

STUDY INTO INJECTION POWDERS USED AS WATER RETAINING AGENTS IN FROZEN CHICKEN BREAST PRODUCTS

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1. EXECUTIVE SUMMARY

1.1 Background

This is an exploratory study into the powders and added proteins used as water-retaining agents in a range of chicken breast products prepared for, and sold mainly into, the catering sector through wholesalers. It follows earlier studies conducted by the Agency in 2001 and 2003, which revealed that undeclared water-retaining hydrolysed proteins derived from pork and beef were being used in tumbled and injected chicken breast products.

The Agency, via its authenticity programme, has commissioned a number of projects whose objective was to develop proteomic methods to detect the species and tissue origin of meat ingredients present in meat products in order to verify the products' labelling. In addition, a number of projects have involved the development of methods for verifying the origin of gelatine used in foods. The work carried out in these projects suggested that a proteomic approach would facilitate analysis of the hydrolysed proteins present in injection powders and this approach was therefore applied as an experimental analysis in this study.

1.2 Sampling

The Agency collected four samples of chicken injection powders, A1, A2, B1 and B2, from 2 premises in the UK which produce chicken breast products injected with added water and other ingredients. Powder sample C was collected from a third premises outside the UK producing injected chicken. Certification for all the powders claimed they contained only hydrolysed poultry or chicken protein.

Powder samples A1 and A2 were collected from one UK producer, samples B1 and B2 from a second UK producer. The two powders are normally made up into an injection brine using different proportions depending on the chicken content and level of added water desired in the final chicken breast product.

The injection powders are not produced by the chicken breast processors themselves but supplied as poultry protein powders from factories in other EU Member States that manufacture hydrolysed proteins. Injection powders A1 and A2 were supplied from a different protein powder manufacturer to injection powders B1 and B2. Powder sample C was produced by the same protein powder manufacturer as sample A1 and was collected to enable confirmation of the findings for sample A1.

In addition to the powder samples, three different brands of frozen chicken breast fillets with added water were collected from a wholesaler. All three brands of chicken products were labelled as containing hydrolysed chicken protein.

1.3 Analyses

The injection powders were subjected to a range of analyses as summarised in Table 1. Selected analyses were applied to certain samples as appropriate therefore, not all analyses were applied to all samples. In general, powders were analysed for nitrogen and hydroxyproline content and amino acid composition to determine the protein and hydrolysed collagen levels. The samples also were analysed for presence of chicken, turkey, pork and beef DNA using the real-time polymerase chain reaction (rtPCR). Samples also were examined for the presence of blood albumin.

Powders also were examined using the newer techniques of metabolomics and proteomics to determine the non-protein and protein constituents present, including the species origin of any proteins. Previous studies to identify the species origin of hydrolysed proteins in injected chicken have used DNA analysis. However, owing to the extreme processing conditions used to produce hydrolysed protein powders the DNA present can be degraded and may be difficult to detect. The application of newer proteomic techniques overcomes this potential limitation as these methods allow the identification of species-specific protein fragments (peptides) directly in a sample.

Proteomic analyses were undertaken by two independent contractors. The first contractor analysed the injection powders by subjecting them to fragmentation by tryptic digest, followed by electrospray tandem mass spectrometry (ES-MS/MS) to determine the mass of each tryptic peptide. The masses of these peptides then were compared with those of peptides in international protein sequence databases and private sequence databases that include animal and bird collagens. Identification of peptides was based on the use of very high peptide ion scores. The masses of the tryptic peptides also were compared with those produced from commercial bovine, porcine and avian gelatines (derived from collagen) and turkey and chicken gelatines produced in-house.

The second contractor used a different methodology. A bovine and an avian/porcine peptide marker (which cannot distinguish avian and porcine collagen) were determined from collagen amino acid sequence information. Presence of these specific peptide markers was identified by subjecting powder samples to fragmentation by partial acid hydrolysis followed by high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) analysis and comparison of the resulting peptide tandem mass spectra with those in an in-house database created from similar treatment of authentic gelatine samples.

Both proteomic approaches were also used to identify species-specific peptides in the drip/thaw exudate of the three chicken breast samples with added water.

1.4 Results

Injection powders: The main results from each type of analysis are summarised in Table 1.

Chicken drip/thaw exudates: The acid digest proteomic approach identified the presence of the bovine collagen-specific marker in chicken sample 1. Tryptic digestion and analysis of the chicken exudates from chicken sample 1 indicated the presence of several bovine collagen-specific peptides, one of which was also identified in powder A1, and two of which were detected in commercial bovine gelatine.

No species-specific collagen peptides could be identified in exudates from the other two chicken samples by either proteomic method.

1.5 Conclusions and discussion

The presence of undeclared mammalian proteins, i.e. from a non-poultry source, was detected in all injection powders tested and in one chicken breast product sampled. The analyses applied indicate the presence of bovine collagen in all the powders and one of the chicken samples. Porcine collagen also may be present in powders B1 and B2.

Despite the presence of chicken DNA in the injection powders, no poultry or chicken-specific peptide markers could be identified using a tryptic peptide shotgun proteomic approach. This suggests that the hydrolysed proteins present are from a non-avian source. Documentation accompanying the analysed powders claim they were produced from a poultry source and contained only hydrolysed chicken protein, no other animal proteins were declared. The analytical results of this study suggest this claim can not be substantiated.

As far as we are aware this initial study represents a novel application of a proteomic approach to identify species-specific peptides present in injection powders. However, the approaches applied were refined and developed as the study progressed. Further characterisation and validation of the proteomic approaches, as applied to these types of samples, are needed before this methodology can be routinely used to determine the species origin of hydrolysed proteins present in injection powders and injected chicken breast products.

Table 1: Summary of results for all powders

Analysis	Powder A1	Powder A2	Powder B1	Powder B2	Powder C
Nitrogen and hydroxyproline	100% protein	17% protein	100% protein	25% protein	No analysis
Real-time PCR	Positive for chicken DNA	No analysis	Positive for chicken and pork DNA	Positive for chicken DNA	No analysis
Albumin	No evidence of bovine, porcine, or poultry blood albumin proteins				No analysis
GC-MS	Urea detected, this can be a result of the breakdown of the amino acid arginine		No analysis	No analysis	No analysis
Amino acid composition	Hydrolysed collagen sole source of protein. Additional glycine, lysine & histidine present	No analysis	No analysis	No analysis	Hydrolysed collagen sole source of protein. Profile similar to A1 but no excess glycine, lysine & histidine
Amino acid racemisation	All powders had undergone extreme treatment relative to commercial gelatine				Powder highly processed, level of racemisation identical to powder A1
Proteomics - both approaches	Protein present in both powders derived from hydrolysed collagen. No chicken collagen-specific peptides detected in either powder.		Protein present in both powders derived from hydrolysed collagen.		
Proteomics (tryptic digest)	Four bovine collagen-specific peptides identified (two of which were found in commercial bovine gelatine)	Two bovine collagen-specific peptides identified	Three bovine collagen-specific peptides identified (two of which were found in commercial bovine gelatine). No chicken-specific peptides identified. Two porcine/non-food animal peptides identified	Three bovine collagen-specific peptides identified (all of which were found in commercial bovine gelatine). No chicken-specific peptides identified. Two porcine/non-food animal peptides identified	Bovine collagen-specific peptides identified. No avian-specific peptides identified
Proteomics (acid hydrolysis)	The bovine collagen/gelatine marker peptide was detected in both powders. The porcine/avian gelatine marker was not detected		The bovine collagen/gelatine marker peptide was detected in both powders. The porcine/avian gelatine marker peptide was detected in both samples		No analysis

2. INTRODUCTION

2.1 Production of chicken breast products

Chicken breast is used widely in cooked dishes; hence there is a substantial trade in frozen chicken breast products sold by wholesalers to caterers. Different products are available that have a chicken content ranging from 100% to 65-55%. The lower chicken contents are obtained by introducing water and a mixture of ingredients to retain this added water during freeze/thawing and cooking by either tumbling or multi-needle injection. Tumbling is generally used to make products with a 80-95% chicken content. Multi-needle injection, on the other hand, can be used to introduce substantial amounts of water in a very short period but requires strong water retaining agents.

The raw material for the injected chicken breast products is imported frozen. The chicken breasts are thawed and injected with varying amounts of brine containing a mixture of ingredients to aid water-holding and add flavour. These ingredients include maltodextrins, sodium citrate, sodium triphosphate, sodium or potassium lactate, xanthan gum, and monosodium glutamate. The main water-retention agent in injection powders is hydrolysed collagen protein. It is common to use two types of injection powders in preparation of the chicken breast products. For products where the final chicken content will be around 80% a powder is used with all the additional ingredients listed above plus around 17-25% hydrolysed protein. When products with a lower chicken content are made i.e. 70% or less, a second powder that is equivalent to 100% protein is added to boost water retention.

2.2 Labelling of chicken breast products

A survey by the Agency published in December 2001 (FSIS 20/01 <http://www.food.gov.uk/science/surveillance/fsis2001/20chick>) highlighted labelling problems in samples of frozen chicken breasts with added water and other ingredients collected in wholesalers. Based on the use of hydroxyproline as a marker for collagen, it was found that around 24% of the samples contained undeclared hydrolysed collagen protein. Coupled with later surveys undertaken by the Food Safety Authority of Ireland and an FSA enforcement exercise in 2003 (<http://www.food.gov.uk/news/pressreleases/2003/mar/chickenwater0303>) that made use of DNA analysis, it was revealed that the likely source of this hydrolysed protein was beef or pork collagen.

