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Date: January 2012

DETERMINATION OF L(-)HYDROXYPROLINE CONTENT

Reference method: NF V04-415

Collagen is one of the few proteins which contains the amino acid hydroxyproline, that's why hydroxyproline is used to estimate the collagen content in meat products including fish and fish derived product.
The method below is the French reference method NF V04-415; it describes the quantitative determination of L(-) hydroxyproline content.

1. PRINCIPLE

L(-)hydroxyproline is released by acid hydrolysis and oxidized with chloramine-T. After the addition of p-dimethylaminobenzaldehyde, a red colour is developed and measured photometrically at 558nm.

2. REAGENTS

2.1 Hydrochloric acid, approx. 6M or sulphuric acid, approx. 3M

hydrochloric acid (ρ_{20} = 1.19g/ml)	500ml
water	s.q.f. 1000 ml
or	
water	750 ml
Slowly add while stirring:	
sulfuric acid (ρ_{20} = 1.84g/ml)	320 ml
Allow to cool down to room temperature	

2.2 Buffer solution, pH 6.0

Dissolve in water:	
monohydrate citric acid	33.0 g
sodium hydroxide	17.0 g
sodium acetate trihydrate	97.0 g
water	approx. 330 ml

Then add:	
1-propanol or 2-propanol	200 ml

Adjust the pH to 6.0 with citric acid and make up to 1000 ml with water.
This solution is stable for 2 months if stored in a dark area below +8°C.

2.3 Chloramine-T reagent

N-chloro-p-toluene sulphonamide, trihydrated sodium salt (chloramines-T)	1.41 g
water	10 ml
Successively add to this solution:	
1-propanol or 2-propanol	10 ml
buffer solution (2.2)	80 ml

This solution is stable for 1 week if stored in a dark area below +8°C.

2.4 Colour reagent

Slowly dissolve:	
p-dimethylaminobenzaldehyde	10 g
in 60% (w/w) perchloric acid	35 ml
Slowly add:	
1-propanol or 2-propanol	65 ml

Prepare this solution on the day of use.

2.5 L(-)hydroxyproline, standard solution

- *Hydroxyproline, stock solution*

hydroxyproline	50mg weighed to the nearest 0.001g
water	s.q.p. 100 ml

This solution is stable for 2 months if stored below +8°C.

- *Intermediate solution*

Stock solution	5 ml
water	s.q.p. 500 ml

- *Standard solution*

Prepare from the intermediate solution a range of calibration solution with a minimum of 4 calibration solutions in the linear zone, i.e between 0 and 5µg L(-)hydroxyproline.

Note: a range from 0.0 µg/ml to 2.5 µg/ml hydroxyproline covers most of this document scope.

The standard solutions are stable for one week if stored below +8°C.

2.6 Nitrogen compound-free paper

2.7 Boiling aid: pumice stone or glass beads for the hydrolysis using hydrochloric acid

2.8 Filter paper for rapid filtration

3. PROCEDURE

3.1 Sample preparation

Weigh, to the nearest 0.001g, 2 g to 8 g (depending on the L(-)hydroxyproline content of the homogenized sample in the hydrolysis tube.

m is the exact mass of the sample.

3.2 Hydrolysis

Add 50 ml to 100 ml of acid solution (2.1) and the boiling aid if using hydrochloric acid (2.7).

Bring to a gentle boil for 16 hours and keep it boiling for 16 hours:

- gentle boiling at 110℃ with hydrochloric acid
- gentle boiling at 107℃ ± 3℃ with sulphuric acid ; the hydrolysate deteriorates when overheated

3.3 Adjustment and filtration

Transfer the hot hydrolysate in a V_0 ml volumetric flask (ex: 200 ml), rinse the hydrolysis tube with hot water, allow to cool down and make up to V_0 ml with water, stir the solution.

Filter the hydrolysate through a filter paper.

The filtered sample is stable for one week if stored in a sealed flask below +8℃.

Make a 1:20 dilution or a 1:50 dilution with the filtered sample. *D* is the dilution (0.05 or 0.02).

The finale concentration should enable a measurement in the linear zone of the calibration curve (2.5).

3.4 Colour development and absorbance measurement

3.4.1 Place in a test tube:

- 5 ml ± 0.1 ml filtered hydrolysate, diluted if necessary, to obtain 2.5 µg to 10µg of L(-)hydroxyproline;
- 2.5 ml ± 0.1 ml chloramine-T reagent (2.3)

Mix and let sit 20 min ± 1 min at room temperature.

3.4.2 Add 2.5ml ± 0.1ml colour reagent (2.4), cover the tube with aluminium foil and stir.

Note: the hydrolysate volume mentioned in paragraph 3.4.1 may be different, the reagent amount should then be changed in the same proportion.

3.4.3 Rapidly put the tube in the hot water bath (60℃ ± 2℃) and heat for 20 min ± 1.

3.4.4 Cool under running water or in a water bath for 3 min minimum and let sit 30 min at room temperature.

3.4.5 Measure the absorbance at 558 nm ± 2 nm against water with a spectrophotometer.

3.4.6 Read the concentration of L(-)hydroxyproline in the hydrolysate, eventually diluted, on the calibration curve, or use the linear regression to calculate it. C is the amount of L(-)hydroxyproline in µg/ml.

3.5 Calibration curve

Perform the steps described in paragraph 3.4 by replacing the hydrolysate by the same volume of each of the calibration solutions, and by the same volume of water for the blank.
Use the absorbance values obtained for each calibration solution concentration to calculate the sample concentration.

3.6 Calculation of the hydroxyproline content

$$\text{L(-)hydroxyproline content in g/100g} = \frac{C \times V_0}{10\,000 \times D \times m}$$

Where:
V_0 is the volume of hydrolysate before filtration (3.3)
C is the concentration obtained in paragraph (3.4.6)
m is the mass of sample used (3.1)
D is the dilution (3.3)
Give the results to 2 decimal places:
If the result is expressed given as wt % collagen, the conventionally used ratio is 8 and the result is given to 1 decimal place.

The method used to measure the content of hydroxyproline in Collactive™ is based on the above reference method number NF V 04-415.

Luce Sergent,
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