fera

The Food and Environment Research Agency

Application of Proteomics to Food Authenticity

Dr Paul Reece,

Summary of presentation

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1. Introduction to food authenticity proteomics
2. Examples of FERA authenticity proteomics projects:

- Detection of meat binding agents.
- Identification of the species of animal protein in animal feed.
- Detection of the species from which gelatine is derived.

Proteomics :

The study of the protein population in biological systems

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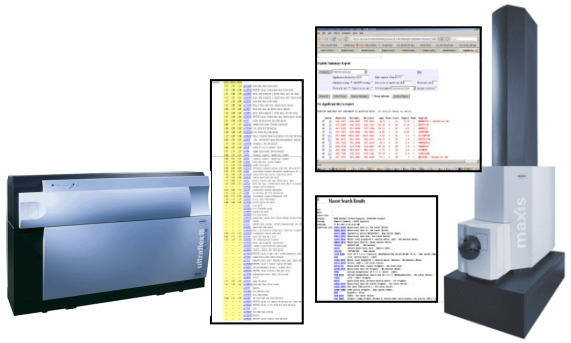
- Rapid growth in clinical, agricultural and nutritional studies:[1,2,3]
- no significant take-up of technology yet in area of food safety and authenticity testing.[4] (ex allergens and toxins [5, 6])
- Complements DNA technology particularly where processing has destroyed DNA or where DNA cannot discriminate target of choice e.g. discriminating tissues in meat products.



1 *Ye X, et al. (2009) Brief Funct Genomic Proteomic: 8(2):126-35*
2 *Lovegrove A, et al. (2009) Methods Mol Biol.;478:273-88.*
3 *Moresco JJ, et al. (2008) Am J Clin Nutr. Sep;88(3):597-604*
4 *M.Lees (2003) Food authenticity and traceability CRC. Woodhead*
5 *S. R. Kalb and J. R. Bar (2009) Anal. Chem., 81 (6), 2037–2042*
6 *L. Monaci and A. Viscontia (2009) Trends in Anal. Chem. On line.*

Application of proteomics to food authenticity

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1. **Protein isolation or enrichment** (e.g. Full purification, affinity enrichment, or no enrichment at all ('*shotgun proteomics*'))

3. **Protein fragmentation** to peptides through digestion with proteases

5. **Comparative studies of peptides** to identify key peptide markers (typically by 1 D or 2D-LC and soft ionisation MS)

6. **Confirmation of peptide identity** (sequence identity by MS/MS)

9. **Transfer of peptide detection system to user friendly format** (triple quadrupole MS, ELISA)

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Example 1

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- **Identifying the species of blood based meat binders by LC-MS**

The problem

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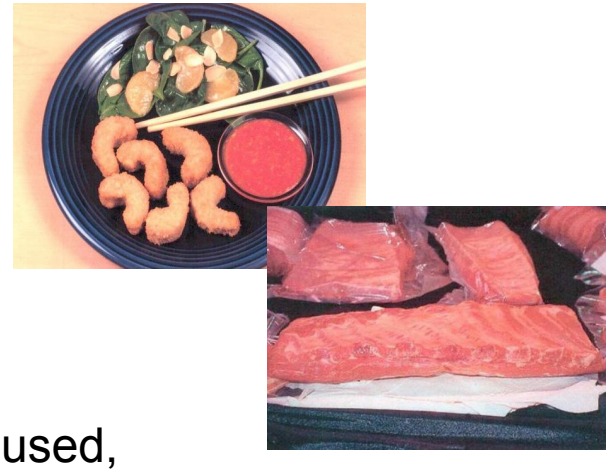
- Cold set meat binding agents based on bovine and porcine plasma or fibrinogen (Fibrimex™) have been approved by EFSA
- UK FSA needed to enforce appropriate labelling legislation on:
  - Species
  - Meat content
  - cut of meat,

  and protect religious sensitivities



The FSA requested a test that could:

- determine where this product has been used,
- determine whether bovine or porcine blood has been used to produce the gelling agent,
- be readily used by enforcement authorities,

