INTERNATIONAL STANDARD

ISO 13496

First edition
2000-03-01

Meat and meat products — Detection of colouring agents — Method using thin-layer chromatography

Viande et produits à base de viande — Détection des agents colorants — Méthode par chromatographie en couche mince

Reference number
ISO 13496:2000(E)

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Printed in Switzerland

Contents

Page

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 3.

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 International Standard may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

International Standard ISO 13496 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Subcommittee SC 6, *Meat and meat products*.

Annex B forms a normative part of this International Standard. Annexes A and C are for information only.

Meat and meat products — Detection of colouring agents — Method using thin-layer chromatography

1 Scope

This International Standard specifies a thin-layer chromatographic method for the detection of synthetic, water-soluble colouring agents in meat and meat products.

The following colouring agents can be detected with the method:

Tartrazine	Patent Blue V
Quinoline Yellow	Indigotine
Sunset Yellow FCF	Brilliant Black PN
Amaranth	Black 7984
Ponceau 4R	Fast Green FCF
Erythrosine	Blue VRS

Synonyms and identity numbers of these colouring agents are listed in annex A.

The plant colours and plant extracts which have been observed not to interfere with this method are listed in Table B.1. Natural colours which in some cases have been shown to interfere with this method are listed in Table B.2.

2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this International Standard. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

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

AOAC 46.1.08:1995, *Official Methods of Analysis* (AOAC International).

3 Term and definition

For the purposes of this International Standard, the following term and definition apply.

3.1
detection of colouring agents
detection of the presence or absence of colouring agents in accordance with the method specified in this International Standard

4 Principle

The colouring agents are extracted from a test portion with hot water and adsorbed onto polyamide powder. The extracted colouring agents are purified by column chromatography and the colours are eluted from the column. The colouring agents are identified by thin-layer chromatography.

5 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified.

5.1 Water, complying with at least grade 3 in accordance with ISO 3696.

5.2 Petroleum ether, boiling range 40 °C to 60 °C.

5.3 Methanol.

5.4 Ammonia, 25 % aqueous solution, ρ_{20} = 0,910 g/ml.

5.5 Acetic acid, 100 % mass fraction, ρ_{20} = 1,050 g/ml.

5.6 Trisodium citrate dihydrate.

5.7 Propan-1-ol.

5.8 Ethyl acetate.

5.9 2-Methyl-2-propanol.

5.10 Propionic acid.

5.11 Eluent solution for column chromatography.

Mix 95 volumes of methanol (5.3) with 5 volumes of ammonia solution (5.4).

5.12 Acetic acid, 50 % solution in methanol.

Mix 1 volume of acetic acid (5.5) with 1 volume of methanol (5.3).

5.13 Polyamide powder, of particle size 0,05 mm to 0,16 mm.

5.14 Sand, fine granular, hydrochloric acid-washed, neutralized and calcinated.

5.15 Standard reference colours.

The purities of the standard colours may vary so it is necessary to know the purity of the colours to be used as standards. The purity shall be determined by the method AOAC 46.1.08.

NOTE Certified food colours may also be used as standards.

5.16 Standard reference solutions for thin-layer chromatography

Separately make solutions in water of each of the standard reference colours (5.15) with a standard colour content of about 1 g/l.

Prepare solutions of indigotine on the day of use. Other solutions will keep for at least 3 months (solutions of erythrosine for 1 month) when stored in the dark.

5.17 Eluent for thin-layer chromatography: solution I

Weigh, to the nearest 0,1 g, 25 g of trisodium citrate dihydrate (5.6) into a 1 000 ml one-mark volumetric flask. Dissolve in water, dilute to the mark with water and mix.

Mix 80 volumes of this citrate solution with 20 volumes of ammonia solution (5.4) and 12 volumes of methanol (5.3).

To avoid or reduce interference from safflor or saffran, it is advisable to use chromatography solution II (5.18).

5.18 Eluent for thin-layer chromatography: solution II

Mix 6 volumes of propan-1-ol (5.7) with 1 volume of ethyl acetate (5.8) and 3 volumes of water.

5.19 Eluent for thin-layer chromatography: solution III

Mix 50 volumes of 2-methyl-2-propanol (5.9) with 12 volumes of propionic acid (5.10) and 38 volumes of water.

6 Apparatus

Usual laboratory apparatus and, in particular, the following.

6.1 Mechanical or **electrical homogenizing equipment,** capable of homogenizing the laboratory sample.

Use a high-speed rotational cutter, or a mincer fitted with a plate with apertures not exceeding 4,0 mm in diameter.

6.2 Centrifuge tubes, of capacity 75 ml, made of glass.

6.3 Flat-bottomed flasks, of capacity 250 ml, with ground glass stoppers.

6.4 Round-bottomed flasks, of capacity 100 ml, with ground glass joint.

6.5 Centrifuge, operating at a radial acceleration of about 2 000 g_n.

6.6 Rotary evaporator.

6.7 Chromatographic column, of glass, with fritted filter and tap, of length about 20 cm, diameter about 30 mm, filter pore size 40 μm to 100 μm (porosity grade P 100 according to ISO 4793 [2]).

