FOOD STANDARDS AGENCY

STANDARD OPERATING PROCEDURE (SOP Q01132-1.0)

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STANDARD OPERATING PROCEDURE FOR THE ISOLATION OF GELATINE FROM CHICKEN FILLET PREPARATIONS

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CONTENTS

1. HISTORY/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, 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 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.

2. PURPOSE

The purpose of the method is to extract and enrich for gelatine protein in exudate isolated from thawing chicken fillets in order to determine the species of origin by mass spectrometry. The methd has been used by participants in an inter-laboratory study for Defra-funded project Q01132.

3. SCOPE

The scope of the method is the extraction and enrichment of gelatine from drip exudates collected from chicken meat to concentrate the sample to a sufficient extent for subsequent mass spectrometric analysis to determine the species of origin of the gelatine.

4. DEFINITIONS AND ABBREVIATIONS

Defra	Department for Environment, Food and Rural Affairs
kDa	kilodalton
MWCO	Molecular Weight Cut-Off
SOP	Standard Operating Procedure
SPE	Solid Phase Extraction

5. PRINCIPLE OF THE METHOD

Proteins in drip exudates from frozen chicken meat are precipitated by incubation at 80°C and by acidification of the extract. Proteins are further concentrated by molecular weight cut-off (MWCO) filtration, solid phase extraction (SPE) and centrifugal evaporation.

Overview of method:

Chicken exudate recovered from meat
↓
10mL aliquot of sample
↓
Incubate at 80°C for 10 minutes
↓
Separate by centrifugation
↓
Retain supernatant and adjust pH to 4.4
↓
Separate by centrifugation
↓
Retain supernatant and apply to MWCO filter
↓
Solid Phase Extraction of the retentate
↓
Retain retentate. Concentrate by centrifugal evaporation

6. MATERIALS AND EQUIPMENT

6.1.Chemicals

6.1.1. Acetic acid, glacial, ≥ 99%, density 1.05 g/mL. Corrosive. For example reference number A/0360/PB15 supplied by Fisher Scientific Limited.

6.1.2. Sodium phosphate monobasic, ≥ 99.0% FW 119.98 g/mol. For example sodium phosphate monobasic, product reference S0751 supplied by Sigma Aldrich.

6.1.3. Sodium phosphate dibasic, Ph Eur FW 141.96 g/mol. For example sodium phosphate dibasic manufactured by Fluka analytical, product reference 56814 supplied by Sigma Aldrich.

6.1.4 Detergent e.g. Decon Neutradecon®. Irritant. For example reference number D/0027/21, supplied by Fisher Scientific Limited. Should be prepared in deionised water (6.2) at a concentration of 5% (v/v).

6.1.5 Formic acid, 98%. Corrosive. For example product reference F/1850/PB08 supplied by Fisher Scientific UK Limited.

6.1.6 Acetonitrile HPLC fluorescence grade. Flammable, harmful. For example product reference A/0630/PB17 supplied by Fisher Scientific UK Limited.

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).

6.3.Solutions, standards and reference materials

6.3.1 4.0 M acetic acid

Add 23 mL of acetic acid, glacial (6.1.1) to 70 mL water (6.2). Make up to 100 mL in a volumetric flask (6.6.2) and store in a glass bottle (6.6.1). The shelf life of this solution is 7 days at room temperature.

6.3.2 1.0M sodium phosphate monobasic

Take 12.0 g of sodium phosphate monobasic (6.1.2) and dissolve into 80 mL of water (6.2). Make volume up to 100 mL with water in a volumetric flask (6.6.2). The shelf life of this solution is 7 days at room temperature.

6.3.3 1.0M sodium phosphate dibasic

Take 14.2 g of sodium phosphate dibasic (6.1.3) and dissolve into 80 mL of water (6.2). Make volume up to 100 mL with water in a volumetric flask (6.6.2). The shelf life of this solution is 7 days at room temperature.

6.3.4 0.1M sodium phosphate buffer pH 7.4

Mix 22.6 mL of 1.0M monobasic sodium phosphate (6.3.2) with 77.4 mL of 1.0M dibasic sodium phosphate (6.3.3). Dilute to 1L with water (6.2) in a measuring cylinder (6.6.3). The shelf life of this solution is 7 days at room temperature.

