

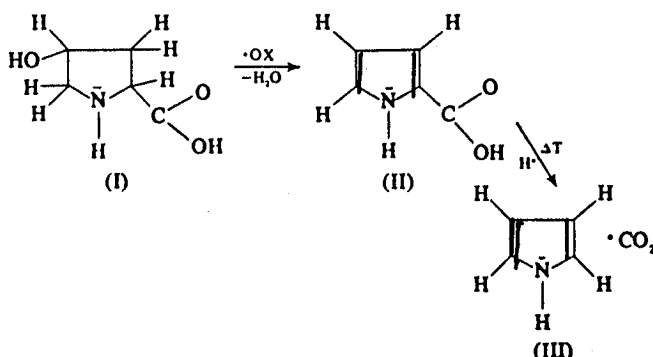
Determination of Hydroxyproline in Materials Containing Collagen

Introduction

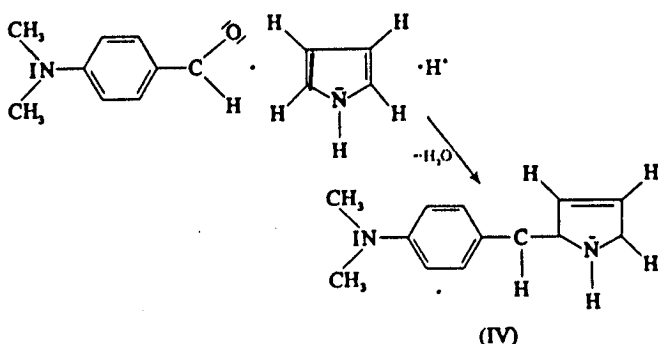
The amino acid hydroxyproline (hypro) is a specific substance of collagen. The hypro content of collagen in hides is fairly constant within one species of animals and for this reason the amount of hypro in leather can be used as a measure of the quantity of hide substance;^{1,2} especially in those cases where non-collagenous nitrogen sources can be expected, the hypro content can give much information.

The determination of hypro is carried out along the following main lines:

- Hypro is liberated from collagen by hydrolysis in 6M hydrochloric acid at elevated temperatures.
- Hypro (I) is oxidised, eg, by addition of chloramin-T, and the oxidation product (II) is decarboxylated in acidic medium at elevated temperatures to provide pyrrole (III).



- The pyrrole formed is coupled in acidic medium with *p*-dimethylaminobenzaldehyde (DMAB) and the quantity of addition product (IV) is determined by measuring the extinction of the solution.



Until now the hypro determination gave problems because the methods were complicated and there was interference by the presence of different materials like vegetable tannins,³ dyestuffs,³ ammonium salts⁴ and formaldehyde.⁴ The greater part of these problems can be overcome by modification of existing methods⁴ by:

- Removing ammonium salts and formaldehyde by evaporation in acid and in alkaline medium.
- Decarboxylation of the oxidation product by steam distillation. At the same time the pyrrole is formed in a well-defined way and is liberated from all non-volatile disturbing materials.⁵

These modification which were introduced by TNO Holland, were published in *Das Leder*, 1973, 24, 113, and details of the method are given here.

The conditions of hydrolysis and the determination as described in the literature cannot easily be carried out in any laboratory. For this reason the hydrolysis - using homemade autoclaves and a stirring device - is modified in such a way that it can be performed with normally available laboratory apparatus; furthermore for the reaction with DMAB a shorter space of time is defined which gives good results with a larger range of DMAB reagents.

1 Detailed description of TNO method for hypro determination

- 1.1 *Apparatus and reagents.* Distillation apparatus: A Büchi distillation apparatus (cup volume 30-100 ml) fed with demineralised water is preferred.
- 1.2 *Hydrolysis tubes.* Test tubes of 25 ml (diameter 15 mm) with silicon-greased grounded glass stoppers and blocking clamps to keep stoppers in position.
- 1.3 *Magnetic stirring rods*, teflon coated, dimensions 5 x 10 mm.
- 1.4 *Magnetic stirring device*, eg, Mettler Kombiplatte LR 13 and *water-bath* with a temperature of 100 °C.
- 1.5 *Drying oven* with a temperature of 100 ± 2 °C.
- 1.6 *Thermostatic bath* with a temperature of 20 ± 0.1 °C.
- 1.7 *Colorimeter.* The Elko II apparatus of Zeiss is used, which gives excellent results only when using filter SS 55 E 61. When using other colorimeters, care should be taken that measurements are carried out at the right wavelength (555 ± 3 nm) otherwise the extinction will be too low and not stable.
- 1.8 *Reagent chemicals* need to be of high purity. Good results are obtained when using the following chemicals.

Product	Manufacturer	Quality
Sulphuric acid (96%)	Baker	Baker analysed
Hydrochloric acid (37%)	Baker	Baker analysed
Stannous chloride (2 aq)	Baker	Baker analysed
Boric acid	Merck	pro analyse
Potassium hydroxide (86%)	Merck	pro analyse
Chloramin-T	Merck	pro analyse
Acetic acid (100%)	Merck	p.a. and stable to chromic acid
p-dimethylaminobenzaldehyde (DMAB)	Riedel or Merck*	pro chromatography or p.a.
Hydroxyproline	Fluka	Puriss
Water		Demineralised

2 Stability of reagents mentioned in the analytical procedure

The oxidation, reduction and DMAB reagents are stable for only one day and thus must be prepared daily. The mixture of 4.5M sulphuric acid and acetic acid, as well as all the other reagents, are stable for several months.

3 Analytical procedure for air-dry material

- 3.1 Mill the material.
- 3.2 Weigh out a quantity containing about 7 mg hypro and transfer into test tube (1.2) containing a teflon-coated stirring rod (1.3).