Specific labelling requirements for meat products looking like a slice, cut, joint or carcass of meat were introduced in 2003 (Meat Products (England) Regulations 2003, SI 2003/2075) to require added water (above 5%) and any different animal ingredients present be declared in the name of the food. Since 2003 various further samples of frozen chicken breast products have been collected, in particular under the Imported Food Sampling Programme and the 2005 EC Co-ordinated Control Programme. These have shown improvement in the labelling of chicken breast products and added protein has been declared as either hydrolysed poultry or chicken protein.

2.3 Development of authenticity methodology

The methods used to measure the chicken content of chicken breast products were detailed in the 2001 survey information sheet (FSIS 21/01) and published in the OJ (Commission Recommendation 2005/175/EC, Annex V, L59/36-39 5/3/2005). Chicken content is measured by determining the nitrogen content and comparing it to a nitrogen factor for 100% chicken breast. The hydroxyproline content is determined and any excess

hydroxyproline is indicative of added hydrolysed collagen proteins. The nitrogen contribution of this added collagen is deducted from the total nitrogen content. Speciation of added ingredients is done by a supplementary DNA analysis on the chicken breast product but can be difficult given the high chicken DNA background.

In recent years there has been considerable progress in the field of proteomics. By cleaving proteins into peptides with proteases it is now possible to identify specific proteins based on the fingerprint of individual peptides as determined by tandem mass spectrometry.

Collagen is the most abundant protein in mammals and humans and is found in bone (predominantly type I collagen), connective tissue, skin, intestines, and eyes (generally type 3 collagen). It is very versatile in its functional properties. When collagen is hydrolysed by acid (or sometimes alkali) it is converted into gelatine. This dissolves in hot water and forms a gel on cooling by holding large amounts of water in a three-dimensional network. Its use as a water-retaining agent in chicken takes advantage of this property. Collagen has a regular arrangement of a limited range of amino acids and the predominant one is hydroxyproline which is unique to collagen. Although the amino acid sequence of collagen is fairly well conserved amongst different animal species there are some differences and hence it is possible to identify the species of origin using peptide fingerprinting by means of mass spectrometry.

3. SAMPLING

3.1 Injection powders

Four samples of injection powders (A1, A2, B3 and B2) used to make a range of injected chicken breast products were collected from two chicken processing premises in the UK. A sample of injection powder C was collected from a separate chicken processing premise outside the UK. Details of the powder suppliers, along with any product specifications were recorded.

Declarations from the powder suppliers indicated that only poultry protein was present. Documentation for powders A1 and C declared the traceable source of the protein as 100% poultry origin. Documentation for powders B1 and B2 stated they were free from porcine or bovine ingredients.

The powders were divided into 200g samples and sent for analyses.

3.2 Chicken breast products

Three 10kg boxes of different brands of frozen chicken breast products (chicken samples 1, 2 & 3) were collected from a catering wholesaler. All three products were labelled as "Chicken breast fillets with added water, Halal slaughtered". Chicken sample 1 declared a 70% meat content. Chicken samples 2 and 3 declared an 80% meat content. The ingredients list for all products declared the presence of hydrolysed protein (chicken).

4. MATERIALS & METHODS

Analyses were carried out as described below. It should be noted that not all methods were applied to all samples. Information on the analyses applied and the corresponding results are detailed in the appendices.

4.1 Nitrogen and hydroxyproline

Nitrogen and hydroxyproline content were determined using International Standards Organisation methods ISO 937 and ISO 3496 respectively. For chicken samples the nitrogen and hydroxyproline content were determined along with moisture (ISO 1442), fat (BS 4401-4) and ash (ISO 936).

4.2 Amino acid composition

Samples were acid hydrolysed at 110°C for 24 hours. The individual amino acids were separated by ion exchange chromatography and detected using a standard photometric detector and ninhydrin reagent. The individual amino acids were quantified using calibration curves for individual amino acids.

4.3 Species-specific real-time PCR

Real-time PCR analysis was conducted on the powders to determine if any species-specific DNA was present. This was done using beef, lamb, pork, chicken and turkey probes according to a method developed and validated in authenticity research project Q01083¹. Water was used as a negative control. Positive controls for each species were raw meat extracts diluted to 0.1 %. DNA was extracted from the powder samples in duplicate and PCR reactions run in triplicate to establish the average crossing threshold (Ct) value and standard deviation. Quantification of the level of species-specific DNA present in samples was not carried out due to the likely degradation of DNA during processing of the powders.

4.4 Analysis of non-protein components

To indicate what ingredients other than hydrolysed proteins were present, powders were examined by a rapid method (gas chromatography – mass spectrometry). The powders were dissolved in methanol to precipitate proteins and an internal standard added. The residue was cleaned up and derivatised using N-methyl-N (trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (MSTFA with 1% TMCS), and then analysed on a gas chromatography-mass spectrometer (GC-MS). Total ion chromatograms were analysed manually and the mass spectra of the predominant components were compared with the National Institute of Science and Technology (NIST) library to facilitate compound identification.

¹ Hird H; Goodier R; Schneede K; Boltz C; Chisholm J; Lloyd J; Popping B; (2004). Truncation of oligonucleotide primers confers specificity on real-time polymerase chain reaction assays for food authentication. *Food Additives and Contaminants* 21(11), 1035-1040.

Hird H; Chisholm J; Sanchez A; Hernandez M; Goodier R; Schneede K; Boltz C; Popping B; (2006). Effect of heat and pressure processing on DNA fragmentation and implications for the detection of meat using real-time polymerase chain reaction. *Food Additives and Contaminants* 23(7), 645-650.

4.5 Blood albumin proteins

The injection powders were tested with anti-albumin antibodies for beef, pork and chicken to determine whether blood albumin proteins were present. Extracts were prepared from powders (5g) by mixing either with 25 ml PBS (phosphate buffered saline) and stirring for 1 hour, or with 25 ml 4 % (w/v) SDS (sodium dodecyl sulphate) and boiling for 15 minutes, before centrifuging at 20,000 x g for 20 minutes. Supernatant fluids were collected and stored at -20°C. Each extract was analysed by Western blotting, and probed with three antibodies (in-house M7B9/D6 for bovine albumin; in-house 8C12/1B9 for porcine albumin and Sigma C1036 for chicken serum).

4.6 Chicken sample exudates

Thaw exudates were collected from each of the frozen chicken breast samples by storing the samples overnight at 4°C and collecting the drip liquid in a beaker. The exudates were then kept frozen until analysis.

4.7 Tryptic peptide shotgun proteomics

Samples were treated with trypsin to digest proteins into peptide fragments. The resulting peptides were separated by high performance liquid chromatography (HPLC) then analysed using electrospray tandem mass spectrometry. The first dimension of the mass spectrometry gave the molecular ion or unfragmented mass of the peptide: the second dimension of the mass spectrometer permitted identification of the probable amino acid sequence of the peptide.

Peptide markers, unique to a particular species were identified by aligning the amino acid sequences of collagens from different species and identifying those regions that were unique to only one species. *In silico* fragmentation of the protein by Trypsin then identified the mass and amino acid sequence of the fragments (peptides) that were unique to each species.

Species-specific peptides were identified in real samples by comparing all the experimental peptide masses to *in silico* tryptic digest of proteins derived from the Uniprot database. This contains the complete amino acid sequences of over 350,000 proteins – and is the worlds' most comprehensive protein sequence resource. The Uniprot database includes the complete collagen sequences from man, rat, mouse, cow, and chicken as well as partial sequences for collagens from a number of other species. In addition, the sequences were matched against a research database of 151 collagen sequences (type I, II and III) from extant and extinct organisms. This research database includes sequences of four domestic species (pig, sheep, goat and turkey) not present in public databases.

Selected peptide masses were then searched using a Mascot database search engine to match the observed MS/MS spectra to the theoretical MS/MS spectra of the species-specific peptides. The Mascot algorithm assigns a peptide score that represents the probability that the observed MS/MS spectrum match between experimental data and a peptide sequence held within the database is a random event. Any value of peptide score above 32-33 has a less than 1% false peptide discovery rate, i.e. 99% confidence level in peptide sequence matching. Only peptides with scores above 40 are presented in this report.

The identification of a protein such as collagen from a particular species depends on the identification of a peptide or number of peptides whose sequences are present only in the

collagen from the one animal species. These peptides are unique to a species insofar as the public and private databases interrogated currently record that sequence as being present only in the collagen from that species and nowhere else.

4.8 Acid hydrolysis proteomic analysis

The second proteomic procedure involves treating samples with hydrochloric acid under controlled conditions in order to achieve partial hydrolysis thereby generating a mixture of peptides. The procedure cleaves the protein molecules at sites different from those in the enzymatic trypsin method described above. Prior interrogation of the collagen sequences in public databases has identified a marker peptide that is unique to bovine and a second marker peptide that is unique to avian / porcine gelatine

The peptide mixture was separated by HPLC and the peptides analysed by ES-MS/MS (ion trap). This enables the first dimension of the mass spectrometer to identify peptides with the mass of the marker peptide, the second dimension of the mass spectrometer enables fragmentation of the selected peptides and recording of specific product ion mass spectra. These reproducible product ion mass spectra function as highly specific “fingerprints” suitable for comparison with spectra stored in a database (mass spectral library searching).

An in-house library of gelatine-related marker peptides was employed for matching and the identities of all marker peptides were confirmed by chemical synthesis. Matching was achieved using the NIST library search algorithm as well as the judgement of experienced analysts. Specificity of analysis was demonstrated by comparison of the HPLC retention times with those of authentic standard peptides.

Examination of the chicken exudates required a solid phase enrichment step to give sufficient hydrolysed collagen protein to enable species origin to be elucidated.

