DEPARTMENT FOR ENVIRONMENT, FOOD AND RURAL AFFAIRS

STANDARD OPERATING PROCEDURE (SOP) FA0122(3) v1.2

Version 1.2, January 2016

STANDARD OPERATING PROCEDURE FOR THE EXTRACTION OF OFFAL MARKER PROTEINS IN RAW AND PROCESSED MEAT PRODUCTS, AND THEIR SUBSEQUENT DETECTION AND SEMI-QUANTIFICATION

Prepared by Lyndsey Durose, Nottingham Trent University **Date** January 2016

Reviewed by Authenticity Methods Working Group **Date** January 2016

This publication is available at www.gov.uk/government/publications

Any enquiries regarding this publication should be sent to us at:

foodauthenticity@defra.gsi.gov.uk

www.gov.uk/defra

CONTENTS

1. HISTORY/BACKGROUND

1.1 Background

The definition of meat for labelling purposes is restricted to skeletal muscle with naturally adherent fatty and connective tissue by the Food Information Regulations 2014 and parallel legislation in Scotland, Wales and Northern Ireland enabling Regulation 1169/2011. These regulations state that other types of muscle, such as heart, tongue, etc., are excluded from this definition. These regulations also state that certain parts of the carcass, such as liver, kidney, lung and heart, have to be explicitly labelled as such. The generic term 'offal' is not permitted. Thus there is a need for robust methods for the detection and quantification of various offals in raw and processed foods. This SOP is a modification of the SOP prepared as part of Food Authenticity Programme project (Q01105) entitled 'Proteomic detection and quantification of offal' and only covers the detection and semi-quantification of offal.

1.2 Changes in current version

- Updated to include the preparation of screening standards containing 2 offals (heart and liver or kidney and lung) to streamline the preliminary offal screening step
- Updated to include PBS 8 M urea extraction of all standards and 'test' samples/products
- Change to gel layout for the preliminary screen for offal in 'test' samples
- Change of blotting apparatus to Bio-Rad Trans blot for detection and semi-quantification of heart and liver
- Change of enhanced chemiluminescence (ECL) substrate supplier
- Change to reporting offal levels in bands rather than absolute values
- Procedure for the mass spectrometry detection of offal is not included in this version but can be followed in the SOP from project Q01105 (SOP for the extraction of offal marker proteins in raw and processed meat products, and their subsequent detection and quantification)

2. PURPOSE

To detect and estimate the amount of specific offals, namely heart, lung, kidney and liver, present in commercial meat products by means of extraction of offal-specific marker proteins from meat products followed by semi-quantitative estimation of the extracted offal-specific proteins by probing Western blots with specific antibodies.

3. SCOPE

To probe Western blots of extracts of meat products with anti-heart specific protein 2 (H-SP2), anti-liver specific protein 3 (L-SP3), anti-lung specific protein 2 (Lu-SP2) and anti-kidney marker protein 1 (K-MP1) antibodies in order to detect and semi-quantify added bovine/ovine/porcine heart, liver, lung and kidney, respectively. K-MP1 is a protein expressed at high levels in kidney, small intestine and placenta and at lower levels in liver, pancreas, prostate, spleen, stomach and large intestine,

however, it is not expressed in skeletal muscle or heart. The SOP can be used to analyse raw and cooked meat products.

4. DEFINITIONS AND ABBREVIATIONS

1-D	One dimensional
AIDA	Advanced Image Data Analyser
APS	Ammonium persulphate
CCD	Charge-coupled device
ECL	Enhanced chemiluminescence
DTT	Dithiothreitol
HRP	Horseradish peroxidase
H-SP2	Heart-specific protein 2
K-MP1	Kidney-marker protein 1
L-SP3	Liver-specific protein 3
Lu-SP2	Lung-specific protein 2
m/s	Metres per second
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
TEMED	N,N,N',N' tetramethylethylenediamine
Tris	Tris(hydroxyethoxy)aminomethane

5. OVERVIEW OF THE PROCEDURE

The overall procedure involves the extraction of offal marker proteins from meat samples (raw or processed), prior to analysis of marker proteins by Western blotting.

