = \frac{c \times V}{m} \times F_d \quad (4)$$

where

- c is the total sum of concentration of each OPEO congener in the sample ($\mu\text{g/ml}$), with: $c = \sum_{n=2}^{16} c_{\text{OPEO}(n)}$;
- n is the number of ethoxylate groups;
- $c_{\text{OPEO}(n)}$ is the concentration of the OPEO congener with n ethoxylate groups in the extract;
- V is the extraction volume according to [8.1](#), in ml;
- m is the mass of the leather sample, in g;
- F_d is the dilution factor (if used).

The APEO content in leather is reported in mg/kg as the sum of the NPEO and OPEO contents determined in [Formulae \(3\)](#) and [\(4\)](#).

9 Test report

The test report shall include at least the following information:

- a) a reference to this document, i.e. ISO 18218-1:2023;
- b) type, origin and denomination of the sample (aliquot, as far as relevant);
- c) the date of the test;
- d) sampling method, if different to ISO 2418;

- e) APEO content, stated as the sum of the NPEO and OPEO content in milligrams per kilogram (mg/kg), as determined in [8.4.4](#).
- f) any deviation from the given procedure, including the use of other commercial standard solutions.

Annex A (informative)

Example of chromatographic method LC-MS/MS

A.1 Preliminary comment

As the LC equipment (5.9) of the laboratories can vary, no general valid instructions can be provided for the chromatographic analysis. The following parameters have been successfully tested and used.

A.2 Chromatographic conditions for the LC-MS/MS method

Eluent 1	5 mM ammonium acetate
Eluent 2	Methanol
Column	Reversed-phase column C18 (5 µm, 2,1 × 50 mm)
Guard column	Reversed-phase guard column C18, 5 µm
Flow rate	1,2 ml/min
Gradient time programme	<ul style="list-style-type: none"> — 0 min, 30 % MeOH — 0 min to 2 min, up to 80 % MeOH — 2 min to 3 min, up to 98 % MeOH — 3 min to 3,5 min, hold 98 % MeOH — 3,5 min to 4,5 min, down to 30 % MeOH — 4,5 min to 8 min, hold at 30 % MeOH
Column temperature	30 °C
Injection volume	20 µl
Detection	Four tandem type pile pole or ion trap mass detector Selected reaction monitoring (SRM) method Product ion mass spectrum
Ionizing	ESI (electro spray ionizing) method and positive/negative ion detection

Annex B (informative)

Characteristic masses for quantification

Table B.1 — Characteristic masses for quantification [M + NH₄]⁺2

NPEO congeners	Q1 m/z	Q3 m/z	OPEO congeners	Q1 m/z	Q3 m/z
NPEO 16	942	925	OPEO 16	928	911
NPEO 15	898	881	OPEO 15	884	867
NPEO 14	854	837	OPEO 14	840	823
NPEO 13	810	793	OPEO 13	796	779
NPEO 12	766	749	OPEO 12	752	735
NPEO 11	722	705	OPEO 11	708	691
NPEO 10	678	661	OPEO 10	664	647
NPEO 9	634	617	OPEO 9	620	603
NPEO 8	590	573	OPEO 8	576	559
NPEO 7	546	529	OPEO 7	532	515
NPEO 6	502	485	OPEO 6	488	471
NPEO 5	458	441	OPEO 5	444	427
NPEO 4	414	397	OPEO 4	400	383
NPEO 3	370	353	OPEO 3	356	227
NPEO 2	326	183	OPEO 2	312	183

Table B.2 — SIM masses of the APEO congeners [M + NH₄]

NPEO congener	SIM mass	OPEO congener	SIM mass
NPEO 2	326	OPEO 2	312
NPEO 3	370	OPEO 3	356
NPEO 4	414	OPEO 4	400
NPEO 5	458	OPEO 5	444
NPEO 6	502	OPEO 6	488
NPEO 7	546	OPEO 7	532
NPEO 8	590	OPEO 8	576
NPEO 9	634	OPEO 9	620
NPEO 10	678	OPEO 10	664
NPEO 11	722	OPEO 11	708
NPEO 12	766	OPEO 12	752
NPEO 13	810	OPEO 13	796
NPEO 14	854	OPEO 14	840
NPEO 15	898	OPEO 15	884
NPEO 16	942	OPEO 16	928