4.9 Racemisation

Chiral amino acid analysis was used to determine the amount of racemisation (conversion of L-form to D-form) of the amino acids (especially glutamic and aspartic acids). *In vivo* amino acids are normally in the L-form and the amount of D-amino acid is either a measure of the age of the sample or a measure of the degree of processing (acid/alkali hydrolysis). Samples were treated with 7M hydrochloric acid (HCl) under nitrogen for 18 hrs at 110°C to release the peptide bound amino acids. The amino acids were separated by reverse phase-HPLC with 0.1mM L-homo-arginine as an internal standard, and measured using a fluorescence detector. The determinations were carried out in duplicate.

APPENDIX 1

5. RESULTS FOR INJECTION POWDER SAMPLES A1 AND A2 AND CHICKEN SAMPLES 1-3.

5.1 Composition of the powders

Table 2 shows the nitrogen, hydroxyproline and protein content of the powder samples and the percentage contribution of the hydroxyproline (HyP) to the nitrogen content.

Table 2: Nitrogen and Hydroxyproline Contents of Powders

Sample	Nitrogen g/100g	HyP g/100g	% Protein	% Contribution of HyP to nitrogen content
A1	16.4	5.8	102.5	45
A2	2.74	1.9	17.1	89

The analyses showed that for powder A1, the nitrogen content indicated a protein content equivalent to 100%. Powder A2 contained only 17% protein indicating that protein was one of several ingredients in the powder mix.

The hydroxyproline analyses strongly suggest the presence of collagen in both powders. In sample A2, the nitrogen contribution from hydroxyproline was nearly 90% of the total nitrogen content. In sample A1 the hydroxyproline content contributed only 45% of the nitrogen content indicating the presence of other nitrogenous compounds in addition to collagen protein.

Results of the amino acid analysis of powder A1 are given in Annex A, Table 3. Based on a comparison with the amino acid profile of bone gelatine, it was estimated that only 35% of this sample was hydrolysed collagen. This was lower than the figure derived from the hydroxyproline analysis but indicated that there were a lot of free amino acids present in the powder, and possibly other nitrogenous compounds as well. The absence of cysteine and tryptophan in the analysis also suggested that the only proteinaceous material present was derived from collagen. This was further substantiated by the absence of any blood albumin protein in samples A1 and A2 which suggests that no blood protein had been added (Annex B, Table 6).

The results of GC-MS analysis of the powders are shown in Annex B (Figure 1, Table 4 and Figure 2, Table 5). This analysis provided putative identification of some of the other non-proteinaceous components of the powders. These non-protein components were derivatised to permit GC analysis. For sample A2, the results indicated the presence of citrate, maltodextrin, xanthan gum, triphosphate, glutamate (all labelled in the ingredients) and urea. For sample A1, the results indicated that the main non-protein components were the amino acids glycine and lysine and this is in agreement with the amino acid analysis. A small but significant amount of urea also was present. This urea could be derived from arginine as a consequence of the hydrolysis conditions applied to the collagen.

5.2 Species origin of the hydrolysed collagen

Powder A1 was subjected to real-time PCR analysis using specific chicken, pork, beef and turkey probes and a consistent signal was obtained only for chicken DNA (Annex C, Table 7). Powder A2 was not analysed by real-time PCR.

Annex D gives the results of the proteomic analysis using a tryptic peptide shotgun proteomic approach. Powders were subject to tryptic digestion followed by ES-MS/MS to identify the most probable amino acid sequence of peptides. The resulting peptides were searched on public and private databases using Mascot software to identify the proteins from which the peptides were derived. Commercial and in-house prepared gelatine samples were similarly analysed in order to confirm that species-specific collagen tryptic peptides found in injection powders also could be detected in commercial gelatine samples.

The mass spectral analysis only identified collagen peptides in the two powders (A1 and A2) examined suggesting that they had been prepared from either collagen or gelatine. Detailed analysis of the identified peptides showed that no avian (chicken or turkey) collagen-specific peptides were present in either of the two powders. In the case of powder A2, two of the peptides identified were unique to bovine collagen and one of these was also present in powder A1 (Annex D, Table 8). Four bovine collagen-specific peptide were identified in powder A1 (Annex D, Table 9) and two of these also were detected in commercial bovine gelatine.

Annex E gives the results of the peptide analysis using acid hydrolysis of powders A1 and A2. Three marker peptides of known sequence, identified in the specific ion chromatograms, were used to confirm species origin. M/z 1044 is a peptide marker common to bovine, porcine and avian collagen. Its presence in powders A1 and A2 confirms that the proteinaceous material present derives from collagen (Annex E, Figure 3). M/z 1028 is found only in bovine gelatine and indicates the presence of hydrolysed bovine type 1 alpha collagen in both A1 and A2. M/z 832 is a peptide marker found in both porcine and avian gelatines, and its absence in both powders confirms avian (or porcine) type 1 alpha collagen, if present, is below the limit of detection. The specificity of these markers was checked against authentic gelatines (Figure 3) and also against synthetic peptide marker standards (Figure 4).

5.3 Species origin of the hydrolysed collagen in chicken breast products

Three exudates of thaw drip liquor from chicken breast samples 1-3 were analysed after both tryptic and acid hydrolysis. In both methods, only peptide markers in chicken sample 1 were identified. This required solid phase enrichment in the case of the acid hydrolysis approach. Three bovine collagen-specific peptides were identified in chicken sample 1 using the tryptic peptide shotgun proteomic approach (Annex D, Table 10). Using the acid hydrolysis approach the bovine marker peptide was clearly identified (Annex E, Figure 5).

5.4 Conclusions

Documentation accompanying powders A1 and A2 claims that they were prepared from pure poultry hydrolysed protein. The source of this hydrolysed protein is declared as being derived from traceable poultry bones. The analyses applied here showed that A2 was a mixture of water retaining ingredients with 17% hydrolysed protein. On the other hand, A1

contained less than 50% hydrolysed protein. The amino acid analysis indicated only 35% was protein, the remainder being free amino acids (mainly glycine and lysine) and other nitrogenous compounds including urea.

DNA analysis of powder A1 gave a consistent signal only for chicken. However examination of the peptide markers using tryptic digest of both powders A1 and A2 indicated the presence of bovine collagen-specific peptides and the absence of any avian-specific peptides. This result was confirmed using the acid hydrolysis method which showed that the two powders contained a bovine collagen peptide marker. Therefore, the claim that powders A1 and A2 derive only from poultry bones could not be substantiated by the analytical techniques applied in this study.

Similar bovine collagen-specific peptides were also confirmed in a chicken breast product with a 70% chicken content declaration. It is possible that the concentration of added hydrolysed collagen protein was too low for identification in the other two chicken samples tested (both with an 80% chicken content), as only one of the injection powders (A2) would be used to make these products and the final concentration of added protein in the chicken product is likely to be around 0.3%. However, in the 70% chicken product (sample 1), it is likely that both powders would have been used giving a final concentration of around 1% added protein.

Table 3: Amino Acid Analysis of Powder A1

Amino acid g/100g powder	ala	gly	val	leu	ile	pro	phe	tyr	ser	thr	cys	met	arg	his	lys	asp	glut	hp
	3.79	26.6	1	1.2	0.6	5.7	0.8	0.1	0.9	0.9	0	0.3	2.9	0.9	16	2.4	4.4	4.6
<i>N content of amino acid (%)</i>	15.75	18.65	11.9	10.7	10.7	12.2	8.42	7.7	13.3	11.8		9.4	26.5	27.1	12.8	10.5	9.57	10.7
	<i>Total 11.1 gN /100g</i>																	
for reference g amino acid/35g commercial bone gelatine	ala	gly	val	leu	ile	pro	phe	tyr	ser	thr	cys	met	arg	his	lys	asp	glut	hp
	3.90	9.39	0.96	1.19	0.53	5.35	0.86	0.08	1.29	0.81	0	0.22	3.11	0.24	1.51	2.31	4.00	4.59

Only 73% of the dry weight of the powder was accounted for by the amino acids.

Based on hydroxyproline content, powder A1 contained approximately 35% gelatine (compared to commercial bone gelatine). Amino acid analysis indicates excess glycine, lysine, histadine when compared to g amino acid/35g commercial bone gelatine.

No tryptophan or cysteine were detected in the powder (separate analysis for tryptophan carried out).

GC-MS ANALYSES OF THE INJECTION POWDERS

Figure 1: GC-MS Chromatogram (TIC) for Methanol-water Extract of Powder Sample A2.

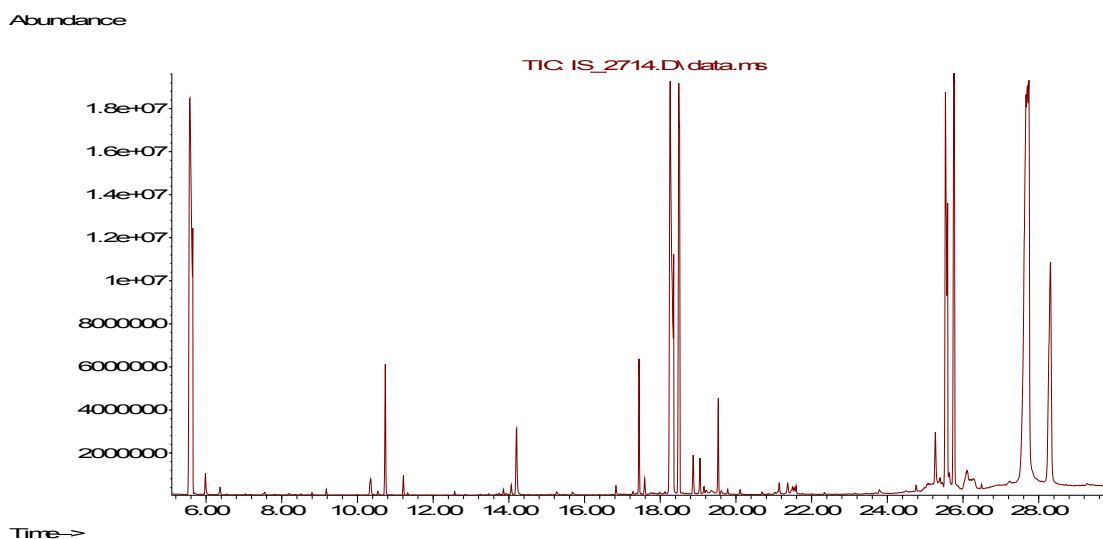


Table 4: Peak Information for the Most Prominent Components of Sample A2.