FOOD STANDARDS AGENCY

Background : blood clotting cascade

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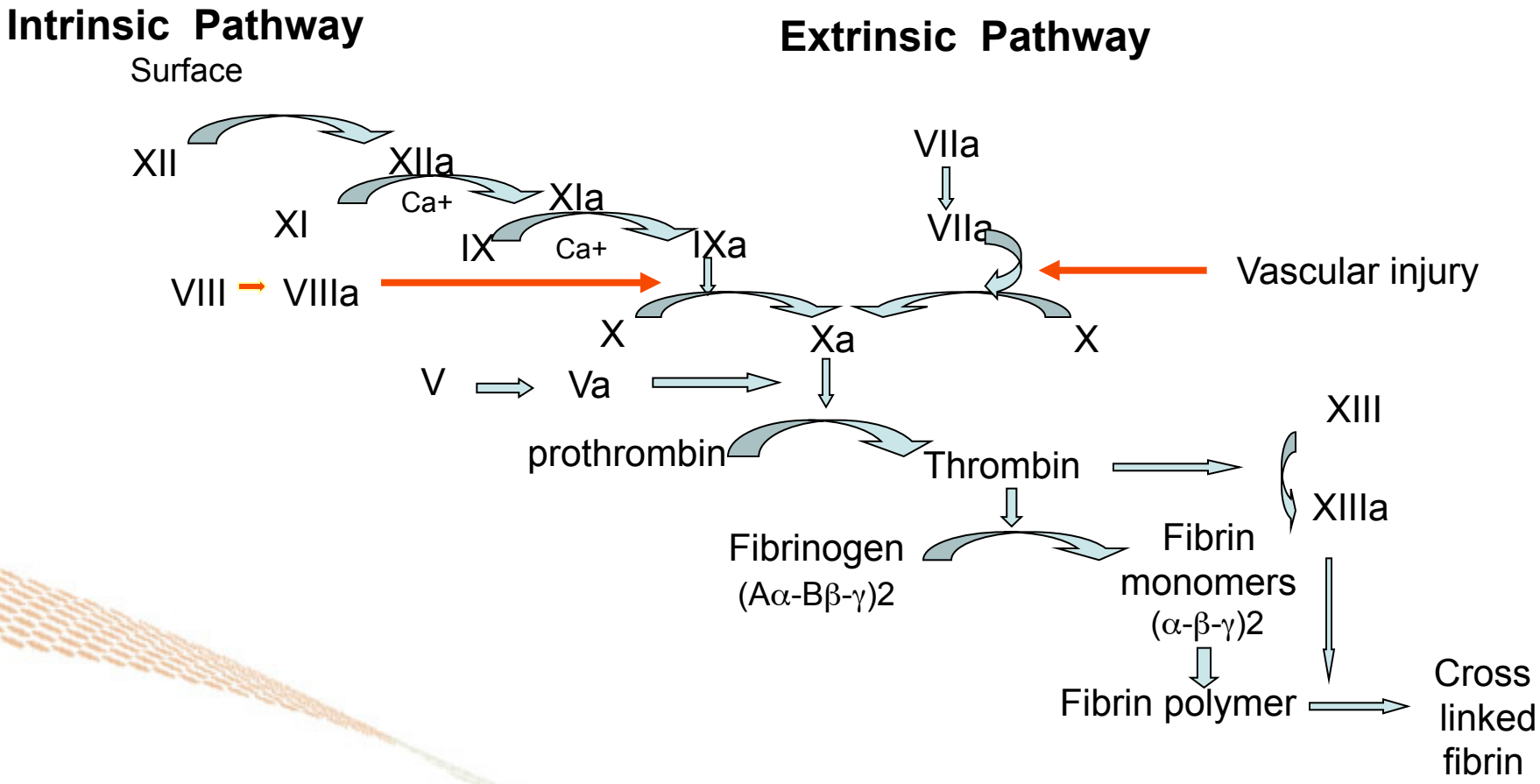
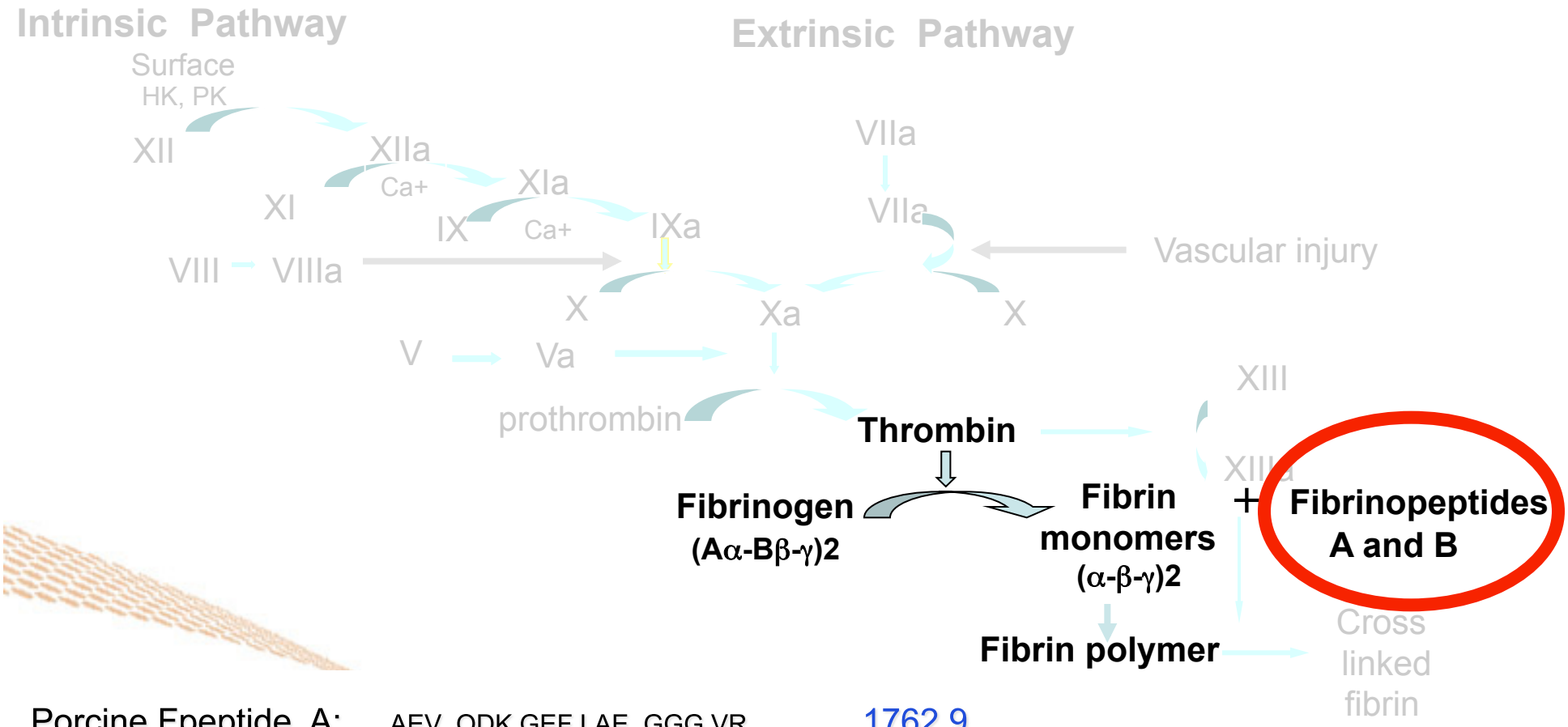


Background : **blood clotting cascade**

The Food and Environment Research Agency



Porcine Fpeptide A:	AEV QDK GEF LAE GGG VR	1762.9
Porcine Fpeptide B:	AID YDE DED GRP KVH VDA R	2201.3
Bovine Fpeptide A:	EDG SDP PSG DFL TEG GGV R	1891.9
Bovine Fpeptide B:	QFP TDY DEG QDD RPK VGL GAR	2364.1 Da

