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Meat and meat products — Determination of L(—)-hydroxyproline content (Reference method)

Viande et produits à base de viande — Détermination de la teneur en L(—)-hydroxyproline (Méthode de référence)

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FOREWORD

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It has been approved by the member bodies of the following countries :

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New Zealand

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Meat and meat products — Determination of L(−)-hydroxyproline content (Reference method)

1 SCOPE AND FIELD OF APPLICATION

This International Standard specifies a reference method for the determination of the L(−)-hydroxyproline content of meat and meat products.

2 REFERENCE

ISO 3100, *Meat and meat products — Sampling.*

3 DEFINITION

L(−)-hydroxyproline content of meat and meat products : The amount of L(−)-hydroxyproline determined according to the procedure specified in this International Standard, and expressed as a percentage by mass.

4 PRINCIPLE

Hydrolysis of a test portion in constant-boiling hydrochloric acid solution containing tin(II) chloride. Filtration and dilution of the hydrolysate. Neutralization, with sodium hydroxide, of an aliquot portion of the diluted hydrolysate. Filtration and dilution. Oxidation of the L(−)-hydroxyproline by chloramine-T, followed by the formation of a red compound with *p*-dimethylaminobenzaldehyde. Photometric measurement at a wavelength of 558 nm.

5 REAGENTS

All reagents shall be of recognized analytical quality. The water used shall be distilled water or water of at least equivalent purity.

5.1 Hydrochloric acid solution containing tin(II) chloride.

Dissolve 7,5 g of tin(II) chloride dihydrate ($SnCl_2.2H_2O$) in water, dilute to 500 ml and add 500 ml of hydrochloric acid (ρ_{20} 1,19 g/ml).

5.2 Hydrochloric acid, approximately 6 M solution.

Mix equal volumes of hydrochloric acid (ρ_{20} 1,19 g/ml) and water.

5.3 Sodium hydroxide, approximately 10 M solution.

Dissolve 40 g of sodium hydroxide in water. Cool and dilute to 100 ml.

5.4 Sodium hydroxide, approximately 1 M solution.

Dissolve 4 g of sodium hydroxide in water. Cool and dilute to 100 ml.

5.5 Buffer solution, pH 6,0.

Dissolve in water :

50 g of citric acid monohydrate ($C_6H_8O_7.H_2O$);

26,3 g of sodium hydroxide;

146,1 g of sodium acetate trihydrate [$Na(CH_3CO_2).3H_2O$].

Dilute to 1 000 ml with water. Mix this solution with 200 ml of water and 300 ml of propan-1-ol.

This solution is stable for several weeks at 4 °C.

5.6 Chloramine-T reagent

Dissolve 1,41 g of *N*-chloro-*p*-toluenesulphonamide, sodium salt trihydrate (chloramine-T) in 10 ml of water and successively add 10 ml of propan-1-ol and 80 ml of the buffer solution (5.5).

Prepare this solution immediately before use.

5.7 Colour reagent

Dissolve 10,0 g of *p*-dimethylaminobenzaldehyde in 35 ml of perchloric acid solution [60 % (*m*/*m*)] and then slowly add 65 ml of propan-2-ol.

Prepare this solution on the day of use.

NOTE — If purification of the *p*-dimethylaminobenzaldehyde is necessary (see note to 8.5), proceed as follows :

Prepare a saturated solution of the *p*-dimethylaminobenzaldehyde in hot 70 % (*V*/*V*) ethanol. Cool, first at room temperature and finally in a refrigerator. After about 12 h, filter on a Buchner funnel. Wash with a little 70 % (*V*/*V*) ethanol. Again dissolve in hot 70 % (*V*/*V*) ethanol. Add cold water and agitate thoroughly. Repeat this procedure until a sufficient quantity of milk-white crystals has been formed. Place in the refrigerator overnight. Filter on the Buchner funnel, wash with 50 % (*V*/*V*) ethanol and vacuum dry over phosphorus(V) oxide.