Retention time	Relative area	NIST match
10.35	0.41	Urea der.
10.54	0.07	Serine der.
10.73	1.65	Glycerol der.
11.21	0.23	Glycine der.
12.57	0.05	Glutaric acid der.
14.06	0.16	Pyroglutamic acid der.
14.20	1.16	N-Acetylglutamic acid der.
16.83	0.15	Phosphoric acid der.
18.87	0.57	Unknown
19.05	0.43	Unknown
17.44	1.69	Citric acid der.
18.26	16.73	Glucose methoxyamine der.
18.49	10.10	Galactose methoxyamine der.
19.53	1.20	D-methylglucopyranoside der.
25.27	0.97	Maltose methoxyamine der.
25.54	12.33	alpha-D-Glc-(1,4)-D-Glc der.
25.77	8.86	alpha-D-Glc-(1,4)-D-Glc der.
26.10	1.23	Maltotriose methoxyamine der.
27.70	33.47	Maltotriose methoxyamine der.
28.31	8.52	Maltotriose methoxyamine der.

Figure 2: GC-MS Chromatogram (TIC) for Methanol-water Extract of Powder Sample A1.

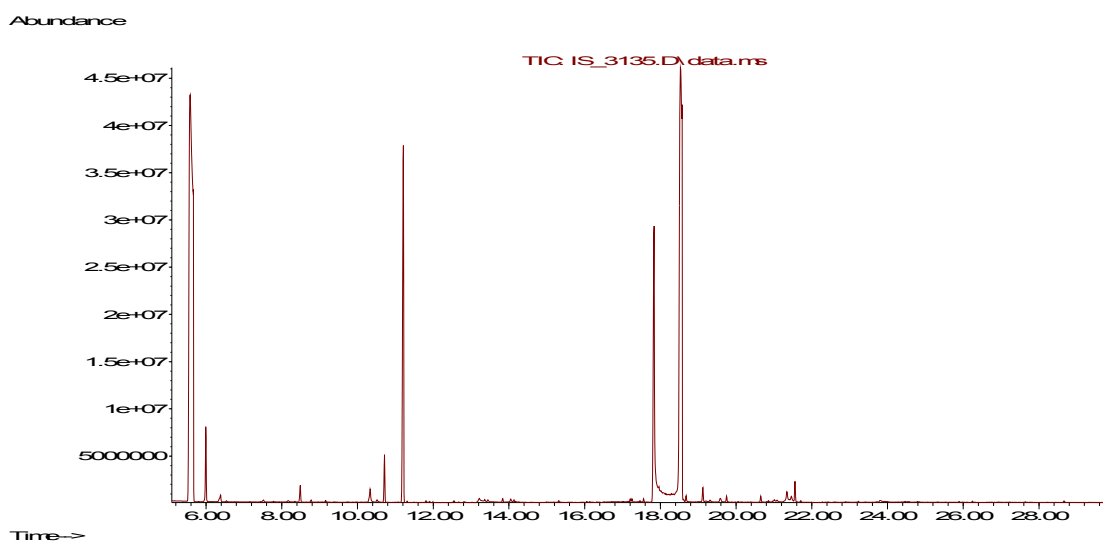


Table 5: Peak Information for the Most Prominent Components of Powder Sample A1.

Retention time	Relative area	NIST match
8.49	0.80	Glycine der.
10.33	0.96	Urea der.
10.71	2.09	Glycerol der.
11.21	22.00	Glycine der
17.84	18.38	Lysine der.
18.53	54.65	Lysine der.
19.13	0.69	5-aminovaleric acid der.
21.56	0.43	Octadecanoic acid der.

BLOOD ALBUMIN ANALYSIS OF INJECTION POWDERS

Table 6: Results of Albumin Immunoassay.

Sample	Porcine albumin	Bovine albumin	Chicken serum
A1	-ve	-ve	-ve
A2	-ve	-ve	-ve

Table 7: Real-time PCR Assay of Injection Powder A1.**ANNEX C**

SAMPLE	CHICKEN			BEEF			TURKEY			PORK			PORK (Repeat)		
	Ct	Av	stdev	Ct	Av	stdev	Ct	Av	stdev	Ct	Av	stdev	Ct	Av	stdev
Powder A1 Extract 1	27.51 27.72 28	27.74	0.25	- - -			- - -			- - 38.38			- - -		
Powder A1 Extract 2	28.21 28.04 28.03	28.09	0.10	- - -			- - -			38.28 - 36.71			- - -		
Negative control	- - -			- - -			- - -			- - -			- - -		
Positive Control	23.24 22.49 23.01	22.91	0.38	21.68 21.58 21.79	21.68	0.11	21.64 20.82 21.38	21.28	0.42	19.67 20.12 20.33	20.04	0.34	18.54 18.52 19.44	18.83	0.53

The smaller the Ct value the higher the DNA copy number, i.e. the more DNA present in the sample.

No beef or turkey DNA was detected in powder A1. A weak and inconsistent signal was detected for pork in the first analysis, the extracts were re-analysed and no pork signal was detected, therefore the sample can be considered negative for pork DNA.

Powder A1 was positive for chicken DNA.

PEPTIDE MARKERS FOUND IN INJECTION POWDERS AND CHICKEN SAMPLES USING TRYPTIC DIGESTS

INJECTION POWDERS

Table 8: Species-specific Peptides Detected in Powder A2

Molecular mass of peptide	Parent protein
1261	Bovine type 1 alpha 2 collagen
1560	Bovine type 1 alpha 1 collagen

Table 9: Species-specific Peptides Detected in Powder A1

Molecular mass of peptide	Parent protein
1435	Bovine type 1 alpha 1 collagen
1261	Bovine type 1 alpha 2 collagen
1266	Bovine type 1 alpha 2 collagen
1427	Bovine type 1 alpha 2 collagen

CHICKEN BREAST EXUDATE SAMPLES

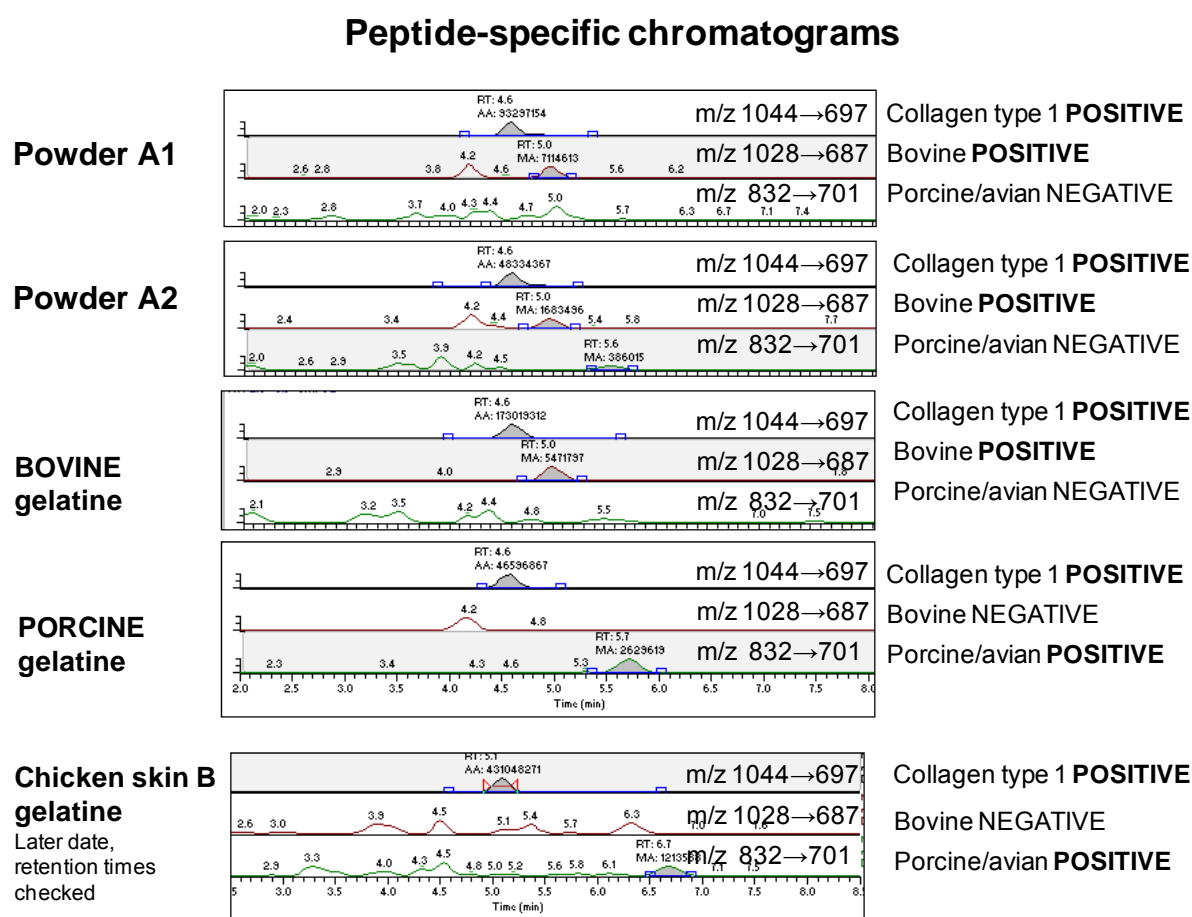
Table 10: Species-specific Peptides Detected in Chicken Sample 1

Molecular mass of peptide	Parent protein
1435	Bovine type 1 alpha 1 collagen
1334	Bovine type 1 alpha 1 collagen
1532	Bovine type 1 alpha 2 collagen

The molecular mass of the peptide is used as a unique identifier. The MS/MS analysis of the peptide was able to provide amino acid sequence information for these peptides and database searching has so far shown these amino acid sequences are only present in the named species and protein.