Meat samples are initially extracted in a buffer and then subjected to fractionation according to molecular mass, using SDS polyacrylamide gel electrophoresis (SDS PAGE). Fractionated proteins are electroblotted onto nitrocellulose to produce a 'Western blot', which is probed using anti-H-SP2, anti-L-SP3, anti-Lu-SP2 or anti-K-MP1 antibodies in order to monitor added heart, liver, lung or kidney, respectively, in the samples.

Once the presence of one of the offals has been detected, semi-quantification can be undertaken using Western blot analysis. However, the he species of the offal needs to be known and, if this is not the case, the species can be determined via mass spectrometric analysis.

6. MATERIALS AND EQUIPMENT

6.1 Chemicals

A number of these chemicals are toxic and/or harmful. Use appropriate personal protective equipment when handling (refer to Appendix 1 for risk phrases).

Name	Formula	Molecular weight	Recommended supplier	Product Code (at time of writing)	Grade	Special Storage	Risk phrases*
Ammonium persulphate	$(NH_4)_2S_2O_8$	228.20	Sigma	A3678	>98%		H272, H302, H315, H317, H319, H334, H335
Bromophenol blue	$C_{19}H_{10}Br_4O_5S$	670.00	Aldrich	114391			none
Copper phthalocyanine 3,4',4'',4''' tetrasulphonic acid (tetrasodium salt)	$C_{32}H_{16}Cu_1N_8O_{12}S_4.4Na$	984.26	Aldrich	245356			H315. H319. H335
Dithiothreitol (DTT)	$C_4H_{10}O_2S_2$	154.20	Melford	MB1015	>99%	4°C	H302. H315 H319. H335
Glycerol	$CH_2OH.CHOH.CH_2OH$	92.10	Fisher	G/0650/08	>99%		none
Hydrochloric acid	HCl	36.46	Fisher	H/1150/PB17			H290 H314. H335.
Marvel milk powder			Premier Brands UK Ltd				none
Sodium dodecyl sulphate	$C_{12}H_{25}O_4SNa$	288.40	Melford	B2008	>99%		H228, H302, H332 H315, H318, H335, H412
Sodium hydroxide	NaOH	40.00	Sigma	S0899	98.4%		H290 H314.
TEMED	$C_6H_{16}N_2$	116.20	Sigma	T8133	99%		H225 H302. H314 H332
Tris base	$C_4H_{11}NO_3$	121.10	Melford	B2005	>99.9%		none
Tween-20			Sigma	P7949	98.4%		none
Urea	CH_4N_2O	60.06	Fisher	BP169-500	>99%		none

* Risk phrases: for definitions see Appendix 1

- 10x Phosphate buffered saline (PBS), pH 7.4, Medicago MC-12-9423-5 (supplied by Cambio Ltd).

6.2 Water

Unless otherwise stated, de-ionised water produced by a reverse osmosis system with a typical resistivity of 18.2 MΩ/cm is used.

6.3 Solutions

1. 40 % (w/v) acrylamide stock [consisting of 29 parts Acrylamide to 1 part Bis-acrylamide] – Commercial product: AccuGel™ 29:1, National Diagnostics EC-852 (supplied by Geneflow A2-0070).

2. ProtoGel® Resolving Buffer (Tris/SDS), National Diagnostics EC-892 (supplied by Geneflow B9-0012).

3. ProtoGel® Stacking Buffer (Tris/SDS), National Diagnostics EC-893 (supplied by Geneflow B9-0014).

4. 10x Tris/Glycine/SDS, National Diagnostics EC-870 (supplied by Geneflow B9-0032).

5. 20 % SDS solution, National Diagnostics EC-874 (supplied by Geneflow B9-0038)

6. Molecular weight markers supplied by Geneflow (BLUeye prestained protein ladder, catalogue number S6-0024). 2 µl used for 1 lane of the mini-gel system.

7. * Mouse monoclonal [4C2] to cTnI supplied by abcam, catalogue number ab10231

8. Mouse monoclonal [L2B10] to liver FABP supplied by abcam, catalogue number ab7366

9. Rabbit polyclonal to liver FABP supplied by abcam, catalogue number ab7847

10. Rabbit polyclonal to surfactant protein A supplied by Millipore, catalogue number AB3424

11. Rabbit polyclonal to SLC9a3r1 supplied by abcam, catalogue number ab3452

12. Goat anti-mouse immunoglobulins labelled with horseradish peroxidase (HRP) supplied by DakoCytomation, catalogue number P0447.

