FOOD STANDARDS AGENCY

STANDARD OPERATING PROCEDURE (SOP Q01132-4.0)

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STANDARD OPERATING PROCEDURE FOR THE LC-MS/MS OF TRYPTIC DIGESTS OF CHICKEN EXUDATE EXTRACTS TO DETERMINE THE SPECIES ORIGIN OF GELATINE

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STANDARD OPERATING PROCEDURE FOR THE LC-MS/MS OF TRYPTIC DIGESTS OF CHICKEN EXUDATE EXTRACTS TO DETERMINE THE SPECIES ORIGIN OF GELATINE

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'. The SOP describes the procedure for liquid chromatography tandem mass spectrometry.

2. PURPOSE

The purpose of the method is to perform the liquid chromatography tandem mass spectrometry (LC-MS/MS) of the gelatine and to analyse the data by database submission. The method has been used by participants in an inter-laboratory study (Defra-funded project Q01132).

3. SCOPE

The scope of the method is the liquid chromatography and subsequent mass spectrometric and data analysis to determine the species of origin of the gelatine. This is a version of a widely accepted and exploited technique for protein identification by searching a sequence database using mass spectrometry data (Perkins *et al.*, 1999).
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4. DEFINITIONS AND ABBREVIATIONS

BSA	Bovine serum albumin
Defra	Department for Environment, Food and Rural Affairs
ETD	Electron transfer dissociation
HPLC	High performance liquid chromatography
LC-MS/MS	Liquid chromatography tandem mass spectrometry
SOP	Standard Operating Procedure

5. PRINCIPLE OF THE METHOD

Gelatine protein previously extracted from exudates of frozen chicken meat and digested with trypsin is analysed by reverse phase LC-MS/MS and the data acquired is submitted to database searches in order to determine the species of origin of the gelatine(s) in the sample.

Overview of method:

Liquid chromatography and mass spectrometry sample analysis

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Acquisition of mass spectra

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Data interrogation

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Data interpretation

6. MATERIALS AND EQUIPMENT

6.1. Chemicals

6.1.1 Acetonitrile. HPLC fluorescence grade (HARMFUL, FLAMMABLE). For example catalogue number A/0630/PB17 supplied by Fisher Scientific Limited.

6.1.2 Formic acid, 98% (CORROSIVE). For example, catalogue number 06440 supplied by Fluka Chemicals.

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 Mobile phase (aqueous). 0.1% formic acid in water. In a 1 L measuring cylinder (6.6.2), measure 999 mL of water (6.2). Add 1 mL of formic acid (6.1.2) using a pipette (6.7.3). Adjust the volume to 1 L with water (6.2) and mix by pouring into a 1 L duran flask (6.6.1). Use within three days of preparation.

6.3.2 Mobile phase (solvent). 95% acetonitrile, 0.1% formic acid. Take 950 mL of acetonitrile (6.1.1) in a 1 L measuring cylinder (6.6.2). Add 1 mL of formic acid (6.1.2) using a pipette (6.7.3). Adjust the volume to 1 L with water (6.2) and mix by pouring into a 1 L duran flask (6.5.1). Use within three days of preparation.

6.3.3 Standard Mixed Protein Digest (lyophilized) manufactured and supplied by Dionex Corporation, part number 161088. Reconstitute the powder in 200µL of re-suspension solution (6.3.4) to obtain a concentration of 0.5pmol/µL of each protein. Aliquot the mixture into appropriate volumes for injection and store at ≤-20°C.

6.3.4 Standard re-suspension solution, 5% acetonitrile, 0.1% formic acid. In an appropriate tube with screw cap (6.5.2), mix 1 mL of acetonitrile (6.1.1) with 19 mL of water (6.2). Add 20 µL of formic acid (6.1.2) using a pipette (6.6.4). Mix by inversion. Use to re-suspend the standard on day of preparation.

6.4 Commercial Kits

No commercial kits are used in this method.

6.5 Polypropylene/Plasticware

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

6.5.2 Polypropylene tube with screw cap. For example, 50 mL centrifuge tube, manufactured and supplied by Greiner Bio-One, catalogue number 210 261.

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

6.6 Glassware

6.6.1 1L Duran flask. For example Bottle vacuum Duran flask 1L, catalogue number BTF-740-010-W supplied by Fisher Scientific Limited.

6.6.2 1L glass measuring cylinder. For example 1L measuring cylinder, manufactured by Azlon, catalogue number CYP-870-070P supplied by Fisher Scientific Limited.

6.7 Equipment

6.7.1 Liquid chromatographer coupled to a tandem mass spectrometer. For example Ultimate 3000 nano LC, supplied by Dionex Corporation coupled to an amaZon electron transfer dissociation (ETD) ion-trap mass spectrometer, supplied by Bruker Corporation. Individual method optimisation is required depending on the sensitivity of the instrument used.

6.7.2 Column: Columns for shotgun proteomics:

- Monolith or packed
- Dionex PepMap (75µm x 25 cm; 3 µm C18 Part no. 164261)
- Waters BEH130 (75µm x 25 cm; 1.7 µm C18 Part no. 186003545)

For example, a nanoACQUITY UPLC BEH C18 Column, 1.7 µm, 75 µm x 250 mm, 10K psi, manufactured and supplied by Waters Corporation, Part Number: 186003545 or a nanoACQUITY UPLC Symmetry C18 Trap (Std Fitting), 5 µm, 180 µm x 20 mm, 10K psi, manufactured and supplied by Waters Corporation, Part Number: 186003514 are both appropriate.