MALDI-TOF MS of synthetic standards

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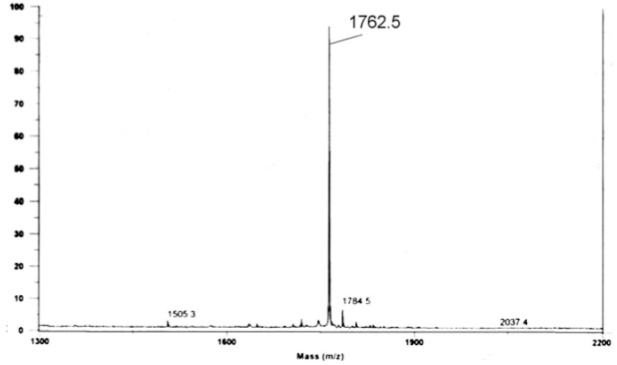
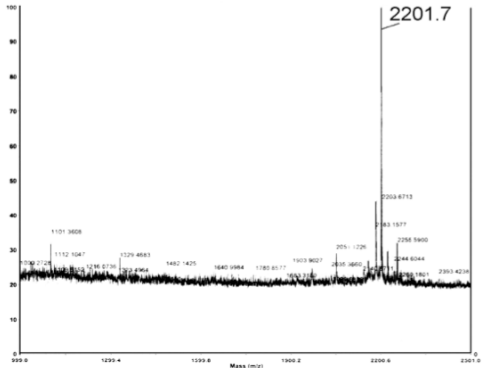
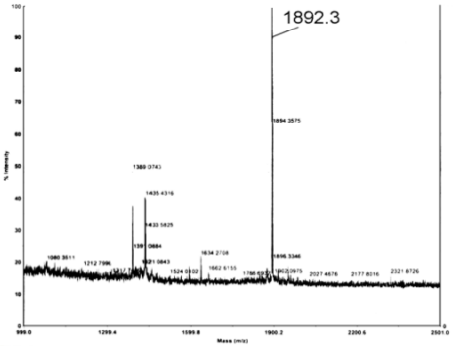
Porcine fibrinopeptide A



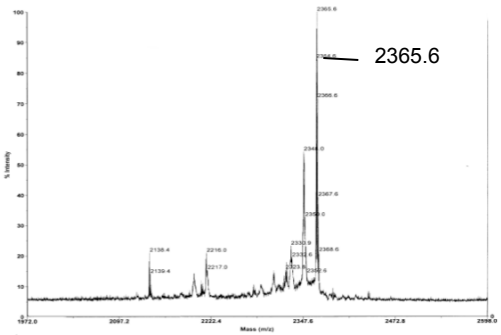
Porcine fibrinopeptide B



Bovine fibrinopeptide A



Bovine fibrinopeptide B



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MALDI-TOF of peptides extracted from plasma

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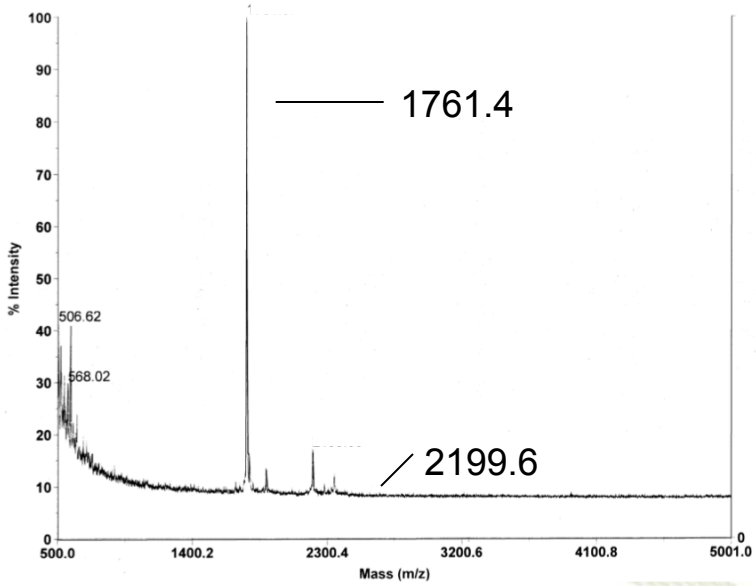
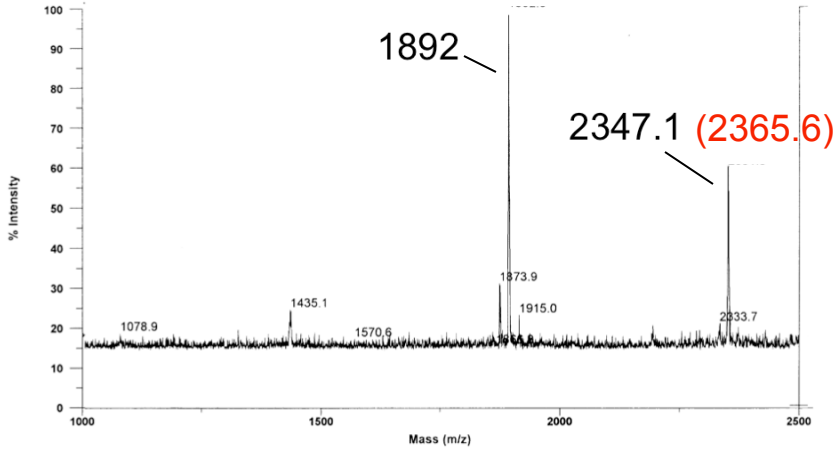
Extraction in TCA then
TCA removal with ether

Porcine plasma



Bovine Fibrimex™



Fibrinopeptide A released before Fibrinopeptide B

Blombäck, B., Hessel, B., Hogg, D., and Therkildsen, L. (1978) Nature 275:501-505.