13. Goat anti-rabbit immunoglobulins labelled with HRP supplied by DakoCytomation, catalogue number P0448.

14. Detergent e.g. Decon 90 supplied by SLS, catalogue number CLE1022.

All the following solutions are produced in house and stored for 4 weeks at 4°C, unless otherwise stated.

15. 10x PBS: prepared using 10x PBS pouches according to manufacturer's instructions (Medicago MC-12-9423-5, store at 20°C

16. 1x PBS: prepared by diluting 10x stock of PBS, store at 20°C

17. PBS containing 8.0 M urea, store at 20°C

18. 2 x concentrated reducing sample buffer:

10 % [w/v] Sodium dodecyl Sulphate	40 ml
Glycerol	30 ml
0.5M Tris-HCl, pH 6.8	20 ml
Dithiothreitol	1.54 g
Bromophenol Blue	0.02 g

Make up to 100ml with distilled water and store in aliquots at -20°C.

19. 10% (w/v) ammonium persulphate (APS), store in aliquots at -20°C.

20. Copper stain: copper phthalocyanine 3,4',4'',4''' tetrasulphonic acid tetrasodium salt [0.05 % (w/v) in 12 mM HCl].

21. Destain for copper phthalocyanine: 12 mM NaOH.

22. PBS-Tween: PBS containing 0.1 % (v/v) Tween-20.

23. Blocking solution: PBS containing 3 % (w/v) Marvel milk powder (prepare prior to use and store at 4°C for up to 24 hours).

24. Primary antibody [diluted in blocking solution, (1:1000), except mouse monoclonal to H-SP2 – (1:20,000) and rabbit polyclonal to L-SP3 – (1:500)] (prepare prior to use).

25. Horseradish peroxidase conjugated secondary antibody [diluted in blocking solution, 1:2000] (prepare prior to use).

6.3.1 Meat and offals

Appropriate offals and meat purchased from a verifiable source for use in the production of standards.

6.4 Commercial kits

1. Bio-Rad Clarity™ Western ECL substrate (Bio-Rad code 1705060).

6.5 Plasticware

1. Pipette tips (0.1 – 10 µl) - e.g. Fisher code FB34521
2. Pipette tips (yellow 200 µl) - e.g. Sarstedt code 70.760.002
3. Pipette tips (blue 1000 µl) - e.g. Sarstedt code 70.762
4. Eppendorf tubes (500 µl) - e.g. Sarstedt code 72.699
5. Eppendorf tubes (1.5 ml) - e.g. Sarstedt code 72.690
6. 50 ml tubes (red topped) - e.g. Sarstedt code 62.547.004
7. 25 ml tubes (yellow topped) - e.g. Sarstedt code 63.9922.254
8. 8 ml flat base tube - e.g. Sarstedt code 63.542.709
9. 25 ml graduated pipettes - e.g. Sarstedt code 86.1685.001
10. 10 ml graduated pipettes - e.g. Sarstedt code 86.1254.001
11. 5 ml graduated pipettes - e.g. Sarstedt code 86.1253.001
12. Square Petri dish (100 x 100 mm) - e.g. Sarstedt code 82.9923.422
13. Gel loading tips - e.g. Star Lab code I 1022 0000

6.6 Equipment

1. Laboratory Blender with small volume capability
2. Heating oven
3. MP FastPrep®-24 - MP Biomedicals
4. SiLi ZY beads (1.4 - 1.6mm) - Sigmund (9615-41)
5. End-over-end tumbler - e.g. Model TM1, Luckham Ltd.
6. Centrifuge - e.g. Eppendorf 5810R
7. Heating block - e.g. Stuart Block Heater SBH 130D
8. Centrifuge for Eppendorf tubes - e.g Hettich Mikro 22R)
9. Orbital shaker - e.g. Stuart SSL1
10. Magnetic stirrer and stirring bar
11. Vortex mixer
12. Bio-Rad Mini Protean III and Mini Protean Tetra electrophoresis kits
13. Power supply (Bio-Rad Powerpac Basic)
14. iBlot® gel transfer device - Invitrogen (IB1001)
15. iBlot® gel transfer stack nitrocellulose - Invitrogen (IB3010-01)
16. Trans-Blot Turbo Transfer System - Biorad (1704155)
17. Trans-Blot consumables nitrocellulose - Biorad (170-4270 & 170-4271)
18. Imaging system eg CCD camera (FUJIFILM luminescent Image Analyser LAS-4000 and associated software)
19. Western blot analysis software e.g. Advanced Image Data Analyser (AIDA) for windows version 4.03 - Raytest
20. Micropipettes
21. Pipette filler
22. Disposable weighing boats (44 x 44 mm, 80 x 80 mm and 140 x 140 mm)
23. Eppendorf tube rack
24. Scissors
25. Disposable nitrile gloves
26. Sterile cork board
27. Scalpels
28. Spatula
29. Aluminium foil
30. Cling film

