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**Leather — Chemical tests for the
determination of certain azo colorants in
dyed leathers —**

**Part 2:
Determination of 4-aminoazobenzene**

*Cuir — Essais chimiques pour le dosage de certains colorants azoïques
dans les cuirs teints —*

Partie 2: Dosage du 4-aminoazobenzène



Reference number
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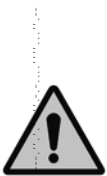
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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

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.

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.

ISO 17234-2 was prepared by the European Committee for Standardization (CEN) Technical Committee CEN/TC 289, *Leather*, in collaboration with the Chemical Test Commission of the International Union of Leather Technologists and Chemists Societies (IUC Commission, IULTCS), in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement). This method is technically similar to the method in IUC 20-2.

IULTCS, originally formed in 1897, is a world-wide organization of professional leather societies to further the advancement of leather science and technology. IULTCS has three Commissions, which are responsible for establishing international methods for the sampling and testing of leather. ISO recognizes IULTCS as an international standardizing body for the preparation of test methods for leather.

ISO 17234 consists of the following parts, under the general title *Leather — Chemical tests for the determination of certain azo colorants in dyed leathers*:

- *Part 1: Determination of certain aromatic amines derived from azo colorants*
- *Part 2: Determination of 4-aminoazobenzene*

Leather — Chemical tests for the determination of certain azo colorants in dyed leathers —

Part 2: Determination of 4-aminoazobenzene

1 Scope

This part of ISO 17234 is supplementary to ISO 17234-1 and describes a special procedure to detect the use of certain azo colorants in commodities, which can release 4-aminoazobenzene. The procedure also detects 4-aminoazobenzene (Solvent Yellow 1) which is already available as free amine in commodities without reducing pretreatment.

Azo colorants that are able to form 4-aminoazobenzene generate, under the conditions of ISO 17234-1, the amines aniline and 1,4-phenylenediamine. The presence of these 4-aminoazobenzene colorants cannot be reliably ascertained without additional information (e.g. the chemical structure of the colorant used) or without a special procedure.

The use of certain azo colorants, which may release, by reductive cleavage of their azo group(s), one or more of the other aromatic amines listed in Annex XVII of Regulation (EC) No. 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), except 4-aminoazobenzene, cannot be determined quantitatively with this method.

2 Normative references

The following referenced documents are indispensable for the application 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 and mechanical and fastness tests — Sampling location*

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

3 General

Certain azo colorants may release by reductive cleavage of their azo group(s), 4-aminoazobenzene, which is proscribed under Annex XVII of Regulation (EC) No. 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH).

Table 1 — 4-aminoazobenzene proscribed under Regulation REACH 1907/2006/Annex XVII

No.	CAS number	Index number	EC number	Substance
22	60-09-3	611-008-00-4	200-453-6	4-aminoazobenzene

4 Principle

After degreasing, the leather sample is treated with sodium dithionite in an alkaline solution at 40 °C in a closed vessel. 4-Aminoazobenzene, which is released in the process, is transferred to a *tert*-butyl methyl ether phase by means of liquid/liquid extraction. An aliquot of the *tert*-butyl methyl ether phase is used for analysis.

The detection and determination of 4-aminoazobenzene is performed using high-performance liquid chromatography (HPLC) with a diode array detector (DAD) or mass selective detector (HPLC/MS), capillary gas chromatography with a mass-selective detector (GC/MS) or capillary electrophoresis with a diode array detector (CE/DAD), or qualitatively with thin-layer chromatography (TLC, HPTLC).

If 4-aminoazobenzene is detected by one chromatographic method, confirmation shall be made using one or more alternative methods. Amine quantification is performed by HPLC/DAD.

5 Safety precautions

5.1 The compound 4-aminoazobenzene is classified as a substance suspected to be a human carcinogen.

Any handling and disposal of this substance shall be in strict accordance with the appropriate national health and safety regulations.

5.2 It is the user's responsibility to use safe and proper techniques when handling materials in this test method. Consult manufacturers for specific details, such as material safety data sheets and other recommendations.

5.3 Good laboratory practice should be followed. Wear safety glasses in all laboratory areas, and a dust respirator and single-use gloves while handling powder colorants and 4-aminoazobenzene.

5.4 Users should comply with any national and local safety regulations.

6 Apparatus

Usual laboratory equipment and, in particular, the following.

6.1 **Suitable reaction vessel**, of temperature-resistant glass with a gastight closure.

6.2 **Heating source**, that generates a temperature of (40 ± 2) °C.