Table B.3 — Molecular weight of the APEO congeners

NPEO congener	Molecular weight	OPEO congener	Molecular weight
NPEO 2	308	OPEO 2	294
NPEO 3	352	OPEO 3	338
NPEO 4	396	OPEO 4	382
NPEO 5	440	OPEO 5	426
NPEO 6	484	OPEO 6	470
NPEO 7	528	OPEO 7	514
NPEO 8	572	OPEO 8	558
NPEO 9	616	OPEO 9	602
NPEO 10	660	OPEO 10	646
NPEO 11	704	OPEO 11	690
NPEO 12	748	OPEO 12	734
NPEO 13	792	OPEO 13	778
NPEO 14	836	OPEO 14	822
NPEO 15	880	OPEO 15	866
NPEO 16	926	OPEO 16	910

Bibliography

- [1] ISO 18218-2, *Leather — Determination of ethoxylated alkylphenols — Part 2: Indirect method*
- [2] ISO 18254-1, *Textiles — Method for the detection and determination of alkylphenol ethoxylates (APEO) — Part 1: Method using HPLC-MS*
- [3] Regulation (EC) No 1907/2006 (REACH), Annex XVII. Available from: <https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2007:136:0003:0280:en:PDF>
- [4] Directive 2003/53/EC of the European Parliament and of the Council. June 2003. Available from: <https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX:32003L0053>
- [5] European Chemicals Agency (ECHA). Substance Information: 4-Nonylphenol, branched and linear, ethoxylated. Available from: <https://echa.europa.eu/en/substance-information/-/substanceinfo/100.239.148>

Sec 1	Quantification by titration
Sec 3	Quantification by atomic absorption spectrometry
Sec 4	Quantification by inductively coupled plasma (ICP)
Part 11	Determination of chromium (VI) content in leather,
Sec 1	Colorimetric method
Sec 2	Chromatographic method
Part 12	Determination of nitrogen content and hide substance by titrimetric method
Part 13	Determination of total silicon content by reduced molybdosilicate spectrometric method
Part 14	Determination of matter soluble in dichloromethane and free fatty acid content

The text of ISO standard has been approved as suitable for publication as an Indian Standard without deviations. Certain conventions and terminologies are, however, not identical to those used in Indian Standards. Attention is particularly drawn to the following:

- Wherever the words 'International Standard' appear referring to this standard, they should be read as 'Indian Standard'; and
- Comma (,) has been used as a decimal marker in the International Standard, while in Indian Standards, the current practice is to use a point (.) as the decimal marker.

In this adopted standard, reference appears to certain International Standards for which Indian Standards also exist. The corresponding Indian Standards, which are to be substituted in their respective places, are listed below along with their degree of equivalence for the editions indicated:

<i>International Standard</i>	<i>Corresponding Indian Standard</i>	<i>Degree of Equivalence</i>
ISO 2418 : 2024 Leather — Chemical, physical, mechanical and fastness tests — Position and preparation of specimens for testing	IS 5868 (Part 2) : XXXX/ISO 2418 : 2023 Leather — Method of sampling: Part 2 Position and preparation of specimens for testing for chemical physical mechanical and fastness tests (<i>second revision</i>) (<i>under preparation</i>)	Identical
ISO 4044 Leather — Chemical tests — Preparation of chemical test samples	IS 16256 : 2022/ISO 4044 : 2017 Leather — Chemical Tests — Preparation of chemical test samples (<i>first revision</i>)	Identical

The Committee has reviewed the provisions of the following International Standards referred in this adopted standard and has decided that they are acceptable for use in conjunction with this standard.

<i>International Standard</i>	<i>Title</i>
ISO 3696	Water for analytical laboratory use — Specification and test methods

In this adopted standard, reference appears to certain International Standards where the standard atmospheric conditions to be observed are stipulated which are not applicable to tropical/subtropical countries. The applicable standard atmospheric conditions for Indian conditions are (27 ± 2) °C and (65 ± 5) percent, relative humidity and shall be observed while using this standard.

In reporting the result of a test or analysis made in accordance with this standard, if the final value, observed or calculated, is to be rounded off, it shall be done in accordance with IS 2 : 2022 'Rules for rounding off numerical values (*second revision*)'.

Bureau of Indian Standards

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Review of Indian Standards

Amendments are issued to standards as the need arises on the basis of comments. Standards are also reviewed periodically; a standard along with amendments is reaffirmed when such review indicates that no changes are needed; if the review indicates that changes are needed, it is taken up for revision. Users of Indian Standards should ascertain that they are in possession of the latest amendments or edition by referring to the website-www.bis.gov.in or www.standardsbis.in.

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Amendments Issued Since Publication

Amend No.	Date of Issue	Text Affected

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