FOOD STANDARDS AGENCY

STANDARD OPERATING PROCEDURE (SOP Q01132-3.0)

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STANDARD OPERATING PROCEDURE FOR THE TRYPTIC DIGESTION OF GELATINE

Prepared by: Helen Grundy, Protein Biochemist, The Defra Food & Environment Research Agency

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Approved by:
Date:

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1. HISTORY

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, along with other food additives, can be added to meats as a water-binding agent to increase the apparent mass of meat at market. Since gelatine can be manufactured from cow, pig, poultry or fish material, there are ethical and religious issues, together with labelling issues, concerned with its undeclared use within the food industry.

Gelatine and associated additives can be recovered from frozen meat by allowing the liquid mixture to drip out of a piece of meat as it thaws. A method has been developed to extract gelatine from this liquid exudate mixture in order that mass spectrometric analysis can take place to identify the species of origin of the gelatine. This SOP has been developed as part of the Department for Environment, Food and Rural Affairs (Defra)-funded project Q01132, entitled "Inter-laboratory trial of a method to determine the species of origin of gelatine found in chicken by mass spectrometry in order that gelatine extracted from chicken exudates can be digested by trypsin in preparation for liquid chromatography mass spectrometry.

2. PURPOSE

The purpose of the method is to digest gelatine isolated from chicken exudate with trypsin in order to subsequently determine the species of gelatine origin by mass spectrometry. The method has been used by participants in an inter-laboratory (Defra)-funded study (project Q01132) entitled "Inter-laboratory trial of a method to determine the species of origin of gelatine found in chicken by mass spectrometry.

3. SCOPE

The scope of the method is the digestion of gelatine isolated from drip exudates collected from chicken meat with trypsin for subsequent mass spectrometric analysis to determine the species of origin of the gelatine.

4. DEFINITIONS AND ABBREVIATIONS

BSA	Bovine serum albumin
Defra	Department for Environment, Food and Rural Affairs
SOP	Standard Operating Procedure
TFA	Trifluoroacetic acid

5. PRINCIPLE OF THE METHOD

Gelatine protein extracted from exudates of frozen chicken meat is digested with trypsin at 37°C for 16 hours to release the constituent peptides. The reaction is then halted by altering the pH with formic acid.

Overview of method:

Reconstitution of trypsin in ammonium bicarbonate

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Reconstitute gelatine sample in ammonium bicarbonate

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Add trypsin to sample

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Digest at 37°C for 16 hours

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Halt digestion by acidification/freezing

6. MATERIALS AND EQUIPMENT

6.1. Chemicals

6.1.1. Trypsin. Promega sequencing grade modified trypsin (V511), supplied in 20µg aliquots must be used. Supplied by Promega Corporation, Catalogue number V511.

6.1.2. Ammonium bicarbonate (also known as ammonium hydrogen carbonate, NH_4HCO_3). For example ammonium bicarbonate (HARMFUL) supplied by Sigma Aldrich, catalogue number A6141. MUST BE PREPARED FRESH EACH DAY.

6.1.3. Trifluoroacetic acid (TFA), ≥99%, protein sequencing grade. (CORROSIVE). For example catalogue number 299537 supplied by Sigma Aldrich.

6.2. Water

HPLC fluorescence grade water (for example catalogue number W/0107/17, supplied by Fisher Scientific) must be used throughout this SOP.

6.3. Solutions, standards and reference materials

6.3.1 The standard that must be used is bovine serum albumin, catalogue number A7030 supplied by Sigma Aldrich.

6.3.2 100mM ammonium bicarbonate. Dissolve 0.395 g of ammonium bicarbonate into a final volume of 50 mL with water using a 100 mL measuring cylinder (6.5.3).

6.3.3 1% (v/v)TFA. Measure 99 mL of water in a 100 mL measuring cylinder (6.5.3) and add 1 mL of TFA (6.1.3). Mix using a magnetic stirrer and bar (6.7.6 and 6.7.7).

6.4. Commercial Kits

No commercial kits were used in this method.

6.5. Polypropylene/Plasticware

6.5.1 Pipette tips, 200 µL. For example TipOne 1-200µL tips, reference number S1122-8810 supplied by StarLab UK Limited.

6.5.2 Eppendorf tube, low binding. For example 1.5 mL boiling tube, reference number AX-MCT-150-C supplied by Thistle Scientific Limited.

