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Meat and meat products — Detection and determination of colouring agents

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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.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 6, *Meat, poultry, fish, eggs and their products*.

This second edition cancels and replaces the first edition (ISO 13496:2000), which has been technically revised. The main changes compared with the previous edition are as follows:

— a new test method, high performance liquid chromatography (HPLC), has been added;

— the order of the clauses has been rearranged;

— the title of the document has been modified;

— the Scope has been modified.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Meat and meat products — Detection and determination of colouring agents

1 Scope

This document specifies a detection method using thin-layer chromatography and a determination method using high performance liquid chromatography (HPLC) for synthetic colouring agents in meat and meat products.

This document specifies the HPLC method as the reference method.

This document is applicable to meat and meat products, including livestock and poultry products.

The method using thin-layer chromatography can detect the following colouring agents:

— Tartrazine
— Quinoline Yellow
— Sunset Yellow FCF
— Amaranth
— Ponceau 4R
— Erythrosine
— Patent Blue V
— Indigotine
— Brilliant Black PN
— Black 7984
— Fast Green FCF
— 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 B.1. Natural colours which in some cases have been shown to interfere with this method are listed in B.2.

The method using HPLC can detect the following colouring agents:

— Tartrazine
— Amaranth
— Ponceau 4R
— Sunset Yellow FCF
— Erythrosine
— Allura Red AC
— Brilliant Blue FCF
— New Red
— Carmoisine
— Indigotine

Chromatograms of these standard reference colours are shown in Annex D.

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

ISO 4793, *Laboratory sintered (fritted) filters — Porosity grading, classification and designation*

AOAC 46.1.08, *Official Methods of Analysis (AOAC International)*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

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

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

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

4 Principle

4.1 Thin-layer chromatography

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.

4.2 HPLC

The colouring agents are extracted from a test portion with hot water and adsorbed onto polyamide powder. The extracted colouring agents are injected into the column and chromatographed in HPLC in reverse phase (RP). The colouring agents are identified according to retention time and quantified with external standard method.

5 Sampling

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.

6 Preparation of test sample

Homogenize the laboratory sample with the appropriate equipment (7.2.1). 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 the composition of the sample are prevented. Analyse the sample as soon as practicable, but always within 24 h after homogenization.

7 Test method of thin-layer chromatography

7.1 Reagents

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

7.1.1 **Water**, conforming to at least grade 3 in accordance with ISO 3696.

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

7.1.3 Methanol.

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

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

7.1.6 Trisodium citrate dihydrate.

7.1.7 Propan-1-ol.

7.1.8 Ethyl acetate.

7.1.9 2-Methyl-2-propanol.

7.1.10 Propionic acid.

7.1.11 Eluent solution for column chromatography.

Mix 95 volumes of methanol (7.1.3) with five volumes of ammonia solution (7.1.4).

7.1.12 Acetic acid, 50 % solution in methanol.

Mix one volume of acetic acid (7.1.5) with one volume of methanol (7.1.3).

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

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

7.1.15 Standard reference colours.

The purities of the standard colours can 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 given in AOAC 46.1.08.

NOTE Certified food colours can also be used as standards.

7.1.16 Standard reference solutions for thin-layer chromatography.

Separately make solutions in water of each of the standard reference colours (7.1.15) 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 three months (solutions of Erythrosine for one month) when stored in the dark.

7.1.17 Eluent for thin-layer chromatography: solution I.

Weigh, to the nearest 0,1 g, 25 g of trisodium citrate dihydrate (7.1.6) 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 (7.1.4) and 12 volumes of methanol (7.1.3).

To avoid or reduce interference from safflor or saffran, it is advisable to use chromatography solution II (7.1.18).

7.1.18 Eluent for thin-layer chromatography: solution II.

Mix six volumes of propan-1-ol (7.1.7) with one volume of ethyl acetate (7.1.8) and three volumes of water.

7.1.19 Eluent for thin-layer chromatography: solution III.

Mix 50 volumes of 2-methyl-2-propanol (7.1.9) with 12 volumes of propionic acid (7.1.10) and 38 volumes of water.

7.2 Apparatus

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

7.2.1 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.

7.2.2 Centrifuge tubes.

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

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

7.2.5 Centrifuge, operating at a radial acceleration of about 2 000*g*.

7.2.6 Rotary evaporator.

7.2.7 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 in accordance with ISO 4793).