MALDI-TOF of peptides extracted from plasma

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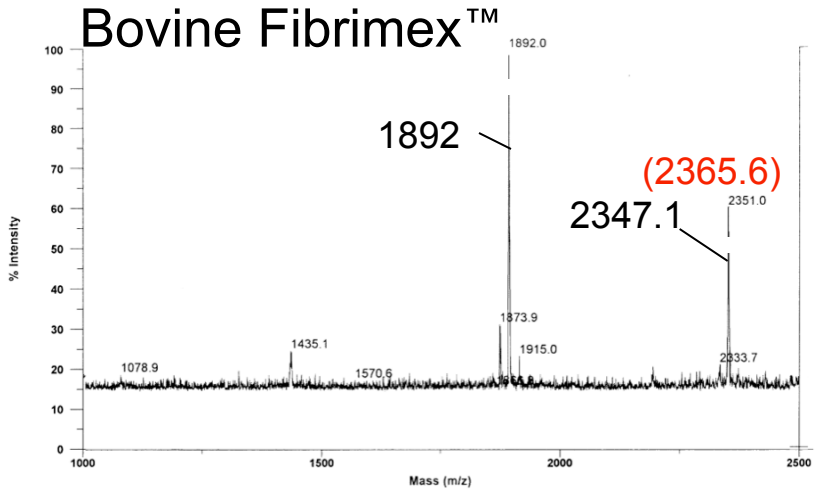
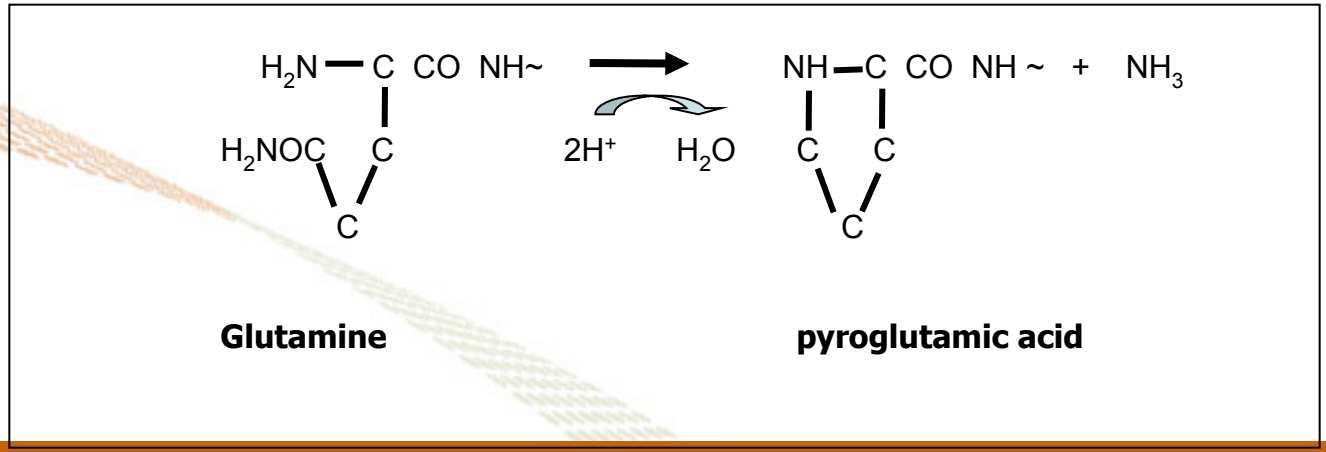
MALDI-TOF post-source decay analysis showed N-terminal glutamine had been cyclized to pyroglutamic acid under low pH conditions. This resulted in a loss of 17Da





Low pH reverse phase separation of synthetic standards

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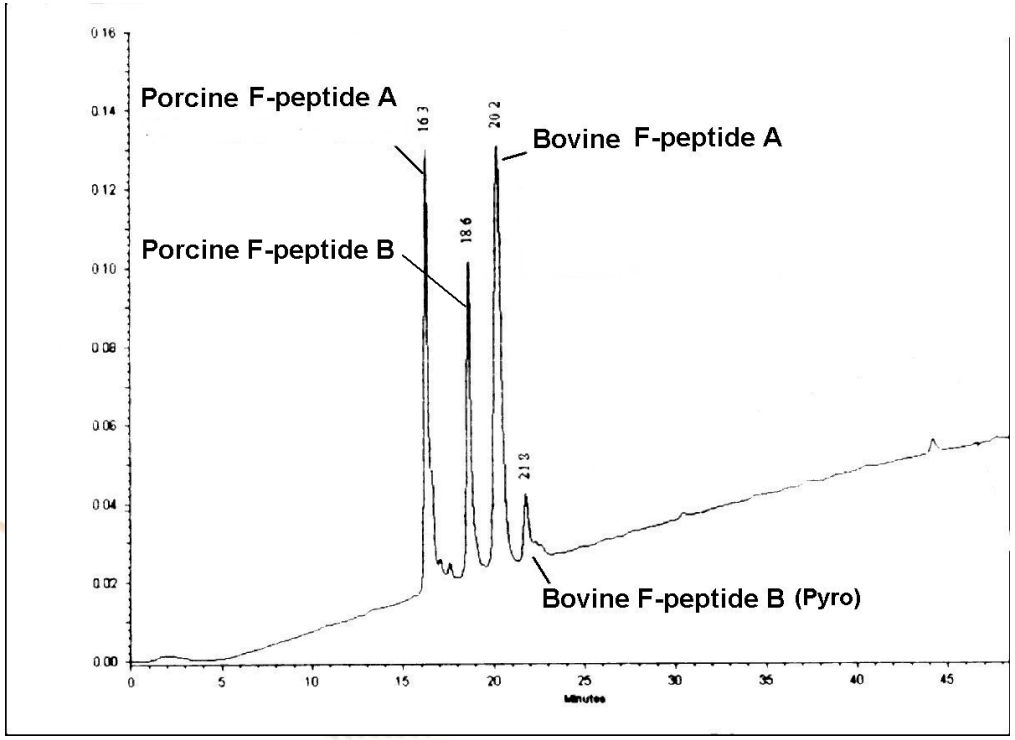
Phenomenex, 10μm, 300Å, C4, 250 x4.6mm
Aqueous 0.1% TFA / Acetonitrile gradient (pH 2.2)
E210nm