6.3.5 Reference materials

Reference materials used were commercial porcine skin gelatine, Type A purchased from Sigma Aldrich, product code G2500, batch 085K0170 and checked by an in-house mass spectrometry method that only porcine collagen peptides were present. Reference materials of different concentrations (0%, 1.15%, 2.30%, 3.45% and 4.60%) were prepared by weighing 0.000 g, 1.150 g, 2.300 g, 3.450 g and 4.600 g on a balance (6.7.11) and dissolving in 0.1M

sodium phosphate buffer pH 7.4 (6.3.4) in a final volume of 100 mL (w/v). The preparations must be stirred at 30-40°C, as verified using a thermometer (6.7.2) on a magnetic stirrer with heating plate (6.7.12) using a magnetic stirring bar (6.7.13) for 10 minutes to dissolve the gelatine. The shelf life of these reference material preparations is 28 days at -20°C. The preparations require incubation for 5 minutes at 37°C in a water bath (6.7.1) followed by agitation by hand to return the preparations to the liquid state following storage at -20°C.

6.3.6 0.1% formic acid in water

Mix 100 mL of water (6.2) with 100µL of formic acid (6.1.5) in a glass flask (6.6.1). The shelf life of this solution is 7 days at room temperature.

6.3.7 5% acetonitrile/0.1% formic acid in water.

In a 100mL measuring cylinder (6.5.8) measure 95 mL of water (6.2). Add 5mL of acetonitrile (6.1.6) to make up to volume and then add 100µL of formic acid (6.1.5) in a glass flask (6.6.1). The shelf life of this solution is 7 days at room temperature.

6.4.COMMERCIAL KITS

No commercial kits were used in this method.

6.5.POLYPROPYLENE/PLASTICWARE

6.5.1 Pipette filter tips, 1 mL. For example TipOne 101-1000uL tips, reference number S1122-1830 supplied by StarLab UK Limited.

6.5.2 Pipette tips, 5 mL. For example 5mL tips reference number I1050-0700 supplied by StarLab UK Limited.

6.5.3 Polypropylene tubes, 15 mL. For example 15mL sterilin tubes, reference code TES-136-016J supplied by Fisher Scientific Limited.

6.5.4 Molecular weight cut-off filters, 30kDa. Vivaspin 500 30kDa MWCO, reference number 28-9322-35 supplied by GE Healthcare, UK must be used for compatibility with the centrifuge conditions described in this SOP.

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

6.5.6 C18 solid phase extraction (SPE) cartridges C18 300Å Orosep 300mg 0.5mL, product code CRC183003A supplied by Orochem Technologies Inc, Illinois must be used.

6.5.7 Syringe, 1 mL. For example Plastipak disposable syringe, 1 mL, reference code SYR6001 supplied by SLS Limited.

6.5.8 Measuring cylinder, 100 mL. For example transparent PMP graduated measuring cylinder, 100 mL, product reference CYL2026 supplied by Scientific Laboratory Supplies Limited.

6.5.9 Pipette filter tip, 200 µL. For example pipette tip Art 200, product reference 732-2305 supplied by VWR International Limited.

6.6.GLASSWARE

All glassware must be cleaned in Decon Neutradecon® (6.1.4) in water of a temperature of at least 60ºC by scrubbing with a bottle brush to remove visible remains of food and gelatine residue. Glassware should then be transferred to an industrial dishwasher (6.6.9) on a cycle which involves a detergent step at ≥ 60ºC.

6.6.1 Bottle with screw cap, 100 mL. For example Duran bottles, reference number 215-1592, supplied by VWR International Limited.

6.6.2 Volumetric flasks, 100mL. For example Volac volumetric flask, reference number 612-1580, supplied by VWR International Limited.

6.6.3 Measuring cylinder, 1 litre. For example 1000 mL measuring cylinder, product reference SMIT1634/AM/1000 supplied by VWR International Limited.

6.6.4 Glass beaker, 500 mL. For example Beaker conical wide mouth borosilicate glass 500ml, Duran, product reference 211414408, supplied by VWR International Limited.

6.7.EQUIPMENT

6.7.1 Water bath capable of 37°C and 80°C. For example, Grant water bath reference number SUB28L, supplied by VWR International Limited.

6.7.2 Thermometer (range of at least 37°C to 80°C in 1 degree increments). For example, reference number THE1080 supplied by Scientific Laboratory Supplies Limited.