6.5.3 100mL measuring cylinder. For example 100mL measuring cylinder, manufacturerd by Azlon, catalogue number CYL2026 supplied by Scientific Laboratory Supplies Limited.

6.6. Glassware

Not applicable

6.7. Equipment

6.7.1 Heating block suitable for Eppendorf® tubes capable of temperatures between 37°C and 65°C. For example, Heating Block Db-2a, catalogue number BLD-701-010X, manufactured by Bibby Scientific and supplied by Fisher Scientific Limited.

6.7.2 Thermometer (range of at least 37°C to 65°C in 1 degree increments). For example, reference number THE1080 supplied by Scientific Laboratory Supplies Limited.

6.7.3 Pipette, 200 µL. For example Pipette Pipet-Lite LTS 20-200µL, supplied by Anachem Limited, reference number L-200.

6.7.4 Laboratory gloves. For example Supreno PF Nitrile Gloves, medium, synthetic durability and strength, reference number SU-INT-M, supplied by StarLab UK Limited.

6.7.5 Balance accurate to 0.001g. For example Mettler Toledo AB204-S, reference number 611-0202 supplied by VWR International Limited.

6.7.6 Magnetic stirrer. For example MSH basic Magnetic Stirrer with stainless steel heating plate, reference code 3131200 supplied by Essex Scientific Laboratory Supplies Limited.

6.7.7 Magnetic stirring bar. For example pivot ring magnetic stirrer, product reference STI5022 supplied by Scientific Laboratory Supplies Limited.

7 PROCEDURE

7.1. Digestion method

7.1.1 Take a gelatine sample of known content/mass of gelatine, for example that prepared/extracted from chicken exudate according to SOP Q01132-1.0 with gelatine content determined according to SOP Q01118-1.2. Also take 5 mg of BSA standard (6.3.1).

7.1.2 In an Eppendorf® tube (6.5.2) reconstitute the sample or standard in 50 mM ammonium bicarbonate (6.3.2) to provide a 5 mg/mL concentration.

7.1.3 Heat at 65°C for 30 minutes using a heating block (6.7.1). Laying a sheet of polystyrene on top of the sample tubes will prevent evaporation of the liquid to the inside of the Eppendorf® lid so that all of the liquid is maintained at the bottom of the tube.

7.1.4 Reconstitute the 20µg trypsin (6.1.1) with 200 µL of 50 mM ammonium bicarbonate (6.3.2) to give 0.1 µg/µL conc. Reconstituted trypsin is then heated to 37°C for 5 minutes on a heating block (6.7.1) prior to mixing with gelatine solution. Reconstituted trypsin can be stored at -20°C or at -80°C for one month.

7.1.5 Remove a 40 µL aliquot of gelatine (200 µg) and mix 1:1 with 40 µL of the trypsin solution (7.1.3), with 4 µg of trypsin in 40 µL of 50 mM ammonium bicarbonate.

7.1.6 Incubate at 37°C for 16 hours in a heating block (6.7.1). Laying a sheet of polystyrene on top of the sample tubes will prevent evaporation of the liquid to the inside of the Eppendorf lid so that all of the liquid is maintained within the digest at the bottom of the tube.

7.1.7 Remove aliquot as required for LC-MS/MS and acidify to 0.1 % TFA using 1% TFA (6.3.3) prior to injection. Freeze the remainder of the digest to halt digestion.

7.2. Quality Assurance

Successful digestion is later verified by LC-MS/MS by checking for products of the digested bovine serum albumin standard (6.3.1) as described in SOP Q01132-3.0.

In brief, the BSA digest is loaded onto the liquid chromatography instrument which is coupled to a mass spectrometer. The LC-MS/MS data is acquired and processed. Results are applied to a Matrix Science Mascot database search. Acceptance criteria are that BSA must have a Mascot score of >1000, with threshold set top=0.05 for both protein and constituent peptides.

8. CALCULATIONS AND DATA ANALYSIS

Not applicable

9. RELATED PROCEDURES

a) Q01118-1.2 Micro-method for the determination of hydroxyproline in gelatine extracts
b) Q01132-1.0 Isolation of gelatine from chicken fillet preparations
c) Q01132-4.0 LC-MS/MS of tryptic digests of chicken exudate extracts to determine the species origin of gelatine

END OF DOCUMENT