Optimising ESI- MS/MS of fibrinopeptides

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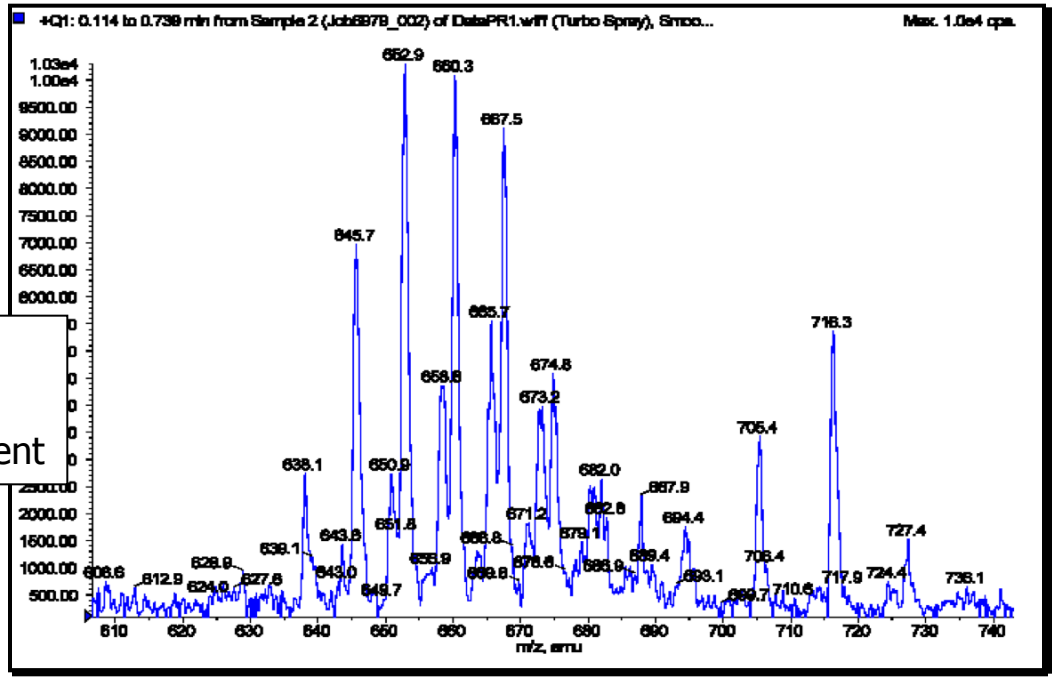
BOVINE
FIBRINOPEPTIDE A

Waters Quattro Ultima
LC- MS/MS
Aqueous Formic acid/ Acetonitrile gradient



The molecular weight of the peptide as determined by MALDI-TOF MS, was 1891.2.
Using this molecular weight, some of the ions were interpreted as follows:

638 = $[M+Na+H_2]^{3+}$

646 = $[M+Na_2+H]^{3+}$

653 = $[M+Na_3]^{3+}$ *and/or unresolved* $[(M-H+Na)+Na_2+H]^{3+}$

660 = $[(M-H+Na)+Na_3]^{3+}$ *and/or unresolved* $[(M-H_2+Na_2)+Na_2+H]^{3+}$

MS/MS Transitions using Selective Reaction Monitoring

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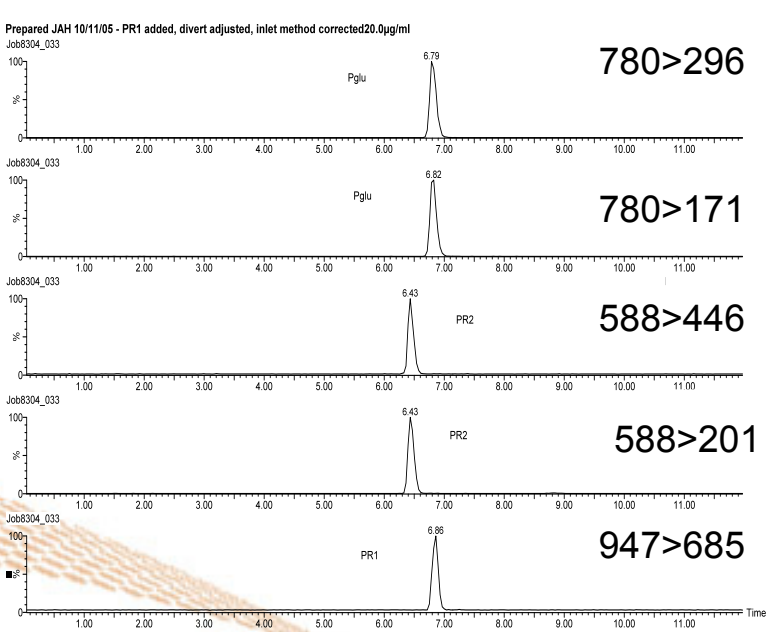

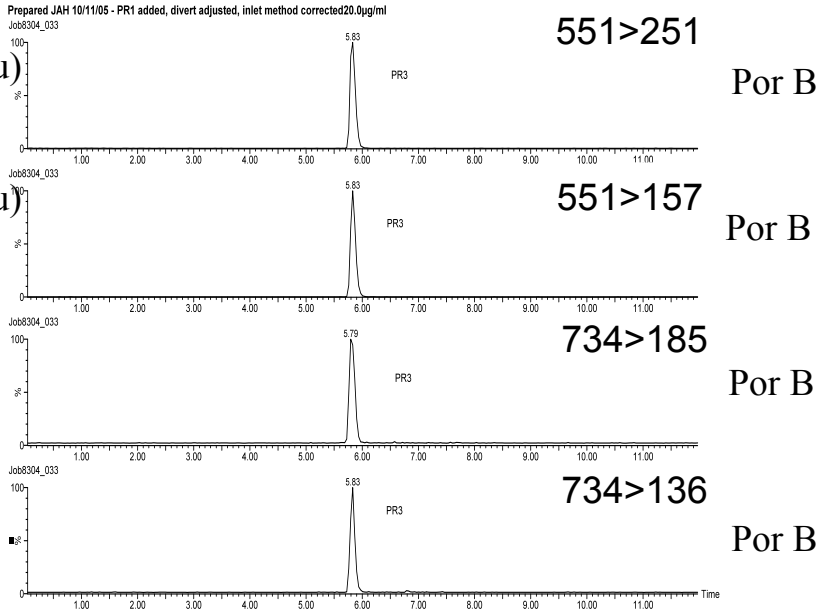

Protocol

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- Extract in trichloroacetic acid.
- Centrifuge to remove proteins.
- Wash in diethyl ether.
- Dry aqueous phase.
- Reconstitute in 100mM phosphate buffer pH 7.2.
- Load sample onto Waters Oasis HLB™ SPE cartridge.
- Wash in 5% methanol.
- Elute in 45% methanol 2% ammonium hydroxide.
- Dry, then dissolve in 0.15ml 5% acetonitrile pH 2.2 with formic acid.
- Apply 10μl to LC-MS/MS