6.7.3 Centrifuge, temperature controlled, capable of centrifuging suitable tubes for precipitation protocol (e.g. 6.5.3) at 4,000 x *g* at 30°C. 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.

6.7.4 pH meter. For example Jenway 2505 portable pH meter, product reference PHK-332-010J supplied by Fisher Scientific Limited. pH meter must be calibrated on day of use to within 0.02 pH units at pH 4.0 and pH 7.0. Examples of calibration solutions are buffer solution pH 4.0, product reference 25858-5000 supplied by Fisher Scientific Limited and buffer solution pH 7.0, product reference 25859-5000, supplied by Fisher Scientific Limited.

6.7.5 Pipette, 1mL. For example StarPet 100uL-1000ul single-channel pipette supplied by StarLab UK Limited, reference number G8900-1000.

6.7.6 Pipette, 5mL. For example 5mL single channel pipette reference number FB60049 supplied by Fisher Scientific Limited.

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

6.7.8 Centrifugal evaporator. For example SPD121P Centrifugal evaporator/vacuum concentrator, product reference LABLSPD121P-230 supplied by VWR International Limited.

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

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

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

6.7.14 Retort stand. For example clamp 3 fingers 100mm, product reference 241-0019 supplied by VWR International Limited.

7. PROCEDURES

7.1 Gelatine extraction

If at any stage the extract solidifies, incubate at 37°C for 2 minutes in a water bath (6.7.1) to re-solubilise the sample before continuing.

7.1.1 Samples of chicken fillet must be stored at -20°C until required. Thaw the meat for 16 hours at 4°C in a glass beaker. Suspend the meat above the beaker for 5 hours at room temperature using a metal clamp on a retort stand (6.7.14). Collect the total exudate resulting from the 16 hour thaw and the 5 hour drip period and transfer to a polypropylene tube (6.5.3). Stored at -20°C until required.

7.1.2 Incubate the sample at 37°C for 5 minutes following storage at -20°C to thaw the sample. Invert twice by hand to mix. Remove 120µL of the sample and retain for later hydroxyproline analysis which will involve 100µL of this aliquot (refer to Section 8). Place the remaining 5 mL of drip exudate into a polypropylene centrifuge tube (6.5.3). Incubate at 80°C in a water bath (6.7.1) for 10 minutes.

7.1.3 Separate the precipitated proteins by centrifugation (6.7.3) at 3220 x g for 5 minutes at 30°C. Retain the supernatant.

7.1.4 Using a pH meter (6.7.4), adjust the pH of the sample with 4.0 M acetic acid (6.3.1) to pH 4.4. Incubate at room temperature (20°C ±5°C) for one hour to allow precipitation to take place. Check every 15 minutes that samples have not solidified. If this occurs, incubate the sample at 37°C for 1 minute (or until the sample liquefies) and agitate by hand.

7.1.5 Separate the precipitated proteins by centrifugation (6.7.4) at 3220 x g for 5 minutes at 30°C. Retain the supernatant. If the centrifuge does not incubate at 30°C, incubate samples for one minute at 37°C immediately prior to centrifugation. In this case, separate the proteins by centrifugation for 2.5 minutes at 3220 x g, re-incubate at 37°C for one minute and then spin again for 2.5 minutes at 3220 x g.

7.1.6 Four 500 µL molecular weight cut-off filters (6.5.4) are required per sample. Wearing gloves to protect samples from cross-contamination (6.7.10), pre-rinse a sufficient number of filters by applying 500 µL of 0.1M sodium phosphate buffer pH 7.4 (6.3.4) to each filter and submitting to centrifugation for 5 minutes at 15000 x g. Discard the filtrate.

7.1.7 Change gloves between samples to prevent cross-contamination. Take 2 mL of the extract and apply 500 µL to each of four pre-rinsed 30kDa molecular weight cut off filters (MWCO filters, 7.1.6). The remaining material (approximately 3 mL) can be stored at -20°C in case they are needed in the future. The samples on the 30kDa MWCO filters are submitted to centrifugation for a total of 20 minutes as follows. Submit to centrifugation at

15000 x g for 10 minutes at 30°C. Then check the condition of the retentate. If the retentate should gelatinise/solidify, incubate at 37°C for two minutes in a water bath (6.7.1). Continue centrifugation for a further 10 minutes at 15000 x g for 10 minutes at 40°C. The remaining volume of sample (approximately 3 mL) can be stored at -20°C or lower for future analysis if necessary.