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

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

7. PROCEDURES

7.1 Liquid Chromatography Method

The liquid chromatography mass spectrometry method requires optimisation for the instrument to be used. An example of suitable conditions is detailed below.

7.1.1 Suitable liquid chromatography columns are detailed in 6.7.2:

7.1.2 Mobile phases

0.1% formic acid and 95% acetonitrile containing 0.1% formic acid as detailed in 6.3.1 and 6.3.2 respectively.

7.1.3 Gradient

A gradient of 0 to 50% acetonitrile, 0.1% formic acid will separate the gelatine peptides. The slope and duration of gradient are sample and system dependent. Less than 30 minutes is suitable for monolith columns and gradients of several hours are necessary for long packed columns.

For example, a nanoACQUITY UPLC BEH C18 Column, 1.7 µm, 75 µm x 250 mm, 10K psi, manufactured and supplied by Waters Corporation, Part Number: 186003545 with a 30 minute 0 to 50% gradient.

The column then must undergo a washing step in the gradient with 95% acetonitrile, 0.1% formic acid before re-equilibration.

7.1.4 Sample loading

Samples, BSA standard and the reference standard (6.3.3) are loaded onto the column for analysis. Samples are analysed in duplicate. Sample loading is

dependent on the sensitivity of the LC-MS system used and will need to be optimised by participants.

For example, for samples it is appropriate to load 100 ng for nanoAcquity-maXis MS and for the reference standard (6.3.3) it is appropriate to load 500fmol.

7.2 Mass spectrometry acquisition

An MS full-scan survey must be performed. The three most intense ions must be selected for subsequent CID-MS/MS on these ions. Collision energy (offset) must be optimised for peptides. It is most appropriate to complete this dynamically by the instrument acquisition software.

7.3 Data Interrogation

A range of databases are available. For example Matrix Science Mascot is appropriate. Instructions regarding the use of Mascot and the required settings are detailed below.

Mascot ion MS/MS submission:

Go to http://www.matrixscience.com/search_form_select.html and select 'MS/MS Ion Search'

- Enter your name, email address and a title for your search.

The following parameters must then be set:

- Database – select SwissProt.
- Fixed modifications
  When analysing the Dionex standard (6.3.3) choose Carboxymethyl (C) only.
  For gelatine samples choose 'none selected'

- Variable modifications:

  For gelatine samples choose all four: Deamidated (NQ)

  Oxidation (K)

  Oxidation (P)

  Oxidation (M)

  For the Dionex standard (6.3.3) choose Oxidation (M)

- Settings for Peptide tolerance, peptide charge, MS/MS tolerance, (also ^{13}C for ion trap) are instrument specific. Click to get help and set the settings for your instrument.
- Data format – Mascot generic
- Instrument: Instrument specific – click for help and select instrument
- Then browse for your data file, containing up to 1200 spectra. This should take a few minutes.

7.4 Data interpretation

For example, for results from Matrix Science Mascot:

To select the summary report, choose, 'select summary (protein hits). Select the following settings:

- Choose Significance threshold of $p<0.05$
- Select Max. no. of hits as AUTO
- Select to Show pop-ups
- Sort unassigned as decreasing score
- Tick Require bold red
- Details of the top scoring peptides for the proteins listed under 'Protein hits' are listed in red.

The species determined is/are listed to the right of the words 'protein hits' in the mascot results.

7.5 Quality Assurance

7.5.1 Successful Mass Spectrometry

Successful mass spectrometry is evaluated by analysis of the reference standards containing 6 digested proteins (6.3.3), namely bovine serum albumin (BSA), transferrin, alcohol dehydrogenase, lysozyme, beta-galactosidase and cytochrome c, all carboxymethylated with iodoacetic acid).

For example on the high sensitivity instrument exemplified in this SOP (6.7.1) a loading of 500 fmol on to the LC is appropriate. The LC-MS/MS data is

acquired and processed. The results are submitted to a Mascot database search again with standard parameters.

The acceptance criteria are:

- All 6 proteins must be identified

- BSA and transferrin must have a Mascot score of >1000, threshold must be set at p=0.05 for both protein and constituent peptides.

At least four peptides must be identified for alcohol dehydrogenase, and at least two peptides for cytochrome c.

7.5.2 Successful digestion

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

The BSA digest is loaded onto the liquid chromatography instrument which is coupled to a mass spectrometer at a suitable loading for the particular 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

Standard Operating Procedure Q01132-1.0 entitled ‘the micro-method for the determination of hydroxyproline in gelatine extracts’ for Defra project Q01132 ‘Inter-laboratory validation of a method to determine the species of origin of gelatine found in chicken by mass spectrometry’.

Standard Operating Procedure Q01132-2.0 for the isolation of gelatine from chicken fillet preparations for Defra project Q01132 ‘Inter-laboratory

validation of a method to determine the species of origin of gelatine found in chicken by mass spectrometry'.

Standard Operating Procedure Q01132-3.0 for the standard operating procedure for the tryptic digestion of gelatine for Defra project Q01132 'Inter-laboratory validation of a method to determine the species of origin of gelatine found in chicken by mass spectrometry'.

10. REFERENCES

Perkins' D.N., Pappin, D.J.C., Creasy, D.M. and Cottrell, J.S. (1999) Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 20 (18) 3551-67.

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