31. Forceps
32. Timer

7. PROCEDURE

The procedure is divided into 4 sections:

7.1 Preparation of skeletal muscle and offal mixtures and 'test' meat samples/products
7.2 Extraction of standards and 'test' meat samples/products
7.3 Preliminary screen for offal in 'test' meat samples/products by Western blot analysis
7.4 Semi-quantification of offal by Western blot analysis

7.1 Preparation of skeletal muscle and offal mixtures and 'test' meat samples/products

Suitable positive and negative controls (standards) need to be prepared to enable the analysis of 'test' meat products/samples with H-SP2 (heart), L-SP3 (liver), Lu-SP2 (lung) and K-MP1 (kidney) antibodies. This is achieved using skeletal muscle samples (bovine, ovine or porcine) spiked with 2 % (w/w) offal; except in the case of pork lung, where 10 % (w/w) lung is required. It is necessary to prepare standards for each species you are screening.

7.1.1 Obtain genuine skeletal muscle of the same alleged species as your 'test' sample (front quarter joints of beef, lamb or pork) from a verifiable source and homogenise in the small mill attachment of the laboratory blender. Offal (heart, liver, kidney and lung from beef, lamb or pork) should also be sourced from a verifiable source. Homogenise at least 50 g of offal for the preparation of standards in the small mill attachment of the laboratory blender for 30 seconds**.**

7.1.2 Prepare "meatballs" containing known amounts of offal (bovine/ovine/porcine heart, liver, kidney and lung, depending on the 'test' sample). The meat and offal are of the same species. See table below for example of beef.

Weight beef meat (g)	Weight beef heart (g)	Weight beef liver (g)	Weight beef kidney (g)	Weight beef lung (g)	Offal content
50.0	0	0	0	0	None
48.0	1.0	1.0	0	0	2 % (w/w) heart and liver
48.0	0	0	1.0	1.0	2 % (w/w) kidney and lung

7.1.3 The standard mixtures of minced meat and homogenised offal are prepared according to the table. Using the small mill attachment of the laboratory

blender, homogenise again in three bursts of 10 seconds to ensure complete mixing and to produce a homogeneous sample.

7.1.4 Transfer the “meatball” to a square of aluminium foil, wrap it securely and cook in a pre-heated oven at 180°C for 30 minutes.

7.1.5 For raw ‘test’ samples, weigh at least 100 g of the product to be tested (this may be 1 or 2 burgers or 2 sausages, for example), into a large weighing boat, break up the product using a spatula and then mix well, form a 50 g test sample into a “meatball” and cook in the same way as the standards.

7.1.6 If the ‘test’ sample is already cooked, homogenise (3 x 10 seconds) the whole product if it weighs less than 50 g (e.g. a whole burger) or homogenise a 50 g representative sample. If the cooked ‘test’ sample contains other ingredients (e.g in the case of lasagne or cottage pie), collect the meat portion from the product and mix well prior to taking a 50 g test sample.

7.2 Extraction of standards and ‘test’ meat samples/products

7.2.1 Allow all cooked standards and ‘test’ samples to cool for at least 1 hour at 20°C.

7.2.2 Take a 5 g representative aliquot from the cooked standard meatball or ‘test’ meat sample/product (duplicate samples of test sample preferred) and homogenise with 15 g SiLi beads into 30 ml PBS 8.0 M urea using MPBio Fast Prep homogeniser.

7.2.3 The MP FastPrep® is used according to the manufacturer’s instructions. The homogeniser is set to 4 Metres per second (m/s) for 30 seconds.