5.8 L(−)-Hydroxyproline standard solutions

Prepare a stock solution by dissolving 50 mg of 4-hydroxypyrrolidine-α-carbonic acid (hydroxyproline) in water. Add 1 drop of the hydrochloric acid solution (5.2) and dilute to 100 ml with water. This solution is stable for at least one month at 4 °C.

On the day of use, dilute 5 ml of the stock solution to 500 ml with water in a volumetric flask. Then prepare four standard solutions by diluting 10 – 20 – 30 and 40 ml of this solution to 100 ml with water to obtain L(−)-hydroxyproline concentrations of 0,5 – 1 – 1,5 and 2 μg/ml respectively.

6 APPARATUS

Ordinary laboratory apparatus, and in particular :

6.1 Mechanical meat mincer, laboratory size, fitted with a plate with holes not exceeding 4 mm in diameter.

6.2 Round or flat bottom hydrolysis flask, capacity about 200 ml, wide-necked, equipped with an air-cooled or water-cooled condenser.

6.3 Electric heating device (for example, heating mantle, hot-plate, or electrically heated sand bath).

6.4 Filter paper disks, diameter 12,5 cm[1].

6.5 pH meter.

6.6 Aluminium or plastic foil.

6.7 Water bath, capable of being thermostatically controlled at 60 ± 0,5 °C.

6.8 Spectrophotometer, capable of being used at a wavelength of 558 ± 2 nm, or **photoelectric colorimeter** with an interference filter with absorption maximum at 558 ± 2 nm.

6.9 Glass cells of 10 mm optical path length.

6.10 Analytical balance.

7 SAMPLE

7.1 Proceed from a representative sample of at least 200 g. See ISO 3100.

7.2 Store the sample in such a way that deterioration and change in composition are prevented.

8 PROCEDURE

8.1 Preparation of test sample

8.1.1 *Raw meat and raw meat products*

Reduce intact meat to small cubes (approximately 0,5 cm^3, i.e. length of side approximately 8 mm) by cutting it while it is cold (just below 0 °C), using a sharp knife.

Either place the sample in a container and seal the latter hermetically, or vacuum pack the sample in heat-resistant plastic film; then heat so as to maintain a temperature of at least 70 °C for at least 30 min; cool and proceed as in 8.1.2.

During the remaining stages of preparation of the test sample and the weighing out of the test portions, ensure that the sample is kept well mixed and, in particular, that any fat or fluid is kept evenly distributed.

NOTE — The heat treatment softens the raw connective tissue and makes it less resistant to homogenization by mincing. However, it may also lead to separation of a fluid containing gelatine. The presence of fat may also demand special attention for the production of a homogeneous test sample.

8.1.2 *Cooked meat and cooked meat products*

Make the sample homogeneous by passing it at least twice through the meat mincer (6.1), and mixing. Keep the homogenized sample in a completely filled, air-tight, closed container and store it in such a way that deterioration and change in composition are prevented. Analyse the test sample as soon as possible, but always within 24 h.

8.2 Test portion

Weigh, to the nearest 0,001 g, about 4 g of the test sample into the hydrolysis flask (6.2). Ensure that none of the sample adheres to the side wall of the flask.

8.3 Hydrolysis

8.3.1 Place some silicon carbide boiling chips in the flask and add 100 ± 1 ml of the hydrochloric acid solution containing tin(II) chloride (5.1). Heat to gentle boiling using the heating device (6.3), and maintain for 16 h under reflux (conveniently overnight).

NOTE — If desired by the analyst, the hydrolysis may alternatively be accomplished in two periods, each of 7 to 8 h, on consecutive days. This alternative procedure has been proved experimentally to yield results that are not significantly different from those obtained with a single hydrolysis period of 16 h.

8.3.2 Filter the hot hydrolysate through filter paper (6.4) into a 200 ml one-mark volumetric flask. Wash the flask and filter paper three times with 10 ml portions of hot hydrochloric acid solution (5.2) and add the washings to the hydrolysate. Make up to the mark with water and mix.

1) For example, S and S No. 287 and Whatman GF/A are suitable.