- 3.3 Add 20 ml of 6M aqueous hydrochloric acid, close tube and transfer into a water-bath on a magnetic stirring device (1.4). Take care that tubes (6-10) are arranged symmetrically and close to the stirring axis to be sure that every tube is stirred.
- 3.4 Heat 16 hours in water-bath at 100 °C and stir the suspension during hydrolysis. After hydrolysis, the following procedure is carried out on the *same day*:
- 3.5 Remove tubes from water-bath and cool. Filter hydrolysate through paper, wash the filter and collect filtrate and washings in a volumetric flask of 50 ml; fill to the mark and mix intensively.
- 3.6 Pipette 2 ml of the filtrate into a beaker of 50 ml; heat on the water-bath (1.4) till dry and be sure that all hydrochloric acid is evaporated (further heating in the drying oven (1.5) at 100 °C for $\frac{1}{4}$ hour and/or neutralisation⁸ to pH 7-8 is recommended).
- 3.7 Dissolve dry residue in 2 ml of aqueous potassium borate solution (50 g potassium hydroxide 86% and 94.8 g H_3BO_3 /litre) and evaporate again till dry on the water-bath (1.4) only.
- 3.8 Dissolve residue in 5 ml aqueous boric acid solution (24.1 g H_3BO_3 /litre) and place during $\frac{1}{4}$ hour in the thermostatic water-bath at 20 ± 0.1 °C.
- 3.9 Add 5 ml oxidation reagent (aqueous solution of 14 g chloramin-T/litre) and leave the beaker for 20 ± 0.5 min in the thermostatic bath at 20 °C (1.6).
- 3.10 Add 5 ml reducing reagent (45 g $SnCl_2$ aq in 1 litre 0.1M aqueous hydrochloric acid). Transfer the mixture into the distillation apparatus (1.1), wash with about 50 ml water, add 5 ml 2M aqueous hydrochloric acid and open steam valve.
- 3.11 Collect distillate in a 10 ml volumetric flask until the mark is reached and mix the distillate in the volumetric flask vigorously.
- 3.12 Transfer into a dry test tube 10 ml DMAB reagent (15.0 g DMAB in a mixture of 850 ml pure acetic acid and 150 ml 4.5M aqueous sulphuric acid), add 5 ml distillate; close the tube with a glass stopper and mix immediately and vigorously.
- 3.13 Measure the extinction of the coloured liquid in a 1 cm cuvet at a wavelength of 555 nm against a blank being a mixture of 10 ml DMAB reagent and 5 ml water. The measurement of the extinction has to take place within the period of time between 45 and 60 min after mixing DMAB reagent and distillate; during this period of time the extinction remains constant if the measurement is carried out at the correct wavelength of 555 ± 3 nm (ELKO II apparatus filter S 55 E 61). Standard deviation of the results should be less than 1% relatively.

4 Standard solutions and standardisation

- 4.1 Calibration with hydroxyproline
100 mg hydroxyproline (calculated on dry substance) and 10 mg sodium azide are dissolved in water and diluted to a volume of exactly 50 ml in a volumetric flask. From the so-prepared solution, 2.50, 3 and 4 ml are diluted to 50 ml in volumetric flasks. The total hydroxyproline content of these standard solutions is 5, 6 and 8 mg respectively. The standard solutions are handled as filtered (and diluted) hydrolysates. Thus 2 ml of each standard solution is treated according to the numbers 3.6 to 3.13 of the analytical procedure. The calibration curve is constructed by plotting the extinction against the amount of hypro standard. Deviation should be <0.5% relatively.
- 4.2 Calibration with hide powder
It is preferable to calibrate the hypro determination using air-dry hide powder* of known moisture ash and fat content. A calibration

curve can be obtained by application of the analytical procedure on amounts of hide powder containing 40, 50 and 65 mg pure (dry, ash- and fat-free) hide powder. The calibration curve is constructed by plotting the extinction against the amount of collagen (= amount of dry hide powder minus ash and fat content). The standard deviation of the results should be smaller than 0.5% relatively.

5 Calculation of the hypro of collagen content of tested materials

From the extinction measured under 3 (the analytical procedure) the amount of hydroxyproline can be found by using the calibration curve of item 4.1. The amount of collagen can be found by using the calibration curve of item 4.2. As the collagen relevant to leather has a hypro content of 12.5%, the conversion factor from hypro to collagen is 8.0.

6 References

- 1 Lollar, *J Am Leather Chem Ass*, 1958, 53, 2.
- 2 Bowes, *J Soc Leather Trades Chem*, 1959, 43, 203.
- 3 Heidemann and Kröll, *Leder*, 1962, 13, 262.
- 4 Langerwerf, *Leder*, 1973, 24, 113.
- 5 Serafini-Cessi and Cessi, *Analyt Biochem*, 1964, 4, 527.

* As DMAB has a tendency to discolour when exposed to sunlight (yellow-reddish), storage in a dark coloured bottle is recommended. Purification of DMAB can be performed by dissolving in 2M hydrochloric acid and fractional precipitation by adding slowly 5M sodium hydroxide solution and stirring. 125 g DMAB can be dissolved in a mixture of 100 ml 12M hydrochloric acid and 600 ml water and afterwards precipitated with about 225 ml 5M sodium hydroxide. The first precipitate is coloured and must be discarded. The middle fraction (about 100 g) is pure; after drying, a melting point of 73 °C can be obtained. The last fractions of precipitate are again less pure (brown discoloration) and must be discarded.

* Neutralisation is essential when non-volatile acidic materials, eg, chromium salts, are present.

* Hide powder must be prepared from flesh split of unlimed cattle hides (chromium content <0.5% Cr₂O₃; eg, Darmstadt hide powder).