7.2.4 Tumble the tubes end-over-end for 17.5 hours at 20°C.

7.2.5 Centrifuge tubes at 1,500 x g for 15 minutes. Harvest the supernatants and store in aliquots at -20°C.

7.3 Preliminary screen for offal by Western blot analysis

Use 10 % or 13 % polyacrylamide gels and 15-well combs (use 10 % gels for Lu-SP2 and K-MP1, for the detection of lung and kidney, respectively, and 13 % gels for H-SP2 and L-SP3, for the detection of heart and liver, respectively). The following procedure is designed for 10 cm x 8 cm gels with 1.5 mm spacers. Four offals of one species can be screened in up to 3 ‘test’ samples using one 10 % gel plus one 13 % gel.

7.3.1 Preparation of extracts for non-continuous SDS PAGE

7.3.1.1 Dilute appropriate standards and ‘test’ samples (in a 0.5 ml Eppendorf tube): PBS 8 M urea extracts of standards and ‘test’ samples are diluted x 12 i.e. 10 µl extract plus 50 µl distilled water plus 60 µl 2 x reducing sample buffer

and load 10 µl. Heavily processed 'test' samples (for example canned foods and ready meals) are diluted x 6 i.e. 20 µl extract plus 40 µl distilled water plus 60 µl 2 x reducing sample buffer and load 10 µl.

7.3.1.2 Place all samples and standards into a heating block at 100°C for 5 minutes to ensure that the proteins are fully dissolved and denatured. Microcentrifuge at top speed (approximately 13,000 x g) for approximately 5 seconds to ensure that all condensation has been incorporated into the sample.

7.3.1.3 Allow the samples to cool for approximately 1 minute before loading into a well of a mini polyacrylamide gel (see section 7.3.2).

7.3.2 Non-continuous SDS PAGE

7.3.2.1 Clean glass gel plates (with 1.5 mm spacers attached) using detergent followed by distilled water.

7.3.2.2 Assemble the gel mould apparatus according to the manufacturer's instructions.

7.3.2.3 Prepare the relevant resolving gel mix in a 50 ml red topped tube (refer to Appendix 2 for gel recipes, making sufficient for one 10 % gels and one 13 % gels).

7.3.2.4 Immediately prior to pouring the gel add the appropriate concentration and amount (see Appendix 2) of ammonium persulphate (APS) and TEMED and mix gently by inverting the tube twice.

7.3.2.5 Pour the relevant gel mix into the gel mould apparatus to a height of 1½ comb depths from the top edge of the plate sandwich.

7.3.2.6 Carefully overlay the gel with 400 µl distilled water to exclude air and ensure the gel surface is flat.

7.3.2.7 Allow polymerisation to proceed for approximately 45 minutes at room temperature.

7.3.2.8 Remove any excess unpolymerised buffer by carefully blotting the top of the gel with filter paper.

7.3.2.9 Prepare the stacking gel mix in a 25 ml tube (refer to Appendix 2).

7.3.2.10 Immediately prior to pouring the stacking gel on top of the resolving gel add the appropriate concentration and amount (see Appendix 2) of APS and TEMED and mix gently by inverting the tube twice.

7.3.2.11 Position the comb (15 well, 1.5 mm thick) in the gel mould apparatus at a 45° angle and carefully add the stacking gel mix to the top of the resolving gel. Then lower the comb into the stacking gel to form individual wells, taking care to avoid the introduction of bubbles.

7.3.2.12 Allow the stacking gel to polymerise for 30 minutes at room temperature before sample application.

7.3.2.13 Remove the comb and wash the formed sample wells with 50 % (v/v) Tris/glycine electrode buffer.

7.3.2.14 Fix the gel into the electrode tank according to the manufacturer's instructions and fill the inner and outer gel reservoirs with electrode buffer.

7.3.2.15 Apply 10 µl of the 'test' samples and standards to the wells of the gel using a microlitre pipette fitted with gel loading tips, according to a documented layout plan. See below for example of a gel layout for detecting the presence of beef heart and beef liver, and beef kidney and beef lung in 3 beef 'test' samples.