6.3 **Separator**, with a revolution speed of more than 3 000 r/min.

6.4 **Vacuum rotary evaporator**.

6.5 **Pipettes**, in required or variable sizes.

6.6 **Ultrasonic bath**, at least 160 W, with controllable heating.

6.7 **Horizontal shaker**, with a sufficient frequency of 5 s^{-1} , path length 2 cm to 5 cm.

6.8 **Polypropylene or polyethylene syringe**, 2 ml.

6.9 **Instrumental equipment**

6.9.1 **Gas chromatography (GC)**, with mass-selective detector (MS).

6.9.2 **High-performance liquid chromatography (HPLC)**, with gradient elution and diode array detector (DAD) or mass-selective detector (MS).

6.9.3 Thin-layer chromatography (TLC), or high-performance thin-layer chromatography (HPTLC) equipment, including relevant detection.

6.9.4 Capillary electrophoresis (CE) with DAD.

NOTE A description of the equipment is given in Annex A.

7 Reagents

Unless otherwise specified, analytical grade chemicals shall be used.

7.1 Sodium hydroxide.

7.2 Aqueous sodium dithionite solution, $\rho = 200 \text{ mg/ml}^1$, freshly prepared, to be used immediately after resting for 1 h in a closed vessel.

7.3 Sodium hydroxide solution, $w = 2 \text{ \%}^2$.

7.4 *n*-Hexane.

7.5 *tert*-Butyl methyl ether.

7.6 Sodium chloride.

7.7 4-Aminoazobenzene, highest purity.

7.8 Internal standards for gas chromatography (IS), e.g.:

- IS1: naphthalene-d8 (CAS No.: 1146-65-2);
- IS2: 2,4,5-trichloroaniline (CAS No.: 636-30-6);
- IS3: benzidine-d8 (CAS No.: 92890-63-6);
- IS4: anthracene-d10 (CAS No.: 1719-06-8).

7.9 Standard solutions

7.9.1 Internal standard solution (IS), in *tert*-butyl methyl ether, $\rho = 10,0 \text{ }\mu\text{g/ml}$.

7.9.2 4-Aminoazobenzene calibration solution, for checking the experimental procedure and preparation of calibration solutions, 4-aminoazobenzene in methanol, $\rho = 500 \text{ }\mu\text{g/ml}$.

8 Sampling

Sample in accordance with ISO 2418 and grind the leather in accordance with ISO 4044. If sampling in accordance with ISO 2418 is not possible (e.g. in the case of leathers from finished products like shoes, garments, etc.), details about sampling shall be given in the test report. Any traces of adhesives shall be removed mechanically.

1) ρ = mass concentration

2) w = mass fraction (percentage by mass)

In the case of leather patchwork fabrics with varicoloured patterns, the various colours have to be taken into account separately as far as possible. For commodities consisting of various leather qualities, specimens of the various qualities shall be analysed separately.

9 Procedure

9.1 Degreasing

Treat more than 1,0 g of leather cut into pieces (25 mm²) or a ground leather sample in a closed 50 ml vessel (6.1) with 20 ml of *n*-hexane (7.4) in an ultrasonic bath (6.6) at (40 ± 2) °C for 20 min.

Decant the *n*-hexane layer from the leather sample. Any loss of leather particles during decanting shall be avoided. Directly after decanting, treat the sample in the same way as before with 20 ml of *n*-hexane. Evaporate the residual *n*-hexane at least overnight in the open vessel.

9.2 Reductive cleavage

Add 9 ml of sodium hydroxide solution (7.3) to the sample. Tightly close the reaction vessel and shake vigorously. All fibres of the leather material should be wetted.

Subsequently, add 1,0 ml of the aqueous sodium dithionite solution (7.2) with a syringe (6.8). The sample and reduction solution is shaken vigorously and immediately kept at (40 ± 2) °C for exactly 30 min. Then cool it to room temperature (20 °C to 25 °C) for 1 min with water or, if necessary, with a cooling mixture of ice, water and salt.

9.3 Separation and concentration of 4-aminoazobenzene

Add 5 ml of *tert*-butyl methyl ether (7.5) or 5 ml of internal standard solution (7.9.1) to the reaction solution. Subsequently, add 7 g of sodium chloride (7.6) and shake the mixture constantly for 45 min; with a shaking frequency of $f = 5 \text{ s}^{-1}$ (6.7). For complete phase separation, centrifuge the mixture after shaking.

The delay time between cooling down and shaking should not be longer than 5 min.