Put some glass wool in the column and add 1 g to 2 g of sand (5.14).

6.8 Plastics container, of volume about 10 ml, with lid.

6.9 Thin-layer plates, coated with a layer of cellulose powder of 0,10 mm thickness, or equivalent.

Ready-to-use plates are suitable.

6.10 Micropipettes, of capacity approximately 5 μl.

6.11 pH-meter, accurate to within 0,1 pH unit.

7 Sampling

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 3100-1 [1].

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage.

Proceed from a representative sample of at least 200 g. Store the sample in such a way that deterioration and change in composition are prevented.

8 Preparation of test sample

Homogenize the laboratory sample with the appropriate equipment (6.1). Take care that the temperature of the sample material does not rise above 25 °C. If a mincer is used, pass the sample at least twice through the equipment.

Fill a suitable airtight container with the prepared sample. Close the container and store in such a way that deterioration and change in composition of the sample are prevented. Analyse the sample as soon as practicable, but always within 24 h after homogenization.

9 Procedure

WARNINGS

If the sample contains indigotine, the temperature shall not at any time during the analysis exceed 35 °C. Indigotine partially decomposes in chromatography solution I, so chromatography solution II shall be used.

Erythrosine is sensitive to light. When pausing in the course of the analysis, solutions and plates shall be stored in the dark. The same also holds for indigotine.

9.1 Test portion

Weigh, to the nearest 0,1 g, 5 g of the prepared test sample (see clause 8) into a centrifuge tube (6.2).

For fatty samples, proceed in accordance with 9.2.

For non-fatty samples, proceed in accordance with 9.3.

9.2 Fatty samples

Add about 20 ml of petroleum ether (5.2) to the centrifuge tube and mix with a glass rod. Decant the petroleum ether.

Repeat this procedure three times.

9.3 Non-fatty samples

Add 25 ml of boiling water (see warning above) and mix. Add 25 ml of the eluent solution(5.11).

Check that the pH is 9 ± 0,5 using the pH-meter (6.11). If not, **adjust the pH** with acetic acid (5.5) or ammonia solution (5.4).

Mix well. Chill the sample in a freezer for 15 min (to prevent turbidity).

Centrifuge (6.5) for 10 min at a radial acceleration of about 2000 g_n.

Decant the clear solution into a flat-bottomed flask (6.3). In the case of indigotine, use a round-bottomed flask (6.4).

Add 5 ml of water to the centrifuge tube containing the residue. Mix and add 10 ml of the eluent solution (5.11). Mix and centrifuge as above.

Repeat the procedure until all colour has been extracted from the sample then combine all extracts.

Evaporate the combined extract on a water bath to about 25 ml in order to remove methanol. In the case of indigotine, use a round-bottomed flask (6.4) and the rotary evaporator (6.6) at 35 °C.

Add 25 ml of boiling water (see warnings) and mix.

9.4 Transfer of the colours to polyamide powder

Using acetic acid (5.5) or ammonia solution (5.4).adjust the pH to between 4 and 5.

Add 1 g of polyamide powder (5.13) to the warm solution (see warnings). Shake vigorously for 1 min.

Allow the powder to form a sediment.

Check that no colour remains in the solution. If the solution is coloured, add some more polyamide powder and shake vigorously.

NOTE Some natural colours (see annex B) are not entirely adsorbed on the polyamide powder, leaving the solution coloured even if all synthetic colours have been completely adsorbed. It is usually possible to decide from the type of sample whether or not such natural colours are present.

Shake and transfer the warm suspension to the chromatographic column (6.7).

Rinse the flat-bottomed flask with three 10 ml portions of hot water (see warnings) and add the rinsings, portion by portion, to the column. Wash the column another three times with 10 ml portions of hot water (see warnings) and finally three times with 5 ml of methanol (5.3). If natural colours are eluted, continue washing the column with methanol until the eluted methanol is colourless.

9.5 Elution and concentration of isolated colours

Place a flask (6.4) under the column and elute the colours from the polyamide powder with 5 ml portions of the eluent solution (5.11), at an elution volume flow rate of 2 ml/min, until the polyamide is colourless.

Evaporate the eluate to dryness using the evaporator (6.6) at a temperature of at most 35 °C (see warnings).

Add 1,0 ml or 2,0 ml of eluent solution (5.11) depending on the amount and number of colours and dissolve the residue. Transfer the colour solution to a plastics container (6.8).

9.6 Thin-layer chromatographic separation

9.6.1 Standard reference plates

Prepare three standard reference thin-layer chromatographic plates. Using a micropipette (6.10), dispense a spot of about 5 µl (diameter < 5 mm) of each standard solution (5.16) separately on each plate (6.9). Develop these separately, one with each chromatography eluent (5.17, 5.18 and 5.19) in an unsaturated tank until the solvent front is about 10 cm to 12 cm from the starting line. Remove the plates from the tank and dry in air under a hood. Store the plates in the dark. The spots, except for that of indigotine, are stable for several years.