13 % gel LANE					
1	2 - 3	4 - 6	7 - 9	10 - 12	13 – 15
Molecular weight marker	S1	S2	U1	U2	U3

10 % gel LANE					
1	2 - 3	4 - 6	7 - 9	10 - 12	13 – 15
Molecular weight marker	S1	S3	U1	U2	U3

S = standard
S1 = 100 % beef (negative control)
S2 = beef meat containing 2 % (w/w) beef heart & 2 % (w/w) beef liver (positive control)
S3 = beef meat containing 2 % (w/w) beef kidney & 2 % (w/w) beef lung (positive control)
U1 = 'test' sample 1
U2 = 'test' sample 2
U3 = 'test' sample 3

7.3.2.16 Place the lid on the tank, connect to the power pack and run the gel at a constant voltage of 180 volts for approximately 45 minutes at room temperature until the tracking dye is approximately 0.3 cm from the bottom of the gel.

7.3.2.17 Electroblot the separated proteins onto nitrocellulose (see section 7.3.3).

7.3.3 Transfer of proteins to nitrocellulose using Invitrogen iBlot® transfer device or Bio Rad Transblot device (electro-blotting to produce a 'Western blot')

Use iblot when blotting and probing for lung-specific and kidney-marker proteins and Trans Blot Turbo when blotting and probing for heart and liver specific proteins.

NB. Always wear gloves when handling gel/filter papers and nitrocellulose.

7.3.3.1 If using Bio-Rad Trans Blot Turbo, dilute the 5x blotting buffer to its working concentration and pre-soak the required number of blotting stacks and nitrocellulose.

7.3.3.2 Meanwhile, assemble the blotting apparatus (iBlot or Trans Blot Turbo) according to the manufacturer's instructions.

7.3.3.3 Wash the electrophoresed gel in 50-100 ml of distilled water to partially remove SDS.

7.3.3.4 Carefully lay the gel onto the iblot or Trans blot turbo stack, taking care to avoid bubbles.

7.3.3.5 Electroblot according to the manufacturer's instructions (7 minutes for iblot, 10 minutes for Trans-blot turbo 1.5 mm gel thickness). Check transfer time is appropriate for the gel thickness and protein of interest (refer to manufacturer's instructions).

7.3.3.6 After transfer, turn off the power supply and disassemble the apparatus.

7.3.3.7 Carefully disassemble the membrane stacks and remove the immobilising membrane for analysis (either immunoprobing or protein staining).

7.3.4 Staining blot to assess electrophoretic transfer

The transfer of proteins to nitrocellulose is assessed using the reversible copper stain (copper phthalocyanine 3,4',4'',4''' tetrasulphonic acid tetrasodium salt).

7.3.4.1 Following transfer, incubate the blot in the copper stain for approximately 5 minutes. This stain will reversibly stain total protein on the nitrocellulose membrane.

7.3.4.2 Wash the blot in distilled water for 5 minutes and obtain a digital image of the stained blot using FUJIFILM Luminescent Image Analyzer LAS-4000 (follow the manufacturer's instructions) or equivalent alternative and then save the digital image as an 8 bit tiff file or other suitable image file format.

7.3.4.3 Cut the nitrocellulose into strips to allow probing for heart and liver (Transblot) and kidney and lung (iblot). For heart detection cut the nitrocellulose above the 37 kDa and on the 20 kDa marker and retain the strip, for liver detection cut the nitrocellulose at the 20 kDa marker and use the bottom strip of the nitrocellulose. For kidney detection cut and retain the

section of nitrocellulose between the orange 75 kDa marker and just below the 48 kDa marker. For lung detection cut the nitrocellulose just below the 48 kDa and on the 25 kDa marker and retain.

7.3.4.4 Destain the blot strips by washing in 12 mM NaOH for approximately 5 minutes and rinse with distilled water before immunoprobing (see section 7.3.5).

7.3.5 Immunoprobing of electroblots

7.3.5.1 Following Western blotting and staining/destaining with copper stain, block the remaining protein binding capacity of the nitrocellulose with blocking solution. Place the nitrocellulose membrane in a square Petri dish with 30 ml of blocking solution on an orbital shaker set at 30 revolutions per minute (rpm) for 1 hour at room temperature.

