

STANDARD OPERATING PROCEDURE Q01132-2.0 MICRO-METHOD FOR
THE DETERMINATION OF HYDROXYPROLINE IN GELATINE EXTRACTS

FOOD STANDARDS AGENCY

STANDARD OPERATING PROCEDURE (SOP) Q01132-2.0

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**STANDARD OPERATING PROCEDURE FOR THE MICRO-METHOD FOR
THE DETERMINATION OF HYDROXYPROLINE IN GELATINE
EXTRACTS**

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STANDARD OPERATING PROCEDURE Q01132-2.0 MICRO-METHOD FOR
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CONTENTS

1.	HISTORY/BACKGROUND	3
2.	PURPOSE.....	3
3.	SCOPE.....	3
4.	DEFINITIONS AND ABBREVIATIONS	3
5.	PRINCIPLE OF THE METHOD	4
6.	MATERIALS AND EQUIPMENT	5
6.1	CHEMICALS.....	5
6.2	WATER	5
6.3	SOLUTIONS, STANDARDS AND REFERENCE MATERIALS	5
6.4	COMMERCIAL KITS	6
6.5	PLASTICWARE	7
6.6	GLASSWARE	7
6.7	EQUIPMENT	7
7.	PROCEDURES	9
7.1	Day 1 SAMPLE HYDROLYSIS.....	9
7.2	Day 2 REAGENT PREPARATION.....	9
7.3	DAY 2 PREPARATION OF SAMPLES.....	10
7.4	DAY 2 PREPARATION OF STANDARDS.....	10
7.5	Day 2 COLORIMETRIC ASSAY.....	10
7.6	QUALITY ASSURANCE.....	11
8.	CALCULATIONS AND DATA ANALYSIS.....	11
9.	RELATED PROCEDURES.....	11

STANDARD OPERATING PROCEDURE Q01132-2.0 MICRO-METHOD FOR THE DETERMINATION OF HYDROXYPROLINE IN GELATINE EXTRACTS

1. BACKGROUND

Gelatine is the cleavage product of collagen, an animal protein present in bones and connective tissue. Gelatine is used in the food industry principally as a thickener for sauces. However, gelatine can be added to meats as a water-binding agent to increase the apparent mass of meat at market. Currently, gelatine is quantified using an Association of Analytical Communities (AOAC) method that detects hydroxyproline, a collagen-specific amino acid. The method entails digestion of the sample in sulphuric acid followed by colorimetric analysis of the released hydroxyproline. However this method requires 4 grammes of solid material as the starting material and is therefore unsuitable for small volume (μL) liquid extracts which result from a gelatine extraction method developed at Fera. Therefore the AOAC method has been modified to accommodate this type of sample. This SOP has been developed as part of Defra-funded project Q01132, entitled "Inter-laboratory validation of a method to determine the species of origin of gelatine found in chicken by mass spectrometry.

2. PURPOSE

The purpose of the method is to determine the hydroxyproline content of samples which are extracted according to the Standard Operating Procedure entitled 'Q01132-2.0 Isolation of gelatine from chicken fillet preparations.'

3. SCOPE

The method described covers the determination of hydroxyproline content in extracts from chicken drip liquor which may have been enriched for gelatine content. The method has been exploited in an inter-laboratory trial. Therefore the scope of the method is limited to the reagents as detailed below and samples prepared as reported in project report FSA Q01132. The limit of detection is $0.6\mu\text{g/mL}$ hydroxyproline.