7.1.8 Recover the retentate from the MWCO filter using a 200 µL pipette tip (6.5.9) or, if appropriate for the MWCO filter of choice, by reversing the filter and centrifuging the retentate into a clean Eppendorf tube (6.5.5). Retain and combine the retentates for each sample into a polypropylene Eppendorf tube (6.5.5). Discard the filtrate.

7.1.9 Dilute the retentate 1:1 with 0.1M NaPO4 pH 7.4 buffer (6.3.4) to reduce viscosity.

7.1.10 Samples are treated by solid phase extraction as follows. To avoid cross contamination, prepare separate tubes of eluant for SPE: for each sample, prepare one eppendorf (6.5.5) tube containing 1mL of 5% acetonitrile, 0.1% formic acid (6.3.7).

7.1.11 Please note that application of bubbles to the C18 cartridge should be avoided as bubbles can disrupt the integrity of the cartridge. Bubbles should be avoided when aspirating liquids into the syringe as the bubbles will later by applied to the cartridge. Further, when the liquid-filled syringe is attached to the cartridge, by ceasing depression of the plunger before the final 10µL (approximately) of liquid is applied to the column, production of bubbles can be avoided.

7.1.12 Take a 1mL syringe and aspirate 1mL of 0.1% formic (6.3.6). Pre-equilibrate a C18 SPE cartridge by attaching this syringe to the cartridge and applying the 1 mL of 0.1% formic acid (6.3.6) slowly to the SPE cartridge (6.5.6) by depressing the plunger. The liquid which exits the cartridge can be disposed of.

7.1.13 Apply the sample to the same cartridge as follows. Remove the syringe from the cartridge and aspirate the sample into the syringe. Attach the syringe to the cartridge and slowly apply the sample to the cartridge. The liquid which exits the cartridge can be disposed of.

7.1.14 Remove the syringe from the cartridge, aspirate the 1mL of 5% acetonitrile/0.1% formic acid from an Eppendorf tube prepared in step 7.1.10 (6.3.7) and re-attach the syringe to the cartridge. Pre-weigh an eppendorf (6.5.5) in grammes on a balance accurate to 0.001g (6.7.11) and note the mass to three decimal places. Position the exit of the cartridge above the clean pre-weighed eppendorf (6.5.5) and slowly depress the plunger of the syringe to elute the sample into the eppendorf. At this stage, stop to perform the hydroxyproline assay (SOP Q01118-1.2) on 100µL of the 1mL sample. The hydroxyproline assay is performed on 100µL of the material from each sample

recovered at this stage and also on 100 µL of material recovered from each sample at stage 7.1.2.

7.1.15 Reduce the eluant volume to zero using a centrifugal evaporator (6.7.8). The mass of the dried sample in the eppendorf can be determined by subtracting the mass of the eppendorf taken in 7.1.14 from the final mass of the tube. Knowledge of the mass of gelatine in the tube is needed in order to carry out the gelatine digestion protocol (SOP Q01132-3.0). The dried samples must be stored at -20°C or less until required. The hydroxyproline content can be measured using the following SOP: 'the micro-method for the determination of hydroxyproline in gelatine extracts' for Food Standards Agency project Q01132 'Inter-laboratory validation of a method to determine the species of origin of gelatine found in chicken by mass spectrometry).

7.2.Quality Assurance

Quality control samples are discussed in the final SOP of this series (Q01132-4.0) which covers LC-MS/MS analysis. For background information only, during method development, the reference materials used were commercial porcine skin gelatine, Type A purchased from Sigma Aldrich, product code G2500, batch 085K0170. This reference standard was analysed by an in-house mass spectrometry method to verify that only porcine collagen peptides were observed. The minimum gelatine yield (as determined by the SOP for the micro-method for the determination of hydroxyproline in gelatine extracts) suitable for subsequent mass spectrometry is dependent on the mass spectrometry instrument used and will be determined for a range of instruments throughout the course of Defra Project Q01132 as part of this inter-laboratory study.

8. CALCULATIONS AND DATA ANALYSIS

Please refer to the SOP for the micro-method for the determination of hydroxyproline in gelatine extracts in order to calculate the yield of hydroxyproline (an amino acid representative of gelatine) in a sample.

9. RELATED PROCEDURES

a) Q01118-1.2 Micro-method for the determination of hydroxyproline in gelatine extracts
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