For subsequent analysis, transfer an aliquot of the *tert*-butyl methyl ether phase into an appropriate vial, which is closed immediately. Perform the detection and determination of 4-aminoazobenzene using the chromatographic techniques listed in 6.9.

RECOMMENDATION — For some analysis techniques, it may be necessary to concentrate the extract from 9.3 or to transfer it to another appropriate solvent (e.g. methanol). It is recommended to concentrate the *tert*-butyl methyl ether extract to about 1 ml (not to dryness) in a rotary vacuum evaporator in a slight vacuum at not more than 50 °C. Then remove the remainder of the solvent very carefully without vacuum by means of a weak flow of inert gas.

NOTE 1 Removal of the solvent (concentration in the rotary vacuum evaporator, evaporation to dryness) can lead to substantial loss of 4-aminoazobenzene if not performed under controlled conditions.

NOTE 3 If possible, avoid changing the solvent as; in the course of the analytical procedure, severe losses of analyte can result due to matrix effects.

9.4 Calibration solution

Add 5 ml of *tert*-butyl methyl ether (7.5) or 5 ml of internal standard solution (7.9.1) to 100 µl of the 4-aminoazobenzene calibration solution (7.9.2). This mixture is used for calibration as the recovery of 4-aminoazobenzene via phase partition according to this procedure is 95 % to 100 %.

9.5 Check of the analytical system

To check the procedure, 100 µl of the 4-aminoazobenzene calibration solution (7.9.2) are treated according to 9.2 and 9.3. The 4-aminoazobenzene recovery rate shall be a minimum of 60 %.

9.6 Chromatographic analyses

The detection of 4-aminoazobenzene can be performed using the chromatographic techniques listed in 6.9. Other validated methods may be used. The quantification of 4-aminoazobenzene is performed by means of HPLC/DAD or GC/MS; where gas chromatography is used, appropriate internal standards (7.8) shall be employed.

10 Evaluation

The amount of 4-aminoazobenzene is usually calculated by means of a software program. The calculation can be carried out manually from the peak areas.

10.1 Calculation with internal standard

The 4-aminoazobenzene level is calculated as a mass fraction, w , in milligrams per kilogram (mg/kg) of the specimen, according to the following equation:

$$w = \rho_C \times \frac{A_S \times A_{ISC} \times V}{A_C \times A_{ISS} \times m_E}$$

where

- ρ_C is the concentration of 4-aminoazobenzene in the calibration solution, in micrograms per millilitre (µg/ml);
- A_S is the peak area of 4-aminoazobenzene in the specimen solution, in area units;
- A_C is the peak area of 4-aminoazobenzene in the calibration solution, in area units;
- A_{ISS} is the peak area of the internal standard in the specimen solution, in area units³⁾;
- A_{ISC} is the peak area of the internal standard in the calibration solution, in area units⁴⁾;
- V is the volume of the specimen according to 9.3 (final specimen volume), in millilitres (ml); here: 5 ml;
- m_E is the mass of the leather specimen, in grams (g).

10.2 Calculation without internal standard

The 4-aminoazobenzene level is calculated as a mass fraction, w , in milligrams per kilogram (mg/kg) of the specimen according to the following equation:

$$w = \rho_C \times \frac{A_S \times V}{A_C \times m_E}$$

3) For quantification by means of GC/MS.

4) For quantification by means of GC/MS.

where

ρ_c is the concentration of 4-aminoazobenzene in the calibration solution, in micrograms per millilitre ($\mu\text{g/ml}$);

A_s is the peak area of 4-aminoazobenzene in the specimen solution, in area units;

A_c is the peak area of 4-aminoazobenzene in the calibration solution, in area units;

V is the volume of the specimen according to 9.3 (final specimen volume), in millilitres (ml); here: 5 ml;

m_E is the mass of the leather specimen, in grams (g).

10.3 Reliability of the method

For the reliability of the method, see Annex B.

11 Test report

The test report shall refer to this official method and state at least the following particulars:

- a) a reference to this International Standard;
- b) kind, origin and designation of the specimen (partial specimen, if applicable);
- c) date of receipt and date of analysis;
- d) sampling procedure;
- e) detection method and quantification method;
- f) results reported as level and detection limit of 4-aminoazobenzene, in milligrams per kilogram (mg/kg). Care should be taken in the interpretation of less than 30 mg/kg of 4-aminoazobenzene (see Annex C).

11

Annex A (informative)

Chromatographic analyses

A.1 Preliminary remark

As the instrumental equipment (6.9) of the laboratories may vary, no generally applicable instructions can be provided for chromatographic analyses. The following parameters have been successfully tested and used.