Continue the determination as soon as possible, but at the latest on the day after hydrolysis.

8.4 Colour development and measurement of absorbance

8.4.1 Using a pipette, transfer into a beaker a volume *V* ml of the hydrolysate (8.3.2) so that, after dilution to 250 ml (see 8.4.2), the L(−)-hydroxyproline concentration will be within the range 0,5 to 2 µg/ml.

NOTE — In most cases, *V* will be of the order of 5 to 25 ml depending on the amount of connective tissue present in the sample.

8.4.2 With the aid of the pH meter (6.5), adjust the pH to 8 ± 0,2 by the addition, drop by drop, first of the 10 M sodium hydroxide solution (5.3) and then, when approaching the required pH, of the 1 M sodium hydroxide solution (5.4). Filter the contents of the beaker into a 250 ml one-mark volumetric flask. Wash the beaker and the tin hydroxide precipitate on the filter paper at least three times with 30 ml portions of water, collecting the washings in the volumetric flask. Make up to the mark with water and mix.

8.4.3 Transfer 4,00 ml of this solution into a test tube and add 2,00 ml of the chloramine-T reagent (5.6). Mix and leave at room temperature for 20 ± 1 min.

8.4.4 Add 2,00 ml of the colour reagent (5.7), mix thoroughly and cap the tube with aluminium or plastic foil (6.6).

8.4.5 Transfer the tube quickly into the water bath (6.7), controlled at 60 ± 0,5 °C, and heat for exactly 20 min.

8.4.6 Cool under running tap water for at least 3 min.

8.4.7 Measure the absorbance at 558 ± 2 nm in a glass cell (6.9) against water, using the spectrophotometer or the photoelectric colorimeter equipped with an interference filter (6.8).

8.4.8 Subtract the absorbance measured for the blank solution (8.5) and read the L(−)-hydroxyproline concentration of the diluted hydrolysate from the calibration graph obtained as described in 8.6.

8.5 Blank test

Carry out in duplicate the procedure described in 8.4.3 to 8.4.8 inclusive, substituting water for the diluted hydrolysate.

NOTE — If the absorbance of the blank exceeds 0,040, a fresh colour reagent (5.7) should be prepared and, if necessary, the *p*-dimethylaminobenzaldehyde should be purified (see note to 5.7).

8.6 Calibration graph

8.6.1 Carry out the procedure described in 8.4.3 to 8.4.8 inclusive, substituting in turn 4,00 ml of each of the four diluted standard L(−)-hydroxyproline solutions (5.8) for the diluted hydrolysate.

8.6.2 Plot the measured absorbance values, corrected for the blank value, against the concentrations of the standard L(−)-hydroxyproline solutions, and construct the best-fitting straight line through the plotted points and the origin.

NOTE — It is necessary to prepare a new calibration graph for each series of analyses.

8.7 Duplicate values

Carry out the procedure on duplicate test portions.

9 EXPRESSION OF RESULTS

9.1 Method of calculation and formula

For each of the two test portions, calculate the L(−)-hydroxyproline content, as a percentage by mass, from the formula

$$\frac{5\,c}{m \times V}$$

where

c is the L(−)-hydroxyproline concentration, in micrograms per millilitre, of the diluted hydrolysate (8.4.2) as read from the calibration graph;

m is the mass, in grams, of the test portion (8.2);

V is the volume, in millilitres, of the aliquot portion of the hydrolysate taken for dilution to 250 ml (see 8.4.1).

Take as the result the arithmetic mean of the two calculated values, provided that the requirement of 9.2 is satisfied. Report the result to the nearest 0,01 %.

9.2 Agreement between duplicates

The difference between the two calculated values obtained simultaneously or in rapid succession from the duplicate test portions by the same analyst shall not exceed 5 % of the arithmetic mean value.

10 TEST REPORT

The test report shall show the method used and the result obtained. It shall also mention any operating conditions not specified in this International Standard, or regarded as optional, as well as any circumstances that may have influenced the result.

The report shall include all details necessary for complete identification of the sample.