4. DEFINITIONS AND ABBREVIATIONS

Defra Department for Environment, Food and Rural Affairs

AOAC Association of Analytical Communities

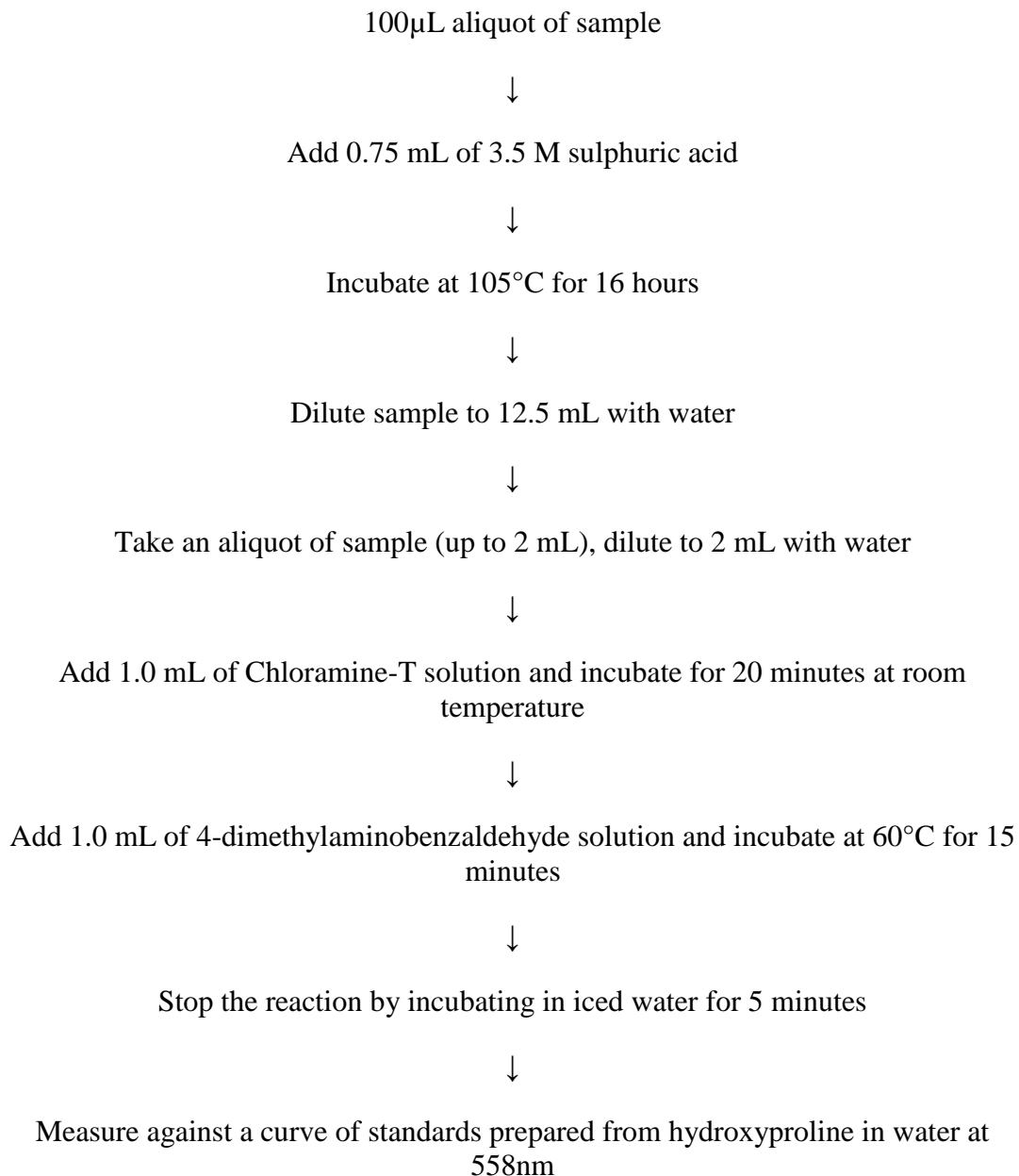
FAPAS Food Analysis Performance Assessment Scheme

SOP Standard Operating Procedure

5. PRINCIPLE OF THE METHOD

Gelatine extract is hydrolysed for 16 hours at 105°C, diluted with water, incubated with Chloramine-T oxidising solution for 20 minutes and then incubated at 60°C with 4-dimethylaminobenzaldehyde solution to form a coloured extract which is measured by a photometric method.

Overview of method:



6. MATERIALS AND EQUIPMENT

6.1. Chemicals

- 6.1.1 Sulphuric acid, concentrated (96%), specific gravity 1.84g/mL, 36.0 N (18M). For example Sulphuric acid 96% ph.eu., reference number 85508.320 supplied by VWR International Limited.

Safety information: Causes severe burns. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Never add water to this product. In case of accident or if you feel unwell, seek medical advice immediately (show the label whenever possible).

- 6.1.3. Detergent e.g. Decon Neutradecon®, reference number D/0027/21, supplied by Fisher Scientific Limited. Should be prepared in tap water at a concentration of 5% (v/v).

6.2. Water

Laboratory grade deionised water (for example sources from an ELGA water purifier UHQ-11 USF ELGA, supplied by VWR International, reference 171-0521 must be used throughout.

6.3. Solutions, standards and reference materials

- 6.3.1 3.5 M Sulphuric acid

This recipe provides sufficient solution for analysis of forty eight samples or standards (or twenty four samples or standards in duplicate). 50 mL of deionised water (6.2) are added to 100 mL volumetric flask (6.6.2) a flask. 37.5 mL of concentrated sulphuric acid (1.84 g/mL) (6.1.1) are added slowly to the flask with agitation (CAUTION: safety glasses and gloves to be worn.). After cooling the volume is made up to 100 mL with deionised water (6.2). Store at room temperature. The shelf life of this solution is seven days. The purity and batch number of the sulphuric acid must be recorded.