PEPTIDE MARKERS FOUND IN INJECTION POWDERS AND CHICKEN SAMPLES USING ACID HYDROLYSIS

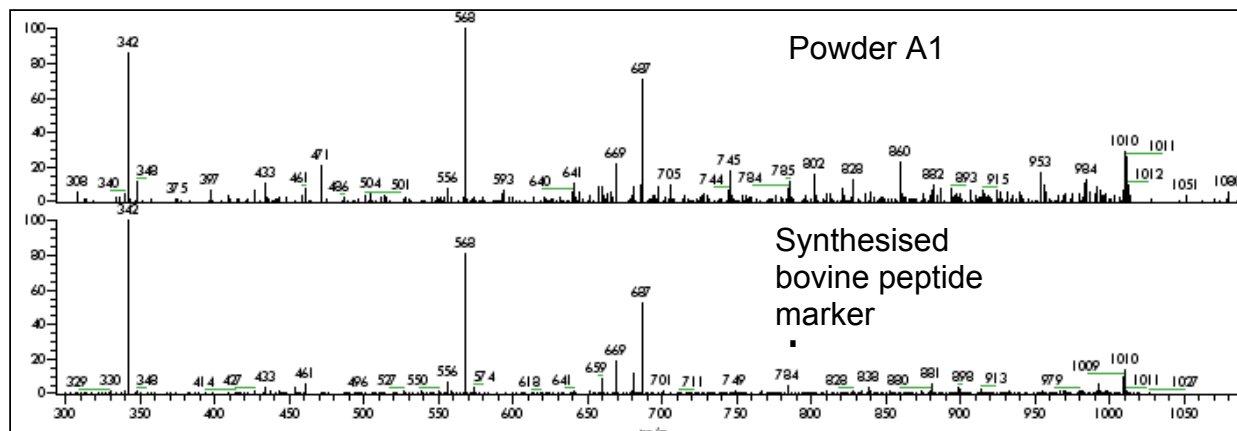
Figure 3: Selected Reaction Chromatograms Obtained from Acid Hydrolysed Samples of Powder A1 and A2, Bovine Gelatine, Porcine Gelatine and a Later Sample of Authentic Chicken Skin Gelatine.



In each case, the upper trace is specific for the type 1 collagen-specific marker m/z 1044, the centre trace is specific for the bovine type 1 collagen marker m/z 1028, and the lower trace is for a porcine/avian-specific marker m/z 832.

Significant peaks are shaded and the corresponding product ion mass spectra have been matched against an in-house database for further confirmation of identity.

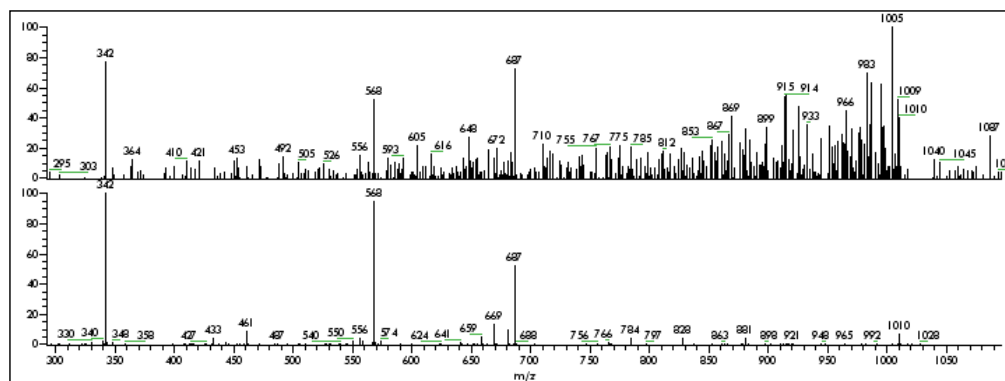
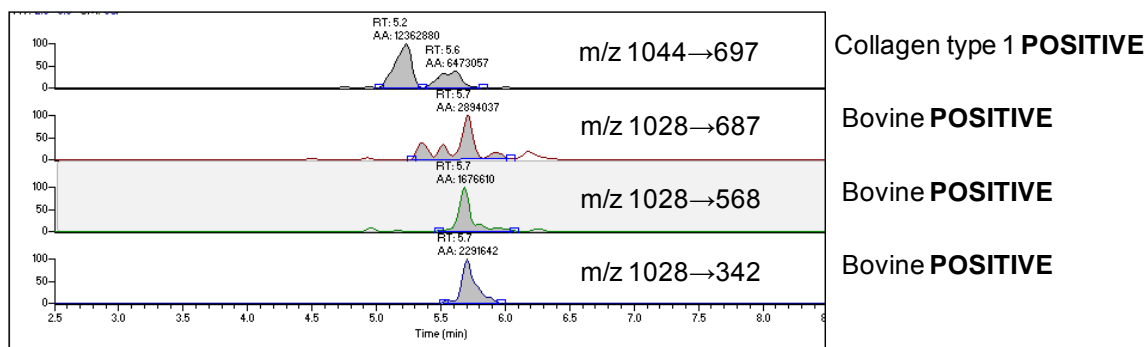
Figure 4: Fragmentation pattern from the synthesised bovine peptide marker standard m/z 1028 compared to the fragmentation pattern of powder A1 sample parent ion m/z 1028. Correspondence of major fragmentations is apparent despite presence of this trace peptide in a complex mixture.



CHICKEN BREAST EXUDATE SAMPLES:

Although following acid hydrolysis and LC/MS/MS, all three exudates showed the presence of the general collagen type 1 marker m/z 1044, other species peptide markers could only be identified in the 70% chicken content sample after enrichment.

Figure 5: Selected reaction chromatograms from acid hydrolysed chicken sample 1 exudate. The fragmentation pattern of the bovine peptide marker m/z 1028 from chicken sample 1 exudate after enrichment compared to the synthesised peptide marker standard is shown below. The bovine peptide marker is clearly identified despite its low level in the complex mixture of fragments, confirming the presence of bovine collagen in the sample.



Fragmentation pattern from chicken sample 1 exudate

Fragmentation pattern of authentic bovine marker peptide m/z1028

6. RESULTS FOR INJECTION POWDER SAMPLES B1 AND B2

6.1 Composition of the powders

Table 11 shows the nitrogen, hydroxyproline and protein content of the powder samples and the percentage contribution of the hydroxyproline to the nitrogen content.

Table 11: Nitrogen and Hydroxyproline Contents of Powders

Sample	Nitrogen g/100g	HyP g/100g	% Protein	% Contribution of HyP to nitrogen content
B1	16.25	11.1	101.6	87
B2	4.06	2.74	25.4	86

The analyses showed that powder B2 contained 25% protein, indicating the collagen protein was one of several ingredients in the mix. For powder B1 the nitrogen content was equivalent to 100% protein. In both powders the nitrogen contribution from hydroxyproline was 86-87% of the total nitrogen content, suggesting that nearly all of the powder was hydrolysed collagen protein.

The results of GC-MS analysis of the powders, to identify non-proteinaceous components, is shown in Annex F (Figure 6, Table 12 and Figure 7, Table 13). These non-protein components were derivatised to permit GC analysis, which indicated the presence of citrate, phosphate, xanthan gum, and maltodextrin (all labelled on the ingredients) in powder B2. For powder B1, the results indicated that the main non-protein components were the amino acid glycine and phosphate. The immunoassay results (Table 14) indicated that no blood albumin was present in powder samples B1 and B2, suggesting no blood protein had been added.

6.2 Species origin of the hydrolysed collagen

Results of the real-time PCR analysis of powders B1 and B2 are given in Annex G, Table 15. Both powders were positive for chicken DNA and negative for turkey and beef DNA. In the case of B2, the pork DNA result was marginal and repeated but not conclusive. B1 on the other hand was positive for pork DNA. A second separate sample of B1 was analysed confirming this result.

Annex H, Tables 16 & 17 give the results of proteomic analysis using a tryptic peptide shotgun proteomic approach. Powders were subject to tryptic digestion followed by ES-MS/MS to identify the most probable amino acid sequences of peptides. The resulting peptides were searched on public and private databases using Mascot software to identify the proteins from which the peptides were derived. Commercial and in-house prepared gelatine samples were similarly analysed in order to confirm that species-specific collagen tryptic peptides found in injection powders also could be detected in commercial gelatine samples.

The mass spectral analysis only identified collagen peptides in the two powders examined (B1 and B2), suggesting that they had been prepared from either collagen or gelatine. Detailed analysis of the identified peptides showed that the great majority were specific for mammals: no poultry (chicken or turkey) collagen-specific peptides were present in either of the two powders.

For powder B2, three bovine collagen-specific peptides were identified, all of which were present in commercial bovine gelatine. Two porcine collagen peptides were identified in powders B1 and B2, however these peptides sequences were shown to occur in other, more exotic species (each one in a different species of animal not commonly used in food production). Powder B1 also contained three bovine collagen-specific peptides, two of which were identified in powder B2 and also present in commercial bovine gelatine.