Matrix effects

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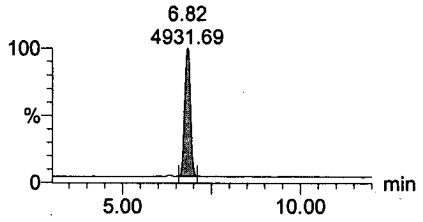
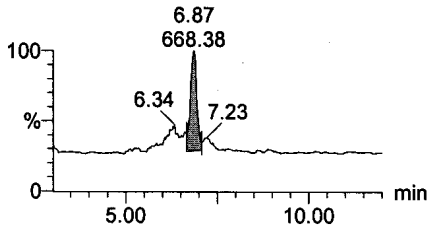
Detection of 5% bovine Fibrimex™ in pork

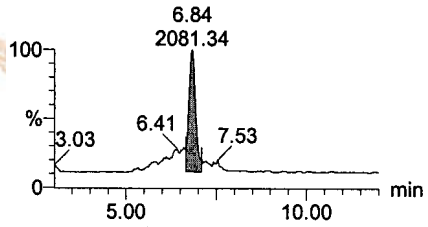
A947>685
(z=2)



B780>296
(z=3)

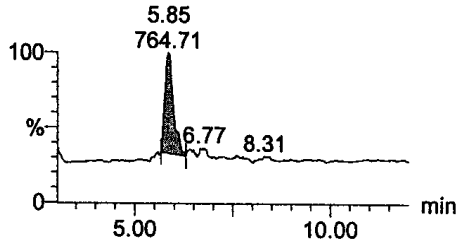


B780>171
(z=3)

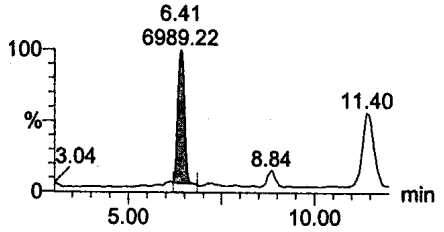


Detection of 5% porcine plasma in beef mince

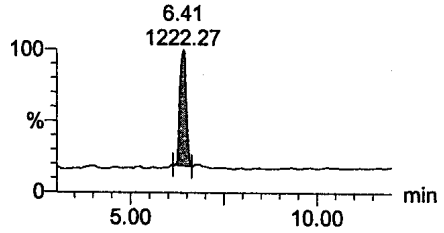
B551>251
(z=4)



A588>201
(*z=3*)



A588>446
(*z=3*)



Matrix effects: Detection of 10% porcine plasma

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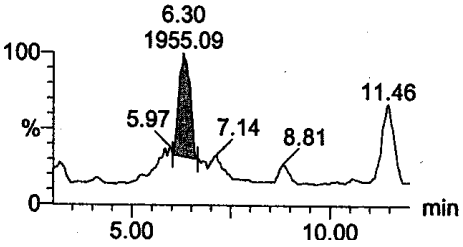
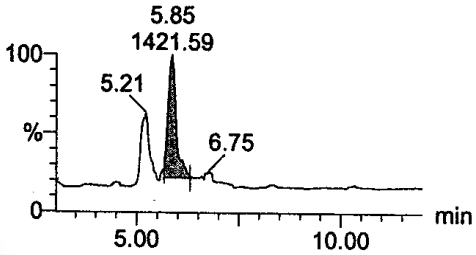
in chicken mince

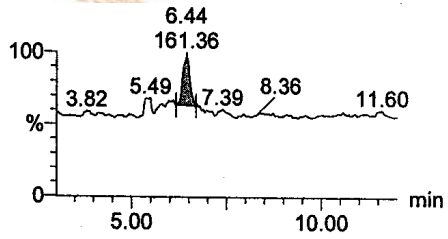
A588>201 *(z=3)*



B551>251 *(z=4)*

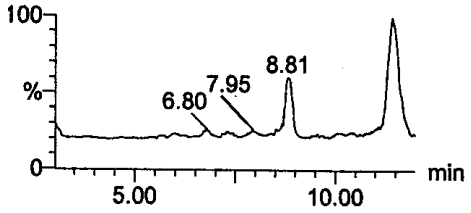


A588>446 *(z=3)*

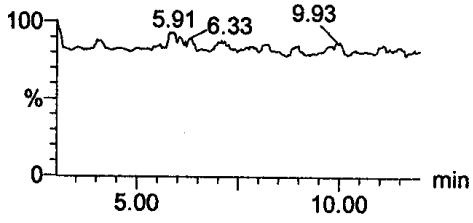


in cod mince

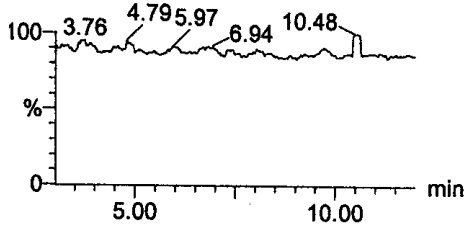
A588>201 *(z=3)*



B551>251 *(z=4)*



A588>446 *(z=3)*



Conclusions

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- Method developed detects the addition of porcine and bovine blood gelling agent to beef, lamb, chicken, pork and tuna when used at commercial levels.
- The addition of porcine or bovine gelling agents to white fish cannot currently be detected using this method
- LC-MS/MS methods on a triple quadrupole platform can be used to detect specific peptides in complex food matrices.

Grundy HH, Reece P, Sykes MD, Clough JA, Audsley N, Stones R. (2007) Rapid Commun. Mass Spectrom. 21(18):2919-25.

Grundy HH, Reece P, Sykes MD, Clough JA, Audsley N, Stones R. (2008) Rapid Commun. Mass Spectrom. Jun;22(12):2006-8.

Example 2

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Identification of the species of animal protein in animal feed

Background

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The BSE crisis lead to a ban on all animal protein in animal feed.