Put some glass wool in the column and add 1 g to 2 g of sand (7.1.14).

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

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

Ready-to-use plates are suitable.

7.2.10 Micropipettes, of capacity approximately 5 µl.

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

7.3 Procedure

WARNING — 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.

WARNING — 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.

7.3.1 Test portion

Weigh, to the nearest 0,1 g, 5 g of the prepared test sample (see Clause 6) into a centrifuge tube (7.2.2).

For fatty samples, proceed in accordance with 7.3.2.

For non-fatty samples, proceed in accordance with 7.3.3.

7.3.2 Fatty samples

Add about 20 ml of petroleum ether (7.1.2) to the centrifuge tube and mix with a glass rod. Decant the petroleum ether.

Repeat this procedure three times.

7.3.3 Non-fatty samples

Add 25 ml of boiling water (see warning above) and mix. Add 25 ml of the eluent solution (7.1.11).

Check that the pH is 9 ± 0,5 using the pH-meter (7.2.11). If not, adjust the pH with acetic acid (7.1.5) or ammonia solution (7.1.4).

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

Centrifuge (7.2.5) for 10 min at a radial acceleration of about 2 000*g*.

Decant the clear solution into a flat-bottomed flask (7.2.3). In the case of Indigotine, use a round-bottomed flask (7.2.4).

Add 5 ml of water to the centrifuge tube containing the residue. Mix and add 10 ml of the eluent solution (7.1.11). Mix and centrifuge as above.

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

Evaporate the combined extracts in a water bath to about 25 ml in order to remove the methanol. In the case of Indigotine, use a round-bottomed flask (7.2.4) and the rotary evaporator (7.2.6) at 35 °C.

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

7.3.4 Transfer of the colours to polyamide powder

Using acetic acid (7.1.5) or ammonia solution (7.1.4) adjust the pH to between 4 and 5.

Add 1 g of polyamide powder (7.1.13) 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 (7.2.7).

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 (7.1.3). If natural colours are eluted, continue washing the column with methanol until the eluted methanol is colourless.

7.3.5 Elution and concentration of isolated colours

Place a flask (7.2.4) under the column and elute the colours from the polyamide powder with 5 ml portions of the eluent solution (7.1.11), at an elution volume flow rate of 2 ml/min, until the polyamide is colourless.

Evaporate the eluate to dryness using the evaporator (7.2.6) at a temperature of at most 35 °C (see warnings).

Add 1,0 ml or 2,0 ml of eluent solution (7.1.11) depending on the amount and number of colours and dissolve the residue. Transfer the colour solution to a plastics container (7.2.8).

7.3.6 Thin-layer chromatographic separation

7.3.6.1 Standard reference plates

Prepare three standard reference thin-layer chromatographic plates. Using a micropipette (7.2.10), dispense a spot of about 5 µl (diameter, d < 5 mm) of each standard solution (7.1.16) separately on each plate (7.2.9). Develop these separately, one with each chromatography eluent (7.1.17, 7.1.18 and 7.1.19) 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.

7.3.6.2 Samples

Using a micropipette (7.2.10), apply to a thin-layer plate (7.2.9) a just-visible amount of sample solution (see 7.3.5). Dry using a hair dryer. In the case of Indigotine, dry in air.

Develop the plate in an unsaturated tank to a height of approximately 10 cm to 12 cm using a suitable chromatography solution (7.1.16, 7.1.17 or 7.1.18), i.e. the solution which gives the best separation of the colours detected in the sample (see Clause 1). Sometimes it will be necessary to prepare a second sample plate and develop this in one of the other two eluents to obtain the best separation.

Remove the plate from the tank and dry in air under a hood.

Compare the sample spots with the appropriate standard reference plate (see 7.3.6.1).

It is recommended that different amounts of sample solutions be applied in the case of mixtures of colorants, because colorants can be present in various concentrations in the concentrate.

Tailing is usually caused by inadequate purification. If this is the case, adsorb the colorant again with the adsorbent, wash with hot water and remove the adsorbent as previously described.

7.3.7 Confirmation

Confirm the identity of the colorants by chromatographing the concentrate (see 7.3.6.2) in a mixture of standards for the colorants identified in the first chromatogram.