Annex I, Figure 8 gives the results of peptide analysis by acid hydrolysis of powders B1 and B2. Three marker peptides of known sequence, identified in the specific ion chromatograms, were used to confirm species origin. M/z 1044 is a peptide marker common to bovine, porcine and avian collagen. Its presence in powders B1 and B2 confirms that the proteinaceous material present derives from collagen (Annex I, Figure 8). M/z 1028 is found only in bovine gelatine and indicates the presence of hydrolysed bovine type 1 alpha collagen in both B1 and B2. M/z 832 is a peptide marker found in both porcine and avian gelatines, its presence in powders B1 and B2 indicates the presence of avian and/or porcine collagen. The specificity of these markers was checked against authentic gelatines (Figure 8) and also against synthetic peptide marker standards (Figures 9 & 10).

6.3 Conclusions

Documentation accompanying powders B1 and B2 claims that they were prepared from pure poultry hydrolysed protein and were free from pork and beef. The analyses applied in this study showed that powder B2 is a mixture of water-retaining ingredients with 25% hydrolysed protein, nearly all of which was hydrolysed collagen protein. Powder B1 appeared to be nearly all hydrolysed collagen protein, although the non-protein components showed a significant level of phosphate.

DNA analysis of both powders indicated the presence of chicken DNA with a confirmed presence of pork DNA in powder B1. Examination of the peptide fragments by tryptic digestion of both powders indicated the presence of bovine-specific collagen peptides. This result was supported by acid hydrolysis proteomic analysis of the powders which showed that powders B1 and B2 contained the bovine collagen marker. In addition, two porcine markers (not specific to pork but found in other animals not commonly used for food) were detected by the tryptic digestion proteomic method. No avian collagen-specific peptides could be identified using this approach. Therefore, the claim that the powders derive only from poultry protein could not be substantiated by the analytical techniques applied in this study.

GC-MS ANALYSIS OF THE INJECTION POWDERS

Figure 6: GC-MS Chromatogram (TIC) for Methanol-water Extract of Powder Sample B2.

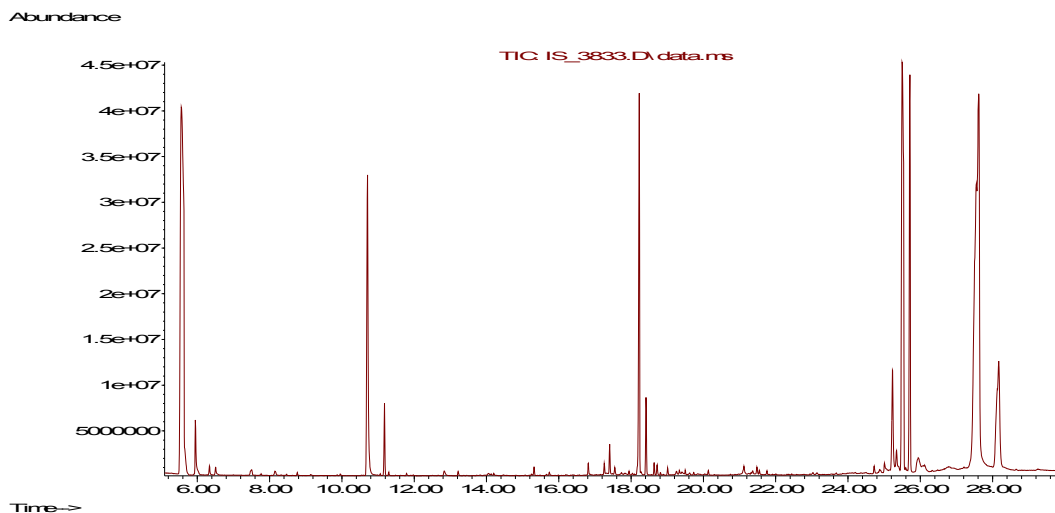


Table 12: Peak Information for the Most Prominent Components of Powder Sample B2.

Retention time	Relative area	NIST match
10.71	8.31	Phosphoric acid der.
11.18	1.29	Glycine der.
12.85	0.18	Unknown
15.33	0.19	Pentanoic acid der.
16.82	0.25	Phosphoric acid der.
17.41	0.53	Citric acid der.
18.23	8.68	Glucose methoxyamine der.
18.42	1.41	Galactose methoxyamine der.
25.23	2.81	Maltose methoxyamine der.
25.50	18.71	Alpha-D-Glc-(1,4)-D-Glc der.
25.72	9.72	Alpha-D-Glc-(1,4)-D-Glc der.
25.95 (*)	1.39	Maltotriose methoxyamine der.
27.60 (*)	38.87	Maltotriose methoxyamine der.
28.17 (*)	7.66	Maltotriose methoxyamine der.

Figure 7: GC-MS Chromatogram (TIC) for Methanol-water Extract of Powder Sample B1.

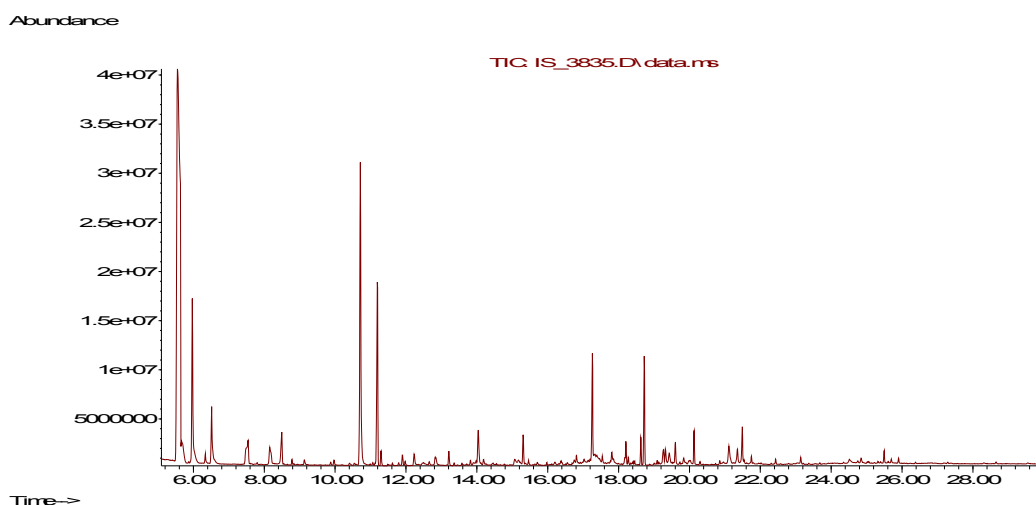


Table 13: Peak Information for the Most Prominent Components of Powder Sample B1.

Retention time	Relative area	NIST match
7.54	4.70	Propanoic acid der.
8.16	3.38	Alanine der.
8.49	1.96	Glycine der.
10.71	21.77	Phosphoric acid der.
11.19	20.66	Glycine der.
11.30	2.50	Succinic acid der.
11.90	0.88	Serine der.
12.23	1.20	Threonine der.
13.21	0.97	Glycine der.
14.04	3.79	Pyroglutamic acid der.
15.31	2.58	5-aminovaleric acid der.
17.26	7.92	Agmatine der.
17.81	0.79	Lysine der.
18.21	1.77	Glucose methoxyamine der.
18.61	1.70	Sorbitol der.
18.73	7.83	Phenylethanolamine der.
19.27	1.32	Unknown
19.32	1.09	Unknown
19.43	0.61	Unknown
19.60	2.02	Unknown
20.13	2.34	Myo-Inositol der.
21.11	2.68	Unknown
21.36	1.97	Unknown
21.49	2.65	Unknown
25.50	0.93	Maltose methoxyamine der.

BLOOD ALBUMIN ANALYSIS OF INJECTION POWDERS

Table 14: Results of Albumin Immunoassay

Sample	Porcine albumin	Bovine albumin	Chicken serum
B1	-ve	-ve	-ve
B2	-ve	-ve	-ve

Table 15: Real-time PCR Assay of Injection Powders

ANNEX G

SAMPLE	CHICKEN			BEEF			TURKEY			PORK			PORK (Repeat)		
	Ct	Av	stdev	Ct	Av	stdev	Ct	Av	stdev	Ct	Av	stdev	Ct	Av	stdev
Powder B2 Extract 1	36.78 38.32 38.9	38.00	1.10	- - -			- - -			- - -			- - -		
Powder B2 Extract 2	36.78 35.92 35.42	36.04	0.69	- - -			- - -			37.03 - 37.31			38.66 - 38.06		
Positive Control	23.24 22.49 23.01	22.91	0.38	21.68 21.58 21.79	21.68	0.11	21.64 20.82 21.38	21.28	0.42	19.67 20.12 20.33	20.04	0.34	18.54 18.52 19.44	18.83	0.53
Powder B1 Extract 1	20.12 20.25 20.01	20.13	0.12	- - -			- - -			28.65 28.30 28.30	28.42	0.20		NA	
Powder B1 Extract 2	20.04 19.98 20.08	20.03	0.05	- - -			- - -			28.44 28.34 28.34	28.38	0.06		NA	
Powder B1 new sample Extract 1	20.04 19.83 19.75	19.87	0.15		NA			NA		27.85 27.97 27.75	27.85	0.11		NA	
Powder B1 new sample Extract 2	19.63 19.68 19.84	19.72	0.11		NA			NA		27.65 27.55 27.63	27.61	0.05		NA	
Negative control	- -			- -			- -			- -			- -		

	-			-			-			-			-		
Positive	24.48	25.05	1.25	23.93	23.68	0.32	25.19	25.14	0.05	22.06	22.17	0.23	24.48	25.05	1.25
Control	24.20			23.79			25.14			22.44			24.20		
	26.49			23.32			25.10			22.01			26.49		

The smaller the Ct value, the higher the copy number, i.e. the more DNA present.

No beef or turkey DNA was detected in powders B1 or B2. Both powders B1 and B2 were positive for chicken DNA.

A weak and inconsistent signal was detected for pork DNA in sample B2, therefore the sample was considered negative for pork DNA.