- 6.3.2 Ice

Crushed ice is required for incubation purposes during the precipitation step of the method.

STANDARD OPERATING PROCEDURE Q01132-2.0 MICRO-METHOD FOR THE DETERMINATION OF HYDROXYPROLINE IN GELATINE EXTRACTS

6.3.3 Reference materials

Reference materials are not available for this procedure. However this method is adapted from AOAC Method 990.26 and from the Standard Operating Procedure entitled Standard Operating Procedure Q01118-1.1 for the detection of gelatine in vegetarian meals for Food Standards Agency project Q01118. The following reference materials were used in the latter SOP: Food Analysis Performance Assessment Scheme (FAPAS) test materials (canned meat) T0147 (0.058-0.091g hydroxyproline/100 g) and T0151 (0.719-0.934g hydroxyproline/100 g).

- 6.3.4 Chloramine T trihydrate, $\geq 98.0\%$. For example, product number 23270, 50 g manufactured by Fluka, supplied by YGS Laboratory Supplies Limited.
- 6.3.5 4-Dimethylaminobenzaldehyde, $\geq 98.0\%$. For example, product number A11712, 100mL manufactured and supplied by Alfa Aesar. (CAUTION: harmful, handle in a fume hood)
- 6.3.6 Citric acid monohydrate, ACS reagent grade. For example product C1909, 500g supplied by Sigma-Aldrich Limited.
- 6.3.7 Sodium hydroxide pH Eur. For example, product number 71694, 1 kg manufactured by Fluka, supplied by YGS Laboratory Supplies Limited.
- 6.3.8 Sodium acetate trihydrate, $\geq 99\%$. For example, product number 71190, 1 kg manufactured by Fluka, supplied by YGS Laboratory Supplies Limited.
- 6.3.9 1-propanol, $\geq 99\%$. For example, product number A19902, 500mL manufactured and supplied by Alfa Aesar. (CAUTION: harmful, flammable, handle in a fume hood)
- 6.3.10 Perchloric acid, ACS reagent grade, 60-62%. For example, product number 033263, 500mL manufactured and supplied by Alfa Aesar. (CAUTION: Corrosive, safety glasses and gloves to be worn).
- 6.3.11 2-propanol, CHROMASOLV. For example product number 34863, 2.5 L manufactured by Sigma-Aldrich, supplied by YGS Laboratory Supplies Limited. (CAUTION: harmful, flammable, handle in a fume hood)
- 6.3.12 Hydroxyproline, laboratory reagent grade. Product number H/2415/44 supplied by Fisher Scientific Limited must be used.

6.4. COMMERCIAL KITS

No commercial kits were used in this method.

STANDARD OPERATING PROCEDURE Q01132-2.0 MICRO-METHOD FOR
THE DETERMINATION OF HYDROXYPROLINE IN GELATINE EXTRACTS

6.5. PLASTICWARE

- 6.5.1 1mL pipette filter tips. For example TipOne 101-1000uL tips, reference number S1122-1830 supplied by StarLab UK Limited.
- 6.5.2 5mL pipette tips. For example 5mL tips reference number I1050-0700 supplied by StarLab UK Limited.

6.6. GLASSWARE

All glassware must be cleaned in Decon Neutradecon® (6.1.3) in water of a temperature of at least 60°C by scrubbing with a bottle brush to remove visible remains of food and gelatine residue. Glassware should then be transferred to an industrial dishwasher (6.6.11) on a cycle which involves a detergent step at $\geq 60^{\circ}\text{C}$.

- 6.6.1 100 mL thick walled bottle with screw cap capable of withstanding 65°C and vigorous shaking. For example Duran bottles, reference number 215-1592, supplied by VWR International Limited.
- 6.6.2 100mL volumetric flasks. For example Volac volumetric flask, reference number 612-1580, supplied by VWR International Limited.
- 6.6.3 100 mL measuring cylinder. For example 100 mL measuring cylinder, reference number HIRS224018027, supplied by VWR International Limited.
- 6.6.4 Pyrex® culture tubes with caps. For example, culture tubes, reference number 1636/24MP from VWR International Limited.
- 6.6.5 100mL amber glass bottle. For example reference number ZPACBGA6100 supplied by VWR International Limited.
- 6.6.6 1L amber glass bottle. For example reference number SCOT218165409 supplied by VWR International Limited.