EC Regulation 1234/2003

- Current scientific evidence suggests only a ban on cannibalism
- Enforcement of a ban on cannibalism requires methods to identify the species of animal protein in the feed
- DNA is almost completely destroyed at the processing temperatures of animal feed (141-145°C)
- DNA tests on low copy number samples are prone to contamination, (PCR reagents contain DNA of domestic animals)

*(Leonard, J. A., Shanks, O., Hofreiter, M., Kreuz, E., Hodges, L., Ream, W., Wayne, R. K. & Fleischer, R. C. 2007 Animal DNA in PCR reagents plagues ancient DNA research. Journal of Archaeological Science **34**, 1361-1366.)*

EU project

SAFEED-PAP

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Detection of presence of species-specific processed animal proteins in animal feed

RIKILT INSTITUTE OF FOOD SAFETY WAGENINGEN UR

CRA-W Gembloux

EUROPEAN COMMISSION DIRECTORATE-GENERAL Joint Research Centre

Veterinary Laboratories Agency

NATIONAL VETERINARY LABORATORY

Ministeriet for Fødevarer, Landbrug og Fiskeri Plantedirektoratet

diagenode

中国动物卫生与流行病学中心 China Animal Health and Epidemiology Center

http://safeedpap.feedsafety.org/

EU project

SAFEED-PAP

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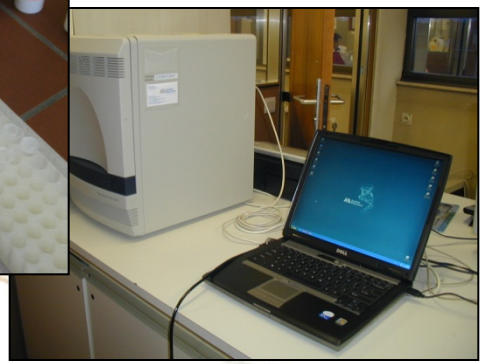
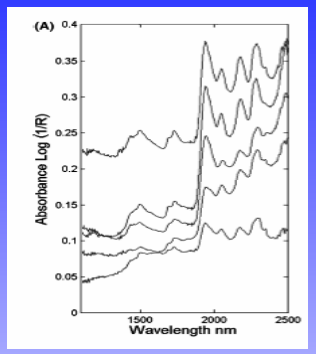
WP2

Evaluate existing dipstick and DNA approaches



WP4

Evaluate NIRM /PCR on bone particles



http://safeedpap.feedsafety.org/

EU project

SAFEED-PAP

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WP3

Identification of species specific proteins from animal feed and development of a confirmatory method to detect and identify the selected targets using HPLC and MS/MS

Observed differences in the amino acid sequence of the first 60 residues of fast skeletal muscle troponin I in 6 animal species (sequence information from NCBI)

mgdeekrnra itarrqhlks vmlqiaatel ekeesrreae kqnylaehcp plhipgsmse	MAN
mgdeekrnra itarrqhlks vmlqiaaqel ekeesrrese kqnylsehcp plhlpgsmse	DOG
msdeekkrra atarrqhlks amlqlavtei ekeaaakeve kqnylaehcp plslpgsmqe	CHICKEN
mgdeekrhra itarrqhlks vmlqiaatel ekevgrrese kqnylsehcp plhlpgsmse	PIG
mgdeekrnra itarrqhlks vmlqiaatel ekeesrrese kenylsehcp plhipgsmse	MOUSE
mgdeekrhra itarrqhlks vmlqiaatel ekeegrreae kqnylsehcp plhlpgsmse	BOVINE

EU project

SAFEED-PAP

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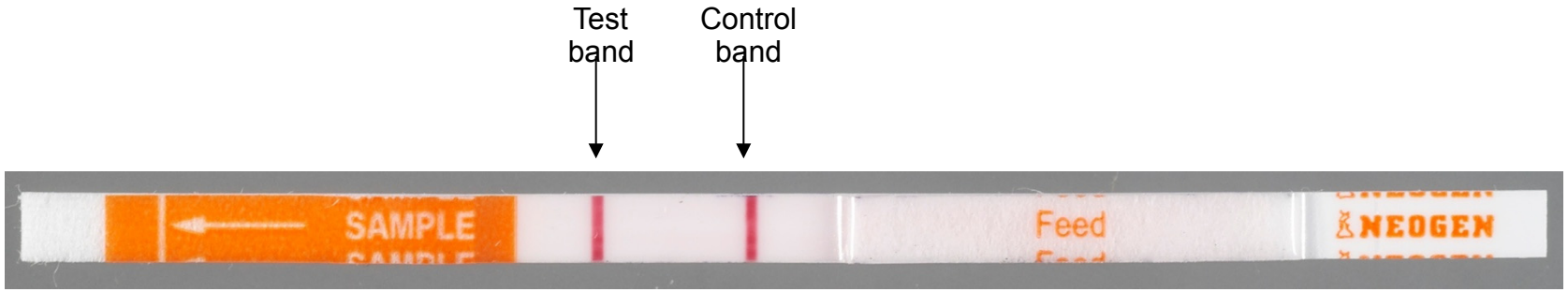

No bovine troponin I tryptic peptides detected by LC-Q-TOF or MALDI-TOF-TOF MS

Observed differences in the amino acid sequence of the first 60 residues of fast skeletal muscle troponin I in 6 animal species (sequence information from NCBI)

mgdeek r nr aitar r qhlk svmlqiaatelek eesr r eaek MAN
mgdeek r nr aitar r qhlk svmlqiaaqelek eesr r esek DOG
msdeek k r r aatar r qhlk samlqlavteiek eaaak evek CHICKEN
mgdeek r hr aitar r qhlk svmlqiaatelek evgr r esek PIG
mgdeek r nr aitar r qhlk svmlqiaatelek eesr r esek MOUSE
mgdeek r hr aitar r qhlk svmlqiaatelek eegr r eaek BOVINE

iTRAQ analysis of Meat meal

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Extracted proteins from Avian meat meal