A.2 High-performance liquid chromatography (HPLC) (quantitative procedure)

A.2.1 High-performance liquid chromatography/diode array detector (HPLC/DAD)

Eluent 1: methanol

Eluent 2: 0,575 g of ammonium dihydrogenphosphate + 0,7 g of disodium hydrogenphosphate in 1 000 ml of water, pH = 6,9

Stationary phase: Zorbax SB-Phenyl®⁵⁾ (5 µm); (250 × 4,6) mm

Flow rate: (0,7 to 1,0) ml/min

Gradient: see Table A.1

Table A.1 — Gradient programme

Time min	Eluent 1 %	Eluent 2 %
0	0	100
5	10	90
10	10	90
27	68	32
29	100	0

Column temperature: 30 °C

Injection volume: 10,0 µl

Detection: DAD, spectrograph

Quantification: at 240 nm, 280 nm, 305 nm and 380 nm

5) Zorbax SB-Phenyl® 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.

A.2.2 High-performance liquid chromatography/mass selective detector (HPLC/DAD/MS)

Eluent 1: acetonitrile
Eluent 2: ammonium acetate in 1 000 ml of water, 5 mmol, pH = 3,0
Stationary phase: Zorbax Eclipse XDB C18⁶⁾ (3,5 µm); (50 × 2,1) mm
Flow rate: 300 µl/min
Gradient: see Table A.2

Table A.2 — Gradient programme

Time min	Eluent 1 %	Eluent 2 %
0	10	90
1,5	20	80
7,5	90	10

Column temperature: 40 °C
Injection volume: 2,0 µl
Detection: quadrupole and/or ion-trap mass detector, scanning mode and/or MS daughter ion MS detection; DAD: for wavelengths, see A.2.1
Spray gas: nitrogen (bottled/generator)
Ionization: API electrospray positive, fragmentor 120 V

A.3 Capillary gas chromatography (GC/MS) (quantitative procedure)

Capillary column: ZB-5-(Zebtron)⁷⁾, length: 30 m, inside diameter: 0,25 mm, film thickness: 0,25 µm
Injector system: splitless
Injector temperature: 250 °C
Carrier gas: helium
Temperature programme: 50 °C (3 min), 50 °C to 280 °C (15 °C/min), 280 °C (6 min)
Injection volume: 1,0 µl, split 1:15
Detection: MS

6) Zorbax Eclipse XDB C18 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.

7) ZB-5-(Zebtron) 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.

A.4 Capillary electrophoresis (CE/DAD) (quantitative procedure)

200 µl of the sample solution (9.3) is mixed with 50 µl HCl ($c = 0,01$ mol/l) and passed through a membrane filter (0,2 µm). This solution is analysed by means of capillary zone electrophoresis.

Capillary 1:	56 cm, uncoated, inside diameter 50 µm, with extended light path
Capillary 2:	56 cm, coated with polyvinyl alcohol (PVA), inside diameter 50 µm, with extended light path
Buffer solution:	phosphate buffer solution ($c = 50$ mmol/l), pH = 2,5
Column temperature:	25 °C
Voltage:	30 kV
Injection time:	4 s
Flushing time:	5 s
Detection:	DAD 214 nm, 254 nm, spectrograph

A.5 Thin-layer chromatography (TLC) (qualitative procedure); HPTLC or TLC only for semi-quantitative confirmation

A.5.1

Plates (HPTLC):	silica gel 60 with fluorescence indicator F254, (20 × 10) cm
Applied volume:	(2 to 5) µl, applied as a dot
Mobile solvent 1:	chloroform/acetic acid (90 + 10) parts per volume

A.5.2

Plates (TLC):	silica gel 60, with fluorescence indicator F254, (20 × 20) cm
Applied volume:	10,0 µl, applied as a line
Mobile solvent 2:	chloroform/ethyl acetate/acetic acid (60 + 30 + 10) parts per volume
Mobile solvent 3:	chloroform/methanol (95 + 5) parts per volume
Mobile solvents 2 and 3:	successively without drying of the plates

A.5.3

Detection:	1. ultraviolet (UV) lamp
	2. after successive treatment with reagents 1 and 2, reaction time approximately 5 min
Reagent 1:	0,1 % NaNO ₂ in HCl ($c = 1$ mol/l)
Reagent 2:	0,2 % α -naphthol in KOH ($c = 1$ mol/l)

A.6 Examples of chromatograms and spectrums

Examples of chromatograms and spectrums are shown in Figures A.1 to A.4.