However, sample B2 was positive for pork DNA. A new sub-sample of powder B2 was extracted and re- analysed confirming the presence of pork DNA.

ANNEX H

PEPTIDE MARKERS FOUND IN INJECTION POWDERS USING TRYPTIC DIGESTS

Table 16: Species-specific Peptides Detected in Powder B2

Molecular mass of peptide	Parent protein
1041	Bovine type 3 alpha 1 collagen
1047	Bovine type 3 alpha 1 collagen
1500	Bovine type 3 alpha 1 collagen
910	Found in porcine collagen but not specific to pork
878	Found in porcine collagen but not specific to pork

Table 17: Species-specific Peptides Detected in Powder B1

Molecular mass of peptide	Parent protein
1031	Bovine type 3 alpha 1 collagen
1041	Bovine type 3 alpha 1 collagen
1047	Bovine type 3 alpha 1 collagen
910	Found in porcine collagen but not specific to pork
1332	Found in porcine collagen but not specific to pork

PEPTIDE MARKERS FOUND IN INJECTION POWDERS B2 AND B1 USING ACID HYDROLYSIS

INJECTION POWDERS:

Figure 8 (over page): Selected reaction chromatograms obtained from acid hydrolysed samples of powders B2 and B1, bovine gelatine, porcine gelatine and a sample of authentic chicken gelatine.

In each case, the upper trace is specific for the type 1 collagen-specific marker (m/z 1044), the centre trace is specific for the bovine type 1 collagen marker (m/z 1028) and the lower trace is specific for the porcine/avian-specific marker (m/z 832).

Significant peaks are shaded and the corresponding product ion mass spectra have been matched against an in-house database for further confirmation of identity.

ANNEX I, Figure 8:

Peptide-specific chromatograms

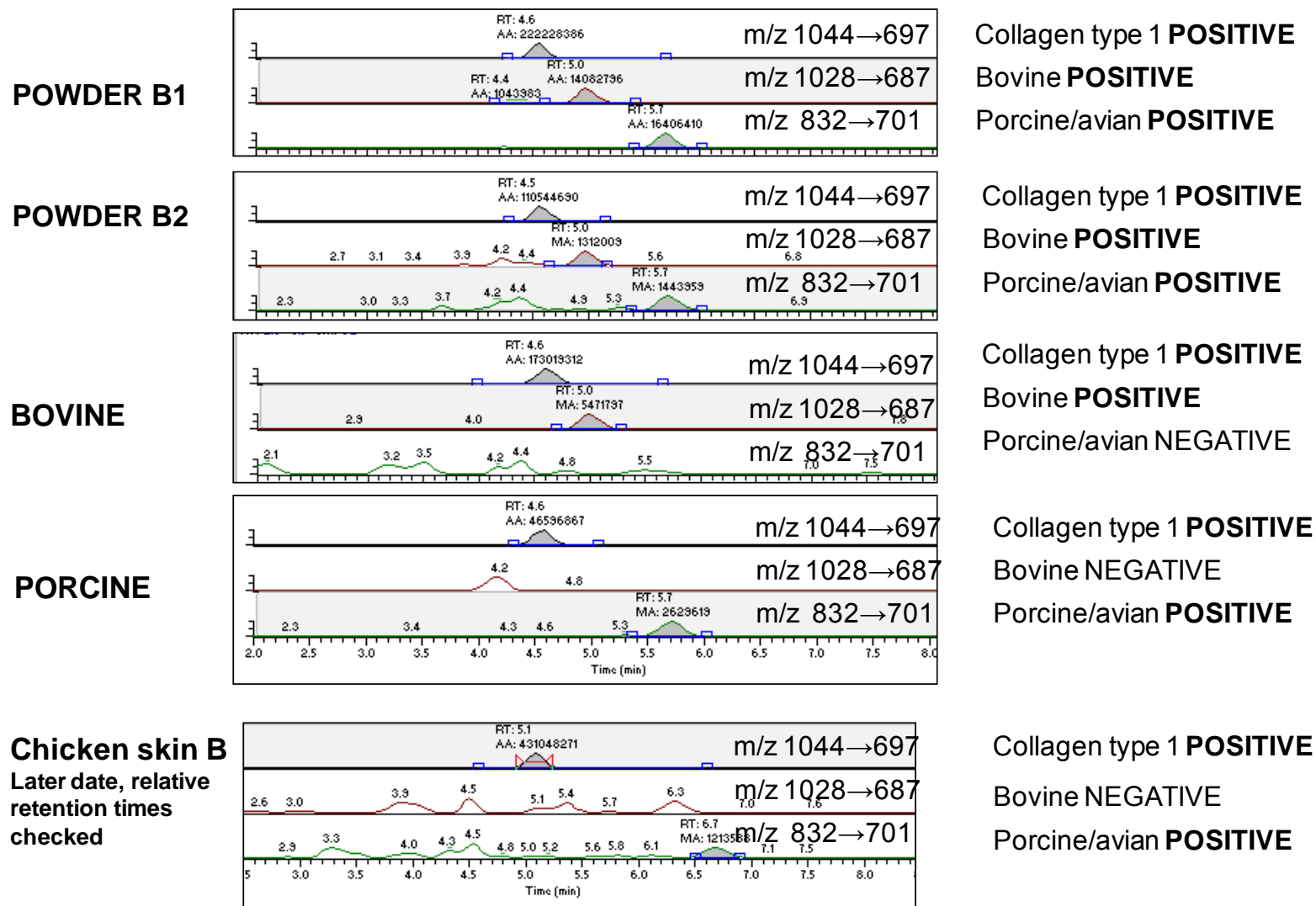


Figure 9: Fragmentation pattern from the synthetic bovine marker peptide standard m/z 1028 compared with fragmentation pattern from powder sample B1 parent ion m/z 1028. Correspondence of major fragmentations is apparent despite presence of trace peptide in a complex mixture.

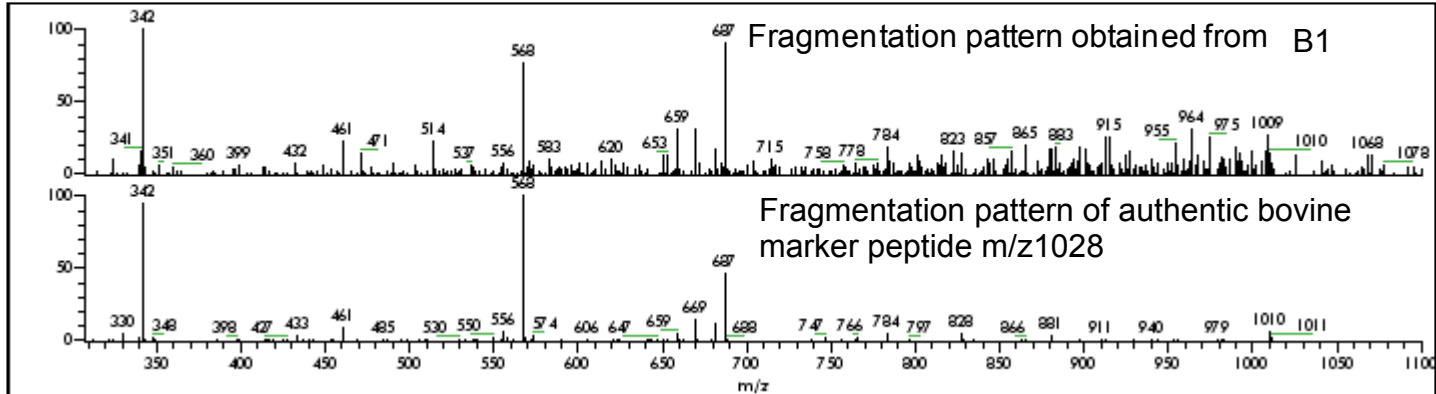
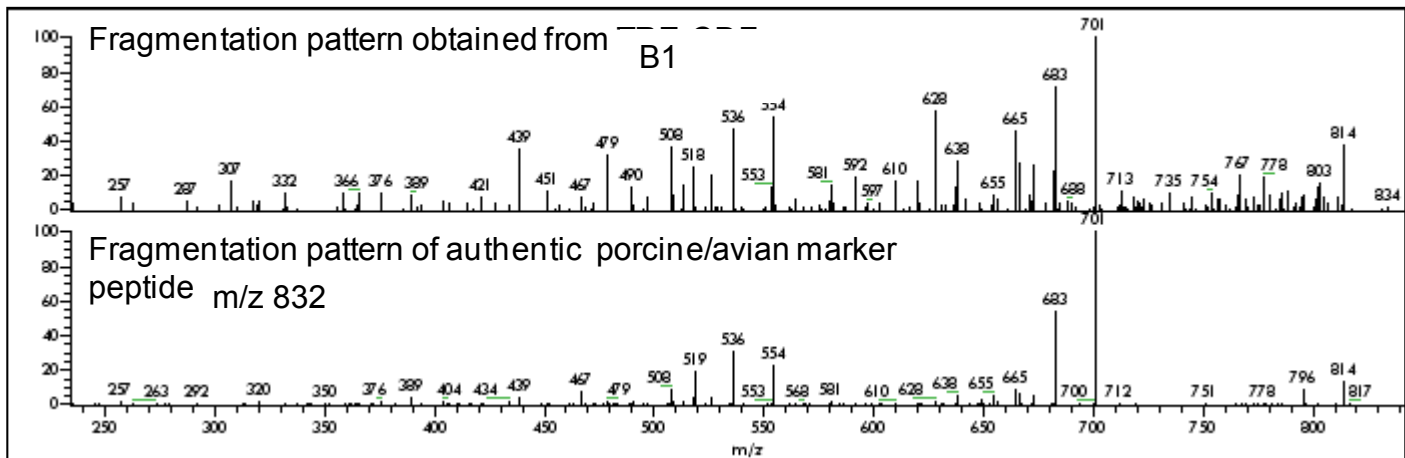


Figure 10: Fragmentation pattern from the synthetic avian/porcine marker peptide standard m/z 832 compared with the fragmentation pattern from powder sample B1 parent ion m/z 832. Correspondence of major fragmentations is apparent despite presence of trace peptide in a complex mixture.