Extracted proteins from Bovine meat meal

Extracted proteins from Ovine meat meal

Extracted proteins from Porcine meat meal

Trypsin digestion

Trypsin digestion

Trypsin digestion

Trypsin digestion

React peptides with iTRAQ™ 114 reagent

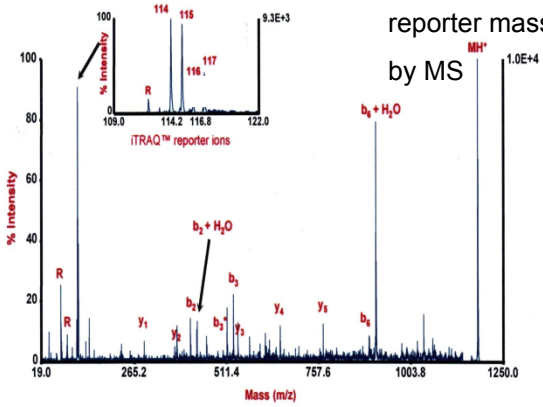
React peptides with iTRAQ™ 115 reagent

React peptides with iTRAQ™ 116 reagent

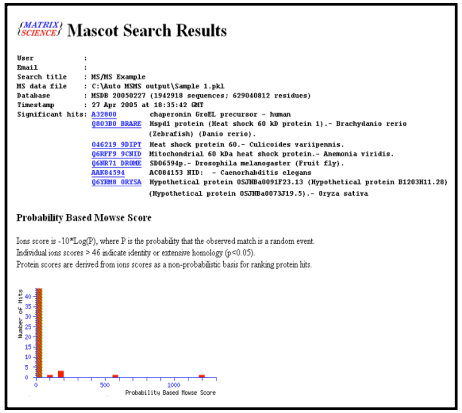
React peptides with iTRAQ™ 117 reagent

MIX 1:1:1:1

Quantitation of peptide reporter masses by MS



Identification of peptide by MS/MS



Major peptides detected in a single iTRAQ analysis of meat meal heated to 141 °C

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	Nº peptides	Species specific peptides
Myosin heavy chain	63	8
Actin	49	0
Collagen	16	2?
Tropomyosin	16	0
Myosin light chain	10	3
Range of muscle enzymes	20	6

Myosin

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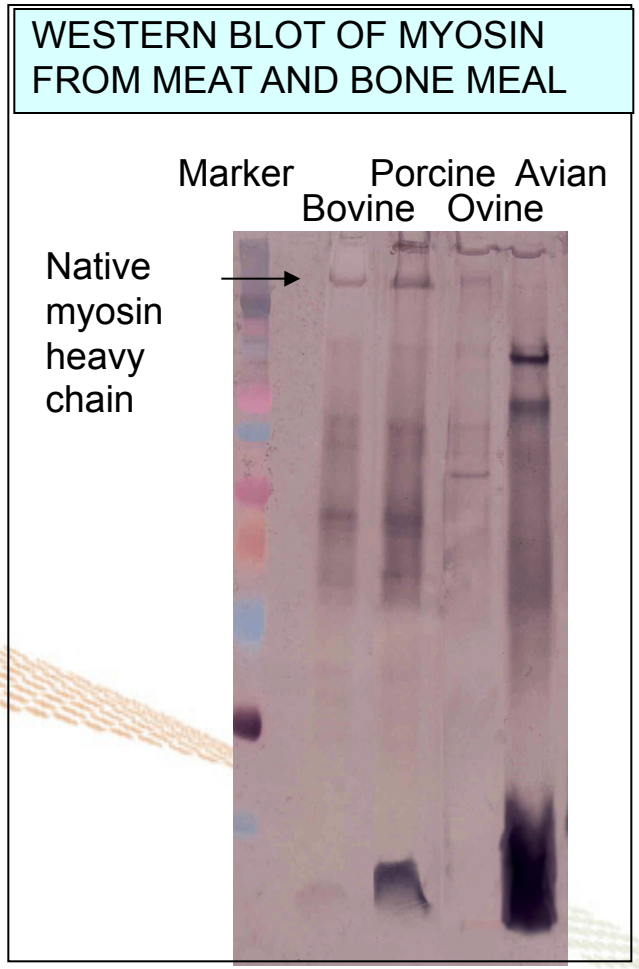

- Simple enrichment of myosin from animal feed by high ionic strength extraction followed by precipitation on dialysis.
- Currently investigating LC–TOF MS profiling of myosin tryptic peptides as a low cost speciation method

Collagen

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- Database of collagen sequences of most domestic animals held by Prof Collins at Dept Archaeology at York University.
- Successful collaboration has lead to the development of a MALDI-TOF-TOF method for domestic species analysing collagen tryptic peptides from individual bone fragments recovered from Meat and Bone meal.
- <1% mixed species can be detected, based on robotic analysis of a large number of bone fragments.

Conclusions

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- Bioinformatic searching is no substitute for mass spec analysis to identify target proteins and peptides.
- Enrichment of parent protein provides much better peptide coverage than a high tech shotgun approach.
- Proteomics offers a direct approach to identifying a wider range of biomarkers in highly processed samples than current DNA approaches.

Example 3

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Identification of the species of gelatin

Gelatin

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- Odourless, colourless food protein used as a processing aid in a wide range of food products from ice cream to pharmaceutical capsules
- Produced by acid treatment of bovine and porcine skin and bone collagen.
- Low pH treatment generally results in complete destruction of DNA

Gelatin

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- The MALDI- TOF-TOF method for collagen was used on in-house prepared gelatins and 21 commercial gelatins in collaboration with Prof Collins.
- Method correctly identified the authentic gelatins, based on the presence of at least one of a number of species-specific peptides
- 2 of the commercial gelatins shown to be mixtures, Bovine (cow +pig) and Avian (avian +pig)
- In many cases we are able to say whether type 1 collagen (bone) or type 2 collagen (skin and connective tissue) had been used to produce the gelatin.

Background

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- In 2001 UK FSA survey of frozen chicken breast found evidence of gelatin addition in ~ 24% samples
  (FSIS 20/01 http://www.food.gov.uk/science/surveillance/fsis2001/20chick)

- Later an FSAI analysis identified traces of beef and pork DNA in some of the samples (questioned whether due to contamination)

- UK labelling legislation revised in 2003 to require labelling of all raw meat with the species of any foreign animal protein

  Meat Products (England) 2003 SI 2003 No 2075

- All subsequent DNA tests have proved negative for pork and beef but gelatin is still added and labelled as 'poultry or chicken hydrolysed protein'

Gelatin in chicken

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- We have extended the collagen method with Prof Collins and have identified porcine and bovine gelatin peptides in extracts from samples of catering packs of frozen chicken breast using LC Q-TOF MS

(some samples labelled as hal al)

- We think this is the first time gelatin in food products has been speciated.

Currently moving this forward with isotopically labelled peptides as standards as a first step in quantifying mixed species of gelatins

Conclusions

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- Proteomics approaches offer opportunity to authenticate the bulk phase of proteinaceous food, eliminating problems of DNA contamination and quantitation based on amplification of a minute component.
- Protein primary sequence more robust than DNA so has application in authenticity of highly processed food components.
- Potential for proteomics technology to trickle down to more routine analytical platforms include MS-TOF for profiling, triple quad MS for specific peptides and peptide dipsticks for screening.

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