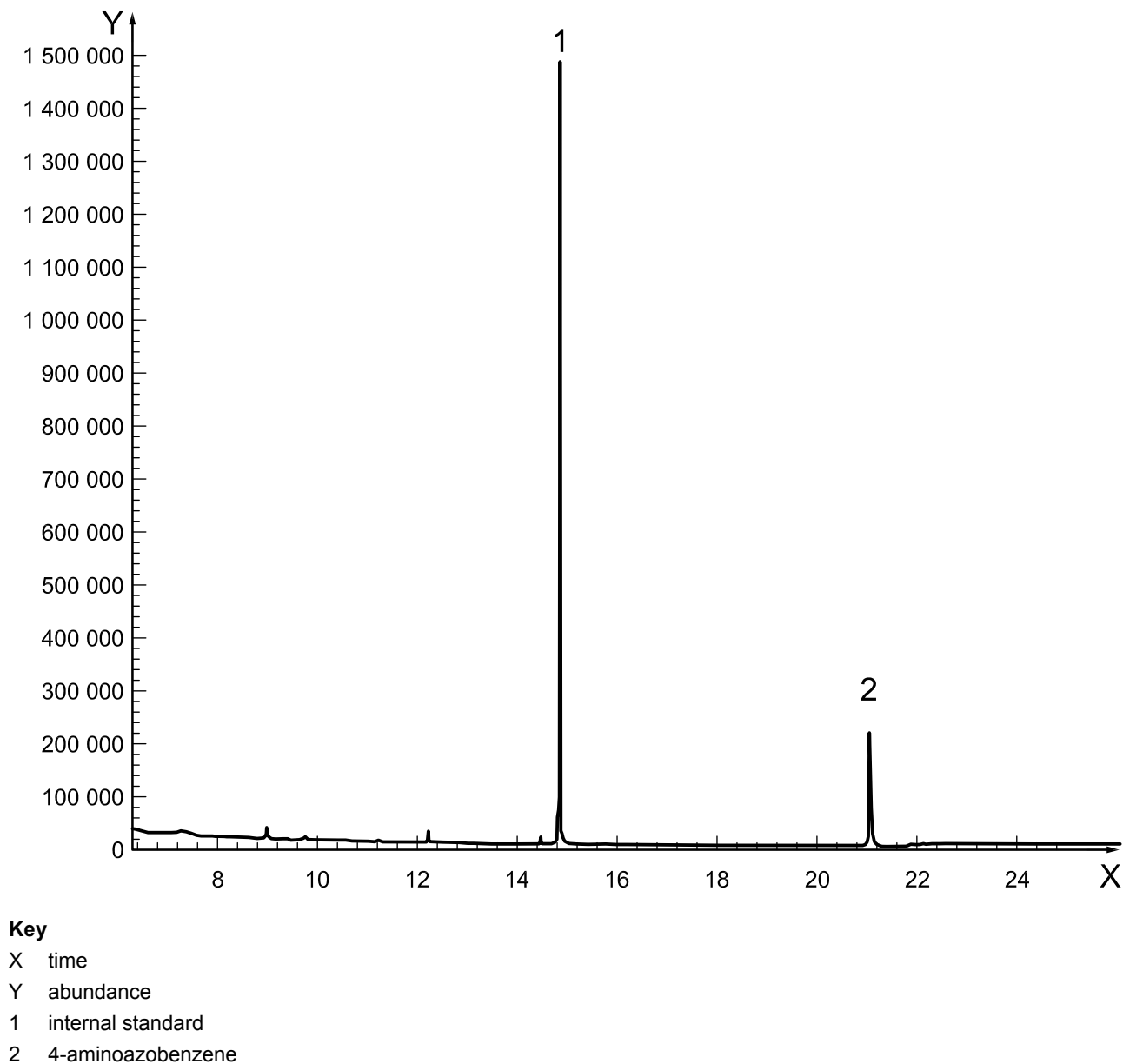


Figure A.1 — Total ion current chromatogram of 4-aminoazobenzene GC/MS

