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Meat and meat products — Detection and determination of colouring agents

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ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Email: copyright@iso.org
Website: www.iso.org

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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.

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).

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, *Food products*, Subcommittee SC 6, *Meat, poultry, fish, eggs and their products*.

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

This edition cancels and replaces the edition (ISO13496-2000), which has been technically revised.

The main changes compared to the previous edition are as follows:

— A new test method - high performance liquid chromatography (HPLC) is added.

— The clauses order of the document has been rearranged.

— The scope of the document has been modified.

— The title of the document has been modified.

Meat and meat products — Detection and determination of colouring agents

1 Scope

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

This document specifies 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	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.

The method using HPLC can detect the following colouring agents:

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

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

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 Terms and definitions

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

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 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, complying with 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, ρ20 = 0,910 g/ml.

7.1.5 Acetic acid, 100 % mass fraction, ρ20 = 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 5 volumes of ammonia solution (7.1.4).

7.1.12 Acetic acid, 50 % solution in methanol.

Mix 1 volume of acetic acid (7.1.5) with 1 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 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.

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 3 months (solutions of erythrosine for 1 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 6 volumes of propan-1-ol (7.1.7) with 1 volume of ethyl acetate (7.1.8) and 3 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

Usual laboratory apparatus and, in particular, the following.

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_n.

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 according to ISO 4793[2]).

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 2000 g_n.

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 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 (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 < 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 (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 (7.3.6.1).

It is recommended that different amounts of sample solutions be applied in the case of mixtures of colorants, because colorants may 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 (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 (0,02mol/l).

Weigh 1,54 g of ammonium acetate (8.1.2), add appropriate water to dissolve, and dilute to 1000 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 (1mg/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).

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

8.2 Apparatus

Usual laboratory apparatus and, in particular, the following.

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, 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 colour solution through 0,45 µm microporous membrane (8.2.2)for injection into HPLC chromatographic system (8.2.1).

8.3.6 HPLC analysis

8.3.6.1 Operating conditions

a) Column: C18 (5 µm, 4,6 x 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-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 determination condition, when the retention time for the peak of analyte in unknown sample is same as the retention time of the standard, we can judge there has synthetical pigments in the sample. Chromatogram of synthetical pigments standard is in Annex D. The method 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 parallel test.

8.3.6.4 Blank test

Except weighing the sample, follow the procedure described above.

8.4 Calculation

The level of colorant is calculated with the aid of the following equation:

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

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 liter (mg/l);

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

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

The result is subtracted from the blank value.

Express the calculation result as the arithmetic average of the two single test results obtained under the repetitive conditions. Express the results with two significant figures.

8.5 Precision

The absolute difference between two single test results obtained under the repetitive conditions must 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, carmosine and indigotine, LOD is 0,15 mg/kg and 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 International Standard;

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

— the test result obtained.

Annex A
(informative)

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

Table A.1 —

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]
Carmosine	Azorubine		14720	E122	3567-69-6
Allura Red AC			16035	E129	25956-17-16
Brilliant Blue FCF			42090	E133	3844-45-9
New Red			[c]	[c]	220658-76-4

[a] C.I.: Identity number according to 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
(normative)

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

β-carotene

β-apocarotenal

β-apocarotenic acid ethyl ester

canthaxanthin

chlorophyll

chlorophyllin copper complex

paprika oleoresin

riboflavin

marigold

beetroot red

mustard

flower of tagetes

tea

tomato

B.2 Colours which may 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 may be affected are given in Table B.1.

Table B.1

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 (5.18).