6.7. EQUIPMENT

- 6.7.1 Balance accurate to 0.001g. For example Mettler Toledo AB204-S, reference number 611-0202 supplied by VWR International Limited.
- 6.7.2 Spatula, stainless steel. For example 230mm SS spatula reference number FB 65043 supplied by Fisher Scientific Limited.

STANDARD OPERATING PROCEDURE Q01132-2.0 MICRO-METHOD FOR THE DETERMINATION OF HYDROXYPROLINE IN GELATINE EXTRACTS

- 6.7.3 Water bath capable of holding up to 24 culture tubes and maintaining a temperature of both 60°C. For example, Grant water bath reference number SUB28L, supplied by VWR International Limited.
- 6.7.4 Thermometer (range -10°C to 110°C in 1 degree increments). For example, reference number THE1080 supplied by SLS Limited.
- 6.7.5 Centrifuge, temperature controlled, capable of centrifuging up to 30 mL aliquots to 4,000 x g at 30°C in glass walled centrifuge tubes. For example Eppendorf centrifuge 5810R, ref. no. 5811 000.061 supplied by Helena Biosciences Limited, with rotor, e.g. swing bucket rotor, reference number 521-0081 supplied by VWR International Limited.
- 6.7.6 Pipette, 1mL. For example StarPet 100uL-1000ul single-channel pipette supplied by StarLab UK Limited, reference number G8900-1000.
- 6.7.7 5mL pipette. For example 5mL single channel pipette reference number FB60049 supplied by Fisher Scientific Limited.
- 6.7.8 200µL pipette. For example Pipette Pipet-Lite LTS 20-200uL, supplied by Anachem Limited, reference number L-200.
- 6.7.9 Laboratory glassware washer. For example Lancer 810LX, ref. no. LABEXIA810LX supplied by VWR International Limited, with Laboratory Machine Detergent e.g. Lancer LCD25, reference number WCK-150-030L, supplied by Fisher Scientific Limited.
- 6.7.10 Laboratory gloves. For example Supreno PF Nitrile Gloves, medium, synthetic durability and strength, reference number SU-INT-M, supplied by StarLab UK Limited.
- 6.7.11 Oven, capable of 105°C. For example Qualivac 63 Litre Vacuum Oven, reference number 322/0532/00 supplied by LTE Scientific Limited.
- 6.7.12 Vortex mixer, capable of 2500 revolutions per minute with a 42 mm orbit. For example Stuart SA8, reference number MPR-558-010F supplied by Fisher Scientific Limited.
- 6.7.13 Spectrophotometer to measure absorbance at 558 nm, with glass cells of 10 mm optical length. For example Ultrospec 2000, reference number 94-0108-29 supplied by GE Healthcare Life Sciences.

7. PROCEDURES

7.1. Day 1. Sample Hydrolysis

- a) Take 100µL of the sample of interest, prepared in steps 7.1.2 and 7.1.14 of SOP Q01132-1.0). In a Pyrex® tube with screw cap (6.6.4), taking care to pipette liquid to

STANDARD OPERATING PROCEDURE Q01132-2.0 MICRO-METHOD FOR THE DETERMINATION OF HYDROXYPROLINE IN GELATINE EXTRACTS

the bottom of the tube rather than liquid running down the sides of the tube, pipette 100 μ L of extracted sample and add 0.75 mL of 3.5 M H₂SO₄ (6.3.1). Mix gently by hand so as to retain the liquid at the bottom of the tube.

b) Incubate for 16 hours at 105°C so that hydrolysis can take place.

c) Allow to cool for 10 minutes. Dilute with 11.65 mL of water and mix using a vortex mixer (6.7.12).

7.2. Day 2. Reagent preparation

Preparation of reagents:

7.1.1. Oxidising solutions

7.1.1.1 Chloramine-T buffer solution (can be prepared in advance)

Mix 30 g citric acid monohydrate (6.3.6), 15 g sodium hydroxide (6.3.7) and 90 g sodium acetate trihydrate (6.3.8) in 500 mL water and adjust the pH to 6.0 with acid or base as necessary. Add 290 mL of 1-propanol (6.3.9) and make up the volume to 1000mL with water. This solution is stable for two months at 4°C in an amber bottle (6.6.6), according to AOAC Method 990.26, so can be prepared in advance.

7.1.1.2 Chloramine-T reagent (can be prepared in advance)

Dissolve 0.705 g of Chloramine-T (6.3.4) into 50mL of buffer solution. This solution is stable for 1 week at 4°C in an amber bottle (6.6.5), according to AOAC Method 990.26, so can be prepared in advance.