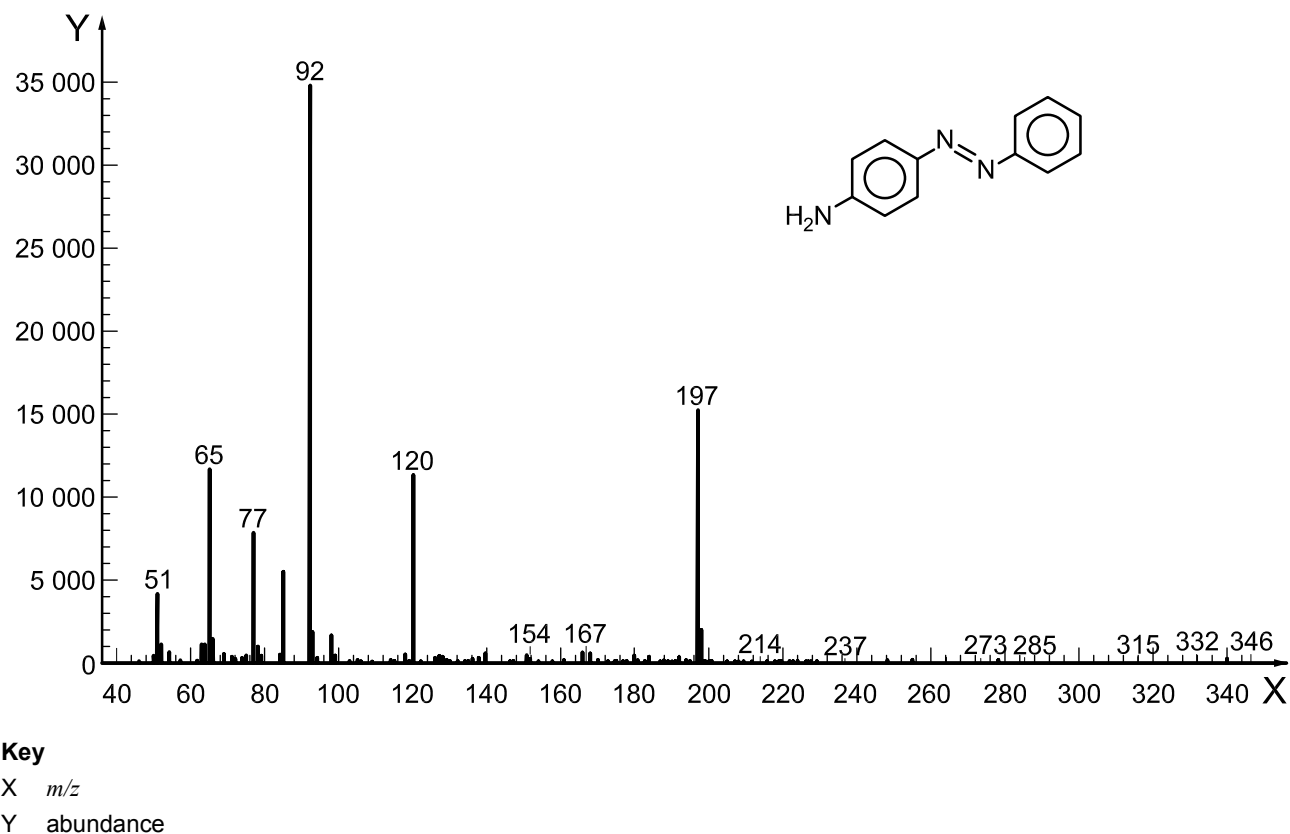
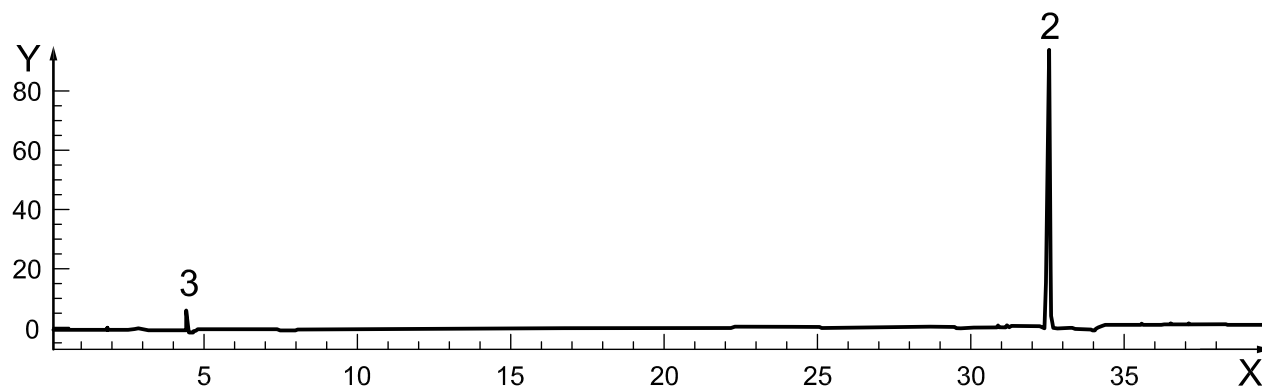
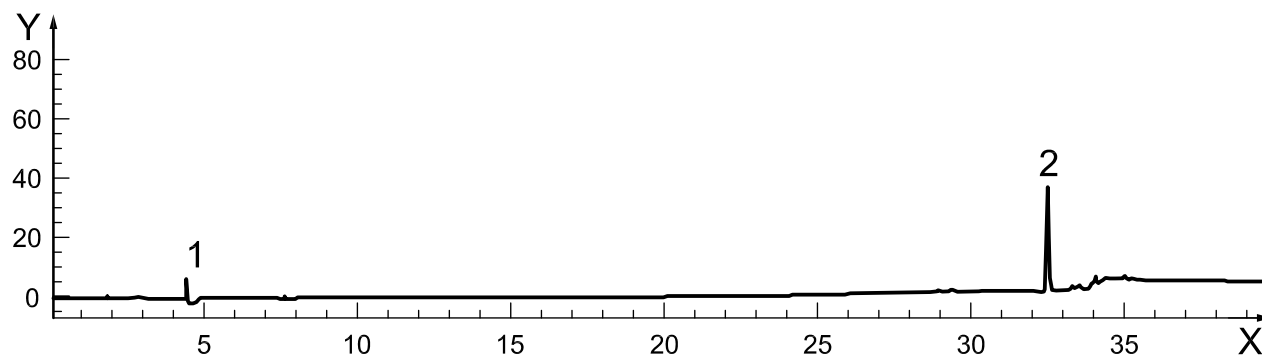