In case of doubt, elute the colorant from the plate with a neutral solution (water or ethanol, or 0,2 g/l ammonium acetate solution), an acid (0,1 mol/l hydrochloric acid) and an alkali (0,1 mol/l sodium hydroxide solution), and compare the absorption spectrum of the colorant to that of the standard. See the absorbance spectra shown in Annex C.

8 Test method of HPLC

8.1 Reagents

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

8.1.1 Acetonitrile, HPLC quality.

8.1.2 Ammonium acetate.

8.1.3 Ammonium acetate solution (0,02 mol/l).

Weigh 1,54 g of ammonium acetate (8.1.2), add appropriate water to dissolve and dilute to 1 000 ml with water. Filter through 0,45 µm microporous membrane (8.2.2).

8.1.4 Methanol, 10 % solution in water.

Mix 10 volumes of methanol (7.1.3) with 90 volumes of water (7.1.1).

8.1.5 Stock solutions (1 mg/ml).

Separately make solutions in 10 % methanol (8.1.4) of each of the standard reference colours (7.1.15) with a standard colour content of about 1 mg/ml.

8.1.6 Working reference solutions (50 µg/ml).

Dilute the 1 mg/ml stock solutions (8.1.5) 20 times with 10 % methanol (8.1.4) and filter through 0,45 µm microporous membrane (8.2.2).

8.2 Apparatus

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

8.2.1 HPLC chromatographic system, with column thermostat and UV/visible or diode array detector.

8.2.2 Micro filters with membranes (diameter of the pores: 0,45 µm).

8.3 Procedure

WARNING — If the sample contains Indigotine, the temperature shall not at any time during the analysis exceed 35 °C.

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

8.3.1 Test portion

Weigh, to the nearest 0,001 g, 5 g of the prepared test sample (see Clause 6) into a centrifuge tube (7.2.2).

For fatty samples, proceed in accordance with 8.3.2.

For non-fatty samples, proceed in accordance with 8.3.3.

8.3.2 Fatty samples

See 7.3.2.

8.3.3 Non-fatty samples

See 7.3.3.

8.3.4 Transfer of the colours to polyamide powder

See 7.3.4.

8.3.5 Elution and concentration of isolated colours

Place a flask (7.2.4) under the column and elute the colours from the polyamide powder with 5 ml portions of the eluent solution (7.1.11), at an elution volume flow rate of 2 ml/min, until the polyamide is colourless.

Evaporate the eluate to dryness using the evaporator (7.2.6) at a temperature of at most 35 °C (see warnings).

Add 1,0 ml or 2,0 ml of water (7.1.1) depending on the amount and number of colours and dissolve the residue. Filter the colour solution through 0,45 µm microporous membrane (8.2.2) for injection into the HPLC chromatographic system (8.2.1).

8.3.6 HPLC analysis

8.3.6.1 Operating conditions

The operating conditions are as follows:

a) Column: C18 (5 µm, 4,6 × 250 mm).

b) Mobile phase:

A: 0,02 mol/l ammonium acetate solution (8.1.3);

B: acetonitrile (8.1.1), elution gradient see Table 1.

c) Column temperature: 35 °C.

d) Flow rate: 1,0 ml/min.

e) Injection volume: 20 µl.

f) Wavelength range of diode array detector: 400 nm to 800 nm, or wavelength of UV detector detection: see Annex D.

Table 1 — Elution gradient

Time, min	**Phase A,** %	**Phase B,** %
0	95	5
3	65	35
7	0	100
10	0	100
10,1	95	5
21	95	5

8.3.6.2 Determination

Under above conditions, when the retention time for the peak of analyte in the unknown sample is the same as the retention time of the standard, the sample can be assumed to contain synthetical pigments. The chromatogram of synthetical pigments standard is given in Annex D. The method is quantified by the external standard curve. The responses of synthetical pigments in the sample solution should be in the linear range of the instrumental detection.

8.3.6.3 Parallel test

According to the above procedure, the same sample was tested in a parallel test.

8.3.6.4 Blank test

Except for weighing the sample, follow the procedure described above.