7.1.2 Colour reagent

Dissolve 2.5 g of 4-dimethylaminobenzaldehyde (6.3.5) in 8.75 mL perchloric acid (6.3.10). Add 16.25 mL 2-propanol (6.3.11) slowly and mix. Prepare solution on day of use (i.e. prepare after sample has been hydrolysed for 16 hours (7.2)).

7.3. Day 2. Preparation of samples

Dilute the hydrolysed gelatine extract (7.1c) with water (6.2) so that the hydroxyproline concentration of the final dilution is in the range 0.3-2.4 μ g/mL. For example dilute 0.3 mL of extract to a final volume of 2 mL. Note the volume of diluted extract used for application of the equation in section 7.5.

STANDARD OPERATING PROCEDURE Q01132-2.0 MICRO-METHOD FOR THE DETERMINATION OF HYDROXYPROLINE IN GELATINE EXTRACTS

7.4. Day 2. Preparation of standards

a) Prepare a stock solution of hydroxyproline (600µg/mL) Dissolve 60 mg of hydroxyproline (6.3.12) in water in a 100 mL volumetric flask (6.6.2). Dilute to volume with water. Solution is stable at 4°C for 2 months.

b) Prepare an intermediate solution (6µg/mL) of hydroxyproline. Mix 1 mL of stock solution (7.1.4a) with 99 mL water in a 100 mL volumetric flask (6.6.2). Prepare on day of use.

c) In Pyrex® tubes with screw caps (6.6.4) prepare five standards from pure hydroxyproline (6.3.12) in water. The concentrations of the standards are 0, 0.6, 1.2, 1.8 and 2.4 µg/mL in a final volume of 2.0 mL to be included in the analysis. i.e. take 0, 200, 400, 600 and 800 µL of intermediate solution (6µg/mL, 7.1.4b) respectively and make up to a final volume of 2.0 mL using a pipette (6.7.6). It is recommended that standards are prepared at, or close to, these values.

7.5. Day 2. Colorimetric assay

Mix the 2.0 mL of diluted hydrolysed extract (7.3) or standard (7.4c) by vortex mixer (6.7.12) with 1 mL of Chloramine-T reagent (7.1.1.2) and incubate at room temperature for 20 minutes in a sealed screw cap culture tube (6.6.4).

Add 1 mL of colour reagent (7.1.2), mix the samples and standards by vortex mixer (6.7.12) and incubate at 60°C (±0.5) for 15 minutes in a water bath (6.7.3).

Allow the samples to cool for 5 minutes in iced water (4°C).

Measure the absorbance of each sample against the zero standard at 558 nm using a spectrophotometer (6.7.13).

Prepare a calibration curve of the standards (total hydroxyproline content in 2 mL of standard against absorbance at 558 nm) and measure samples against this curve. If the absorbance of a sample falls outside the range of the calibration curve then repeat the analysis using a more appropriate dilution.

To calculate the hydroxyproline content of a sample, please refer to Section 8 below.

7.6. QUALITY ASSURANCE

Reference materials used were available to purchase from the FAPAS Proficiency Testing Group, Sand Hutton, York as detailed in 6.3.3. The acceptable levels of compliance for these test materials are 0.058-0.091g hydroxyproline/100 g for

STANDARD OPERATING PROCEDURE Q01132-2.0 MICRO-METHOD FOR
THE DETERMINATION OF HYDROXYPROLINE IN GELATINE EXTRACTS

reference material T0147 and 0.719-0.934g hydroxyproline/100 g for reference material T0151.

8. CALCULATIONS AND DATA ANALYSIS

The hydroxyproline content of the samples (H) is calculated as follows:

$$H, \text{ g/100g} = (h \times 2.5)/(m \times V)$$

Where $h = \mu\text{g}/2 \text{ mL}$ filtrate, read from the curve

$V =$ volume, mL of diluted sample taken in section 7.3 and multiplied by 50. For example, if the volume of diluted sample used is 0.1mL, $V=5$.

$m =$ volume of original sample hydrolysed, mL

Finally, the gelatine or collagen content of the sample (g/100g) is equivalent to $H \times 8$.

9. RELATED PROCEDURES

- a) Q01132-1.0 Isolation of gelatine from chicken fillet preparations
- b) Q01132-3.0 Tryptic digestion of gelatine
- c) Q01132-4.0 LC-MS/MS of tryptic digests of chicken exudate extracts to determine the species origin of gelatine

END OF DOCUMENT