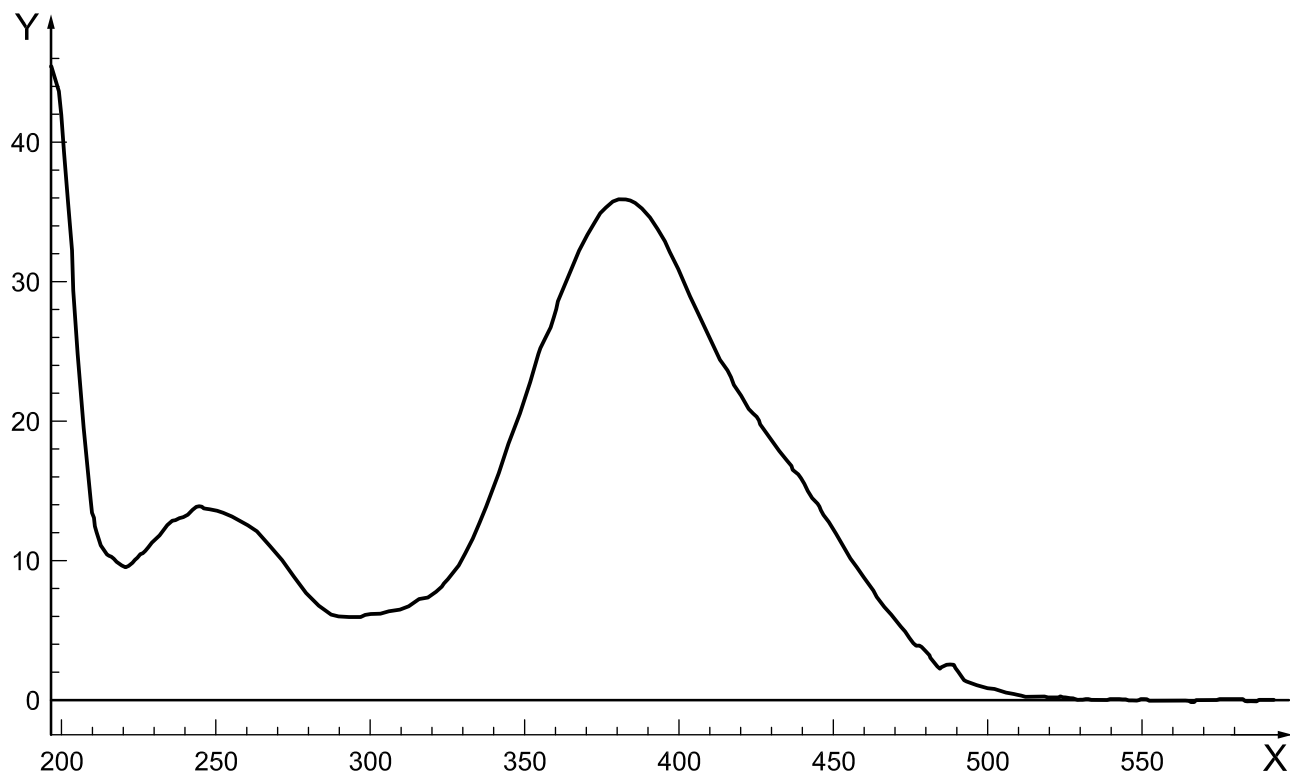
Figure A.2 — GC/MS 70 eV spectrum and structure of 4-aminoazobenzene