7.3.5.2 Incubate the blot with 5 ml of the appropriately diluted primary antibody solution overnight at 4°C (anti-H-SP2, anti-L-SP3 (use the mouse monoclonal antibody for beef or lamb and rabbit polyclonal antibody for pork), anti-Lu-SP2 or anti-K-MP1 antibodies for heart, liver, lung and kidney, respectively) in a square Petri dish. Place on an orbital shaker set at 30 rpm.

7.3.5.3 Wash off any unbound antibody with 30 ml of PBS-Tween (3 times, for 10 minutes each time) whilst shaking on an orbital shaker set at 70 rpm at room temperature.

7.3.5.4 Incubate with 10 ml of the appropriately diluted HRP-conjugated secondary antibody, with slow (30 rpm) shaking on an orbital shaker for 1.5 hours at room temperature.

7.3.5.5 Wash for 30 minutes with PBS-Tween (3 times, for 10 minutes each time), shaking on an orbital shaker set at 70 rpm at room temperature.

7.3.5.6 Rinse the blot in PBS for 5 minutes.

7.3.5.7 Transfer the blot to a large weighing boat (140 x 140 mm) and pipette 0.5 ml of substrate solution (Clarity Chemiluminescence Substrate) onto the blot and incubate in the dark for 5 minutes at room temperature.

7.3.5.8 Tip off the excess substrate and place the nitrocellulose membrane onto a non-reflective plate.

7.3.5.9 Place the plate on a tray inside the CCD camera and set the required exposure time and blot area using the FUJIFILM Image reader software. Exposure time will vary depending on antibody titre and sample, but is generally in the region of 10 seconds to 6 minutes for anti-H-SP2, anti-Lu-SP2 and anti-K-MP1; and 2 to 20 minutes for anti-L-SP3. Always start with a short exposure time and increase accordingly.

7.3.5.10 Save a digital image of the exposed blot as an 8 and 16 bit tiff file for analysis using AIDA software or equivalent.

7.3.5.11 If a band, of the same molecular weight as the band in the positive control lanes is present in any of the 'test' samples, offal is present. The mouse monoclonal anti-L-SP3 detects beef and lamb L-SP3, the rabbit polyclonal anti-L-SP3 antibody detects pork L-SP3 and to a lesser extent beef and lamb L-SP3. The other antibodies (anti-H-SP2, anti-Lu-SP2 and anti-K-MP1) recognise beef, lamb and pork offal tissue.

7.4 Semi-quantification of offal by Western blot analysis

If the species of origin of the offal is known, semi-quantification can be conducted immediately when a positive reading has been obtained in the preliminary screen. If the species is not known, MS analysis is required (see section 7.5 for heart and section 7.6 for liver, lung and kidney of the standard operating procedure for the extraction of offal marker proteins in raw and processed meat products, and their subsequent detection and quantification). MS analysis can also be used to confirm the presence of offal tissue, limit of detection is 1 % (w/w) heart, 1- 2 % (w/w) liver and approx. 10 % (w/w) lung and kidney.

Suitable standards need to be prepared to enable the analysis of 'test' meat products/samples with H-SP2 (heart), L-SP3 (liver), Lu-SP2 (lung) and K-MP1 (kidney) antibodies. This is achieved using skeletal muscle spiked with known amounts of offal. It is necessary to prepare standards for each species and offal you are screening.

7.4.1 Obtain genuine skeletal muscle of the same alleged species as your 'test' sample (front quarter joints of beef, lamb or pork) from a verifiable source and homogenise in the small mill attachment of the laboratory blender. Offal (heart, liver, kidney and lung from beef, lamb or pork) should also be sourced from a verifiable source. Homogenise at least 50 g of offal for the preparation of standards in the small mill attachment of the laboratory blender for 30 seconds**.**

7.4.2 Prepare "meatballs" containing known amounts of offal (bovine/ovine/porcine heart, liver, kidney or lung, depending on the positive 'test' sample). See table for example of beef heart.

Weight beef meat (g)	**Weight beef heart (g)**	**Offal content**
50.0	0	None
49.5	0.5	1 % (w/w) heart
49.0	1	2 % (w/w) heart
47.5	2.5	5 % (w/w) heart
45.0	5	10 % (w/w) heart
42.5	7.5	15 % (w/w) heart

7.4.3 The standard mixtures of minced meat and homogenised offal are prepared according to the table. Using the small mill attachment of the laboratory blender, homogenise again in three bursts of 10 seconds to ensure complete mixing and to produce a homogeneous sample.