8.4 Calculation

The level of colorant is calculated as shown by Formula (1):

$$X = \frac{C \times V}{m} \tag{1}$$

where

X is the content of the colorant in the sample, in grams per kilogram (mg/kg);

C is the concentration of the colorant in the sample solution, in milligrams per litre (mg/l);

V is the final diluted volume of the sample solution, in millilitres (ml).

m is the sample mass, in grams (g);

The result is subtracted from the blank value.

Express the calculation result as the arithmetic average of two single test results obtained under repetitive conditions. Express the results to two significant figures. Interlaboratory testing results are shown in Annex E.

8.5 Precision

The absolute difference between two single test results obtained under repetitive conditions shall not exceed 10 % of the arithmetic mean.

8.6 Limit of detection (LOD) and limit of quantification (LOQ)

For Tartrazine, Amaranth, Ponceau 4R, Sunset Yellow FCF, Erythrosine, Allura Red AC, Brilliant Blue FCF, New Red, Carmoisine and Indigotine, the LOD is 0,15 mg/kg and the LOQ is 0,5 mg/kg.

9 Test report

The test report shall specify:

— all information necessary for the complete identification of the sample;

— the sampling method used, if known;

— the test method used, with reference to this document including its year of publication, i.e. ISO 13496:—;

— all operating details not specified in this document, or regarded as optional, together with details of any incidents which can have influenced the test result;

— the test result obtained, including a reference to the clause which explains how the results were calculated;

— the date of the test.

Annex A
(informative)

Synonyms and identity numbers of synthetic, water-soluble colouring agents

Table A.1 — Synonyms and identity numbers of synthetic, water-soluble colouring agents

Name	Synonym 1	Synonym 2	C.I.[a]	E No.[b]	CAS No.[d]
Tartrazine	FD&C Yellow No. 5		19140	E 102	1934-21-0
Quinoline Yellow			47005	E 104	8004-92-0
Sunset Yellow FCF	FD&C Yellow No. 6		15985	E 110	2783-94-0
Amaranth	FD&C Red No. 2	Naphthol Red S	16185	E 123	915-67-3
Ponceau 4R	New coccine	Cochineal Red A	16255	E 124	2611-82-7
Erythrosine	FD&C Red No. 3		45430	E 127	16423-68-0
Patent Blue V			42051	E 131	3536-49-0
Indigotine	FD&C Blue No. 2		73015	E 132	860-22-0
Brilliant Black PN			28440	E 151	2519-30-4
Black 7984			27755	E 152	2118-39-0
Fast Green FCF	FD&C Green No. 3		42053	[c]	2353-45-9
Blue VRS			2045	[c]	[c]
Allura Red AC			16035	E129	25956-17-16
Brilliant Blue FCF			42090	E133	3844-45-9
New Red			[c]	[c]	220658-76-4
Carmoisine	Azorubine		14720	E122	3567-69-6

[a] C.I.: Identity number according to the Colour Index[4].

[b] E No.: Current number within the European Community (EC).

[c] E No. is not available.

[d] CAS No.: Chemical abstracts service number.

Annex B
(informative)

Possible interference by colours

B.1 Colours which do not interfere

The following plant colours or plant extracts have been observed not to interfere with this method:

- alfalfa
- annatto (bixin and norbixin)
- anthocyanins
- beetroot red
- β-apocarotenal
- β-apocarotenic acid ethyl ester
- β-carotene
- canthaxanthin
- chlorophyll
- chlorophyllin copper complex
- flower of tagetes
- marigold
- mustard
- paprika oleoresin
- riboflavin
- tea
- tomato

B.2 Colours which can interfere

In some cases, natural colours have been observed to interfere with this method. Their uses in foods and the synthetic colours of which the determination can be affected are given in Table B.1.

Table B.1 — Colours which can interfere

Substance	Use in foods	Interferes with analysis of
Curcumin	Spice, also used as yellow colour	Quinoline Yellow (E 104)[a] Brilliant Black PN (E 151)[a] Black 7984 (E 152)[a]
Saffran	Spice, too expensive for use as a colour	Erythrosine (E 127)[b] Quinoline Yellow (E 104)[a,b] Brilliant Black PN (E 151)[a,b] Black 7984 (E 152)[b]
Safflor	Substitute for saffran	Tartrazine (E 102)[b]

[a] The interferences are minor and may be considered negligible.

[b] To avoid or reduce interference from safflor or saffran, it is advisable to use chromatography solution II (7.1.18).