Key

- X min
- Y mAU
- 1 DAD A, Sig = 240
- 2 4-aminoazobenzene
- 3 DAD B, Sig = 380

Figure A.3 — Chromatogram of 4-aminoazobenzene HPLC/DAD; detection at 240 nm and 380 nm



Key
X mn
Y mAU

Figure A.4 — HPLC/DAD spectrum of 4-aminoazobenzene

Annex B (informative)

Reliability of the method

Table B.1 refers to the interlaboratory trials carried out for the determination of 4-aminoazobenzene in leather.

Table B.1 — Results from interlaboratory trials — Determination of the formed level of 4-aminoazobenzene in leather^[1]

Parameter	Analytical procedure	
	GC/MS	HPLC
Number of participating laboratories	10	11
Number of outliers	2	2
Number of laboratories after elimination of outliers	8	9
Mean value, \bar{x} , mg/kg	81,9	91,2
Repeatability, r , mg/kg	21,4	27,1
Standard deviation of the repeatability, s_r , mg/kg	7,6	9,7
Reproducibility, R , mg/kg	32,5	35,9
Standard deviation of the reproducibility, s_R , mg/kg	11,6	12,5

This official method was developed by the working group “Analytic of proscribed azo colorants” of the German BVL and assayed in a ring test with 11 participants.

- a) Regarding the evaluation of repeatability and reproducibility of the ring test results, the following has to be considered:
- 1) The ring test demonstrated that the ratio of colorant to reducing agent and the age of the reducing agent can have a decisive influence on the quantitative result. Therefore, it is recommended to perform the reductive cleavage in strict accordance with the conditions described in 9.2 (time, temperature and amount particulars).
 - 2) Another important fact is liquid/liquid extraction, e.g. the separation of aqueous and organic phases to prevent further reaction of the azo link cleavage of 4-aminoazobenzene. Therefore, it is strictly recommended that the conditions described in 9.3 be accurately observed.
- b) The leather sample for the ring test was from a typical commodity, cut and mixed with an uncoloured leather. Thereby, the homogeneity of the material can be reduced.
- c) Application of other appropriate internal standards may lead to higher reliability of the GC/MSD procedure. This term, however, was not considered in the evaluation of the ring test.

Annex C (informative)

Assessment guide — Interpretation of analytical results

Since the derivation of amines in very small amounts may lead to false positive results, Annex XVII of the Regulation REACH 1907/2006 defines a limit value of 30 mg/kg of sample material. This value only applies to sample material that is homogenous in matrix and colouring, but not to a mixed sample of heterogeneous composition.

If the detected amount of 4-aminoazobenzene is over 30 mg/kg, it must be assumed that a certain azo colorant was used. Below 30 mg/kg it is at present not possible to make a reliable statement on the use of certain azo colorants without further information such as the type and/or purity of the colorants used or the other raw materials used.

In this context, it is recommended to report the analytical results as follows:

In the case of formed levels of 4-aminoazobenzene \leq 30 mg/kg

- According to the analysis carried out, azo colorants that can release 4-aminoazobenzene by reductive cleavage of their azo group/s were not detected in the commodity submitted.

In the case of formed levels of 4-aminoazobenzene $>$ 30 mg/kg

- According to the analysis carried out, it is suggested that the commodity submitted has been manufactured or treated using azo colorants that are proscribed under Annex XVII of Regulation REACH 1907/2006.

Bibliography

- [1] Amtliche Sammlung von Untersuchungsverfahren nach § 64 LFGB B 82.02-9:2006-09 / BVL B 82.02-9:2006-09 Berichtigung 2008-04 – Nachweis der Verwendung von Azofarbstoffen, die 4-Aminoazobenzol freisetzen können
- [2] Regulation (EC) No. 1907/2006 of the European Parliament and of the Council concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), Annex XVII

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