7.4.4 Transfer the “meatball” to a square of aluminium foil, wrap it securely and cook in a pre-heated oven at 180°C for 30 minutes.

7.4.5 Extract proteins from standards as described in section 7.2.

For each offal, run a gel containing an appropriate range of standards plus the ‘test’ sample loaded in triplicate.

7.4.6 Prepare standards and ‘test’ samples for SDS PAGE (as described in section 7.3.1).

7.4.7 Fractionate standards and ‘test’ samples on 10 % (for lung and kidney) or 13 % (for heart and liver) gels, as described in section 7.3.2. An example of a gel layout for the semi-quantification of beef heart in 2 ‘test’ samples containing > 2 % beef heart is given below.

LANE								
1	2	3	4	5	6	7	8-10	11-13
Molecular weight marker	S1	S2	S3	S4	S5	S5	U1	U2

S1 = 100 % beef meat
S2 = 1 % (w/w) beef heart
S3 = 2 (w/w) beef heart
S4 = 5 % (w/w) beef heart
S5 = 10 % (w/w) beef heart
S6 = 15 % (w/w) beef heart
U1 = ‘test’ sample containing heart
U2 = ‘test’ sample containing heart
U3 = ‘test’ sample containing heart

7.4.8 Transfer proteins to a nitrocellulose membrane, to produce Western blots, and visualise transfer using copper staining (as described in sections 7.3.3 and 7.3.4).

7.4.9 Immunoprobing of electroblots/Western blots. Probe the blots with the appropriate antibodies (as described in section 7.3.5).

7.4.10 Calculations and data analysis

The digital image is analysed using imaging analysis software (eg. AIDA image analysis software).

7.4.10.1 For each gel, obtain the densities of the main band for both the standards and unknowns (using the 16 bit image). Subtract the background signal from all the standards and unknowns. Once all the values have been obtained further analysis can be performed in a suitable line fitting software package e.g Excel.

7.4.10.2 Using a suitable line fitting software, arrange the results for each gel so that the standards and samples are labelled. Prepare a standard plot by subtracting the blank (100 % beef meat) signal from the other standards.

7.4.10.3 For a linear fit, produce the standard graph by using an x/y scatter plot. Produce a trend line and, using the equation of the line, calculate the percentage of H-SP2, L-SP3, Lu-SP2 or K-MP1 in the ‘test’ sample(s). For non-linear data use a polynomial fit; produce a standard plot using a suitable curve fitting software e.g.GraphPad Prism software, and calculate the amount of H-SP1, L-SP3, Lu-SP2 or K-MP1 in the ‘test’ sample(s).

7.4.10.4 Results should be reported as follows: < 4 % (w/w) offal, 4 % - 12 % (w/w) offal or > 12 % (w/w) offal.

8. APPENDICES

Appendix 1

Risk phrases are a system of codes and phrases for labeling hazardous chemicals and compounds.

H225 Highly flammable liquid and vapour

H228 Flammable solid.

H290 May be corrosive to metals.

H301 Toxic if swallowed

H302 Harmful if swallowed

H302 + H332 Harmful if swallowed or if inhaled

H311 Toxic in contact with skin

H314 Causes severe skin burns and eye damage.

H315 Causes skin irritation.

H318 Causes serious eye damage.

H319 Causes serious eye irritation

H331 Toxic if inhaled

H332 Harmful if inhaled

H335 May cause respiratory irritation.

H370 Causes damage to organs.

H412 Harmful to aquatic life with long lasting effects.

Appendix 2

Gel mixtures

Resolving gel

To make 10 ml (sufficient for one gel)

	10%	13%
40% Acrylamide stock	2.5 ml	3.25 ml
ProtoGel resolving gel buffer	2.5 ml	2.5 ml
Distilled water	5.0 ml	4.25 ml

To polymerise 10 ml add	TEMED	10 µl
	Ammonium persulphate [(APS) 10% w/v]	50 µl

Stacking gel mix 4%

To make 10 ml

40% Acrylamide stock	1 ml
ProtoGel stacking gel buffer	2.5 ml
Distilled water	6.4 ml

To polymerise 10 ml	TEMED	20 µl
	Ammonium persulphate [(APS) 10% w/v]	50 µl