7. RESULTS FOR INJECTION POWDER SAMPLE C

7.1 Composition of the powder

Annex J, Table 16 gives the results of the amino acid analysis of powder C, and also powder A1 for comparison. Sample C was calculated as equivalent to 79% gelatine (based on the hydroxyproline content) in comparison with the amino acid profile of commercial bone gelatine. The amino acid content of powder sample C follows fairly closely the amino acid profile of bone gelatine. This is in comparison with sample A1, which had a large excess of the free amino acids glycine, lysine and histidine. When these excess amino acids were subtracted from the powder sample A1 profile, the ratio of the remaining amino acids is similar. This implies that the excess free amino acids may be added to sample A1, but the base hydrolysed protein in both samples is similar, and similar to bone gelatine. The absence of cysteine in the injection powders suggests that the only source of protein was collagen and not muscle/blood etc.

7.2 D-Amino acid content (racemisation)

Annex K, Figure 11 gives the results of the D amino acid contents of total aspartic and glutamic acids in powder samples A1 and C and authentic gelatines. The diagram shows the degree of chemical degradation as measured by the racemisation of the two amino acids (from the L-form to the D-form). The figure indicates, from its position on the curve, that powder C was highly degraded compared with commercial bovine, porcine or avian gelatines. Powder C coincides with the previous UK sample (powder A1) on the figure, which would suggest a standardised hydrolysis treatment of the collagen since production dates were at least nine months apart.

7.3 Species origin of the hydrolysed collagen

Annex L, Table 19 gives the results of the proteomics analysis using the tryptic peptide shotgun proteomic approach. Powders were subject to tryptic digestion followed by ES-MS/MS and identified peptides were searched against the publicly available international protein databases and research collagen-specific databases compiled at York University. The table shows that in powder C, 29 peptide fragments were identified as having collagen origin. No other proteins were identified. Of these, 15 peptides were bovine collagen-specific, whereas no avian collagen-specific peptides were detected. Four of the bovine collagen-specific peptides were the same as those identified in sample A1, where only seven bovine collagen-specific peptides were identified.

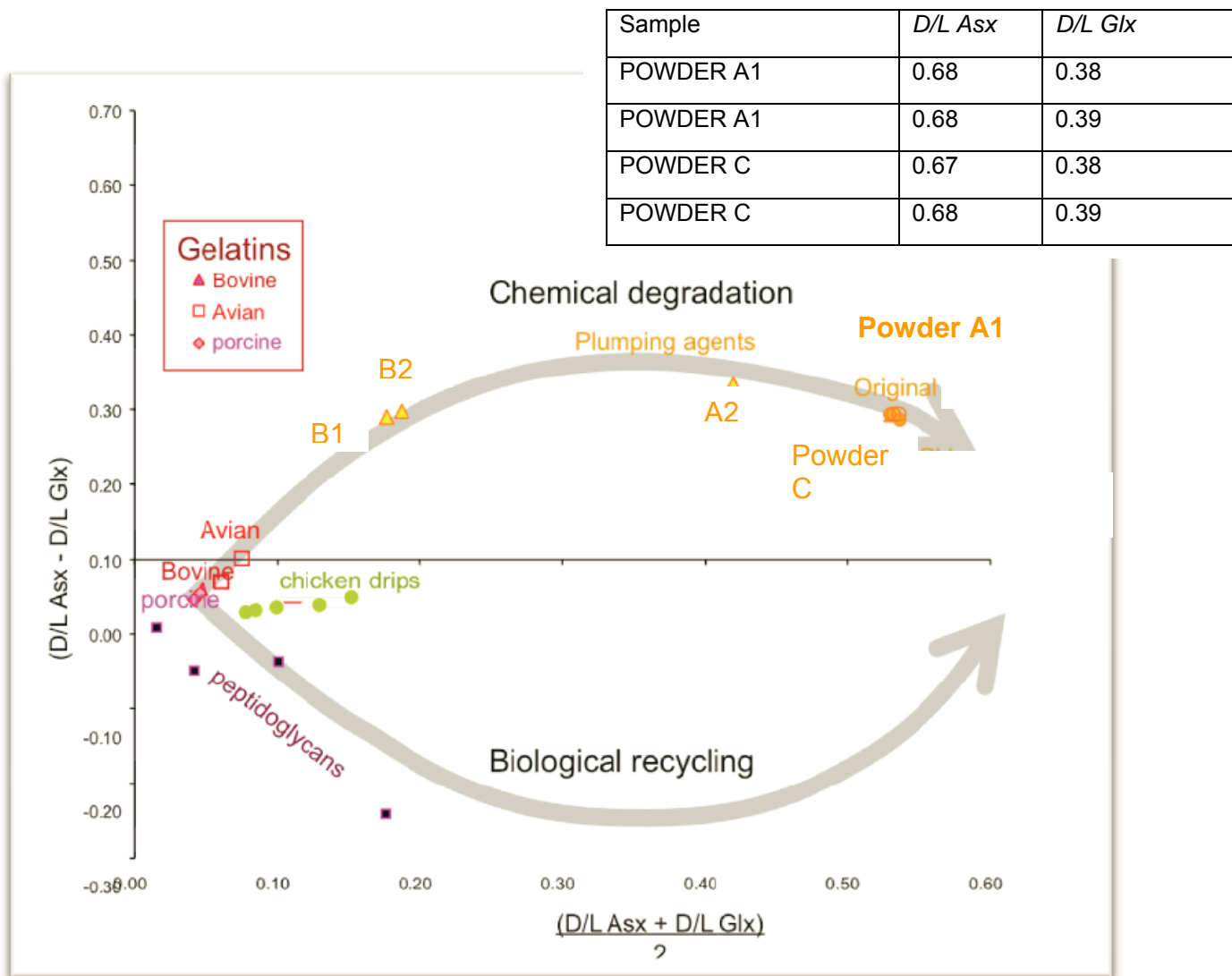
7.4 Conclusions

Three tests were applied to sample C, and compared with sample A1. Although there are some differences in amino acid profile, the base hydrolysed collagen in both appears similar. The D amino acid content indicates that the heavily processed collagen in both powders was given a standardised chemical process. Only bovine collagen-specific peptides were identified in powder C. No avian collagen-specific peptides were identified. This agrees with the earlier conclusions for powder sample A1 that the claim that powder C is manufactured wholly from a poultry source can not be substantiated by the analytical techniques applied in this study.

Table 18: Amino Acid Analysis of Injection Powders A1 and C

Amino acid	Powder C g amino acid/100g powder	Powder A1 g amino acid/100g powder	g amino acid /35g commercial bone gelatine*
Alanine	7.27	3.79	3.90
Glycine	17.00	26.6	9.39
Valine	1.94	1.0	0.96
Leucine	2.38	1.21	1.19
Isoleucine	1.13	0.58	0.53
Proline	11.10	5.73	5.35
Phenylalanine	1.63	0.81	0.86
Tyrosine	0.17	0.14	0.08
Serine	1.79	0.92	1.29
Threonine	1.57	0.89	0.81
Cysteine/cystine	0.00	0.00	0.00
Methionine	0.61	0.31	0.22
Arginine	5.97	2.94	3.11
Histidine	1.73	0.92	0.24
Lysine	2.59	16.00	1.51
Aspartic acid	4.63	2.39	2.31
Glutamic acid	8.53	4.36	4.0
Hydroxyproline	9.11	4.59	4.59
Total	79.2	72.3	

Figure 11: D Amino Acid Contents of Powders C and A1



During sample preparation glutamine and asparagine degrade to glutamic and aspartic acids. Hence D/LAsx and D/LGlx are combined D/L values of aspartic acid and asparagine, and glutamine and glutamic acid respectively.

The results show that a higher D amino acid content in A1 and C indicate a high degree of chemical degradation compared with either other injection powders examined or commercial gelatines.

The D/L Asx/Glx values of the two samples A1 and C are, within experimental error, identical. This implies that the chemical process given to raw material to produce the two samples was standardised.

Table 19: Peptide Markers Found in Powders A1 and C Using Tryptic Digestion

Collagen peptides detected by MS/MS and their presence in the collagen sequence of selected animals is shown; each row represents a unique peptide. Expect score is the probability that the observed match between MS/MS spectra and peptide sequence is a random event.

Collagen Peptides	POWDER C			POWDER A1				
	Expect score	Avian	cow	pig	Expect score	Avian	cow	pig
Type I								
					3.3e-04		•	•
	4.5e-04		•	•				
	4.9e-05		•	• ^a				
	2.4e-04		•	•	2.4e-06		•	•
	3.4e-04		•	•	1.2e-05		•	•
					1.1e-05		•	•
	6.8e-04		•					
	4.0e-05	•	•	•				
					1.9e-05		•	•
	1.5e-05		•	•	1.9e-09		•	•
	1.9e-04		•	•				
					1.8e-02		•	•
	5.8e-04	•	•	•	2.1e-05	•	•	•
					1.4e-06		•	
	3.1e-04		•					
					1.9e-03	•	•	•
	8.1e-07	•	•	•				
	1.2e-08		•	•				
	1.4e-05		•		2.0e-02		•	
	3.8e-07		•		3.0e-05		•	
	4.2e-05		•					
	3.6e-05		•		6.8e-06		•	
	1.0e-06		•					
					3.2e-04		•	
					2.4e-03		•	•
					3.1e-02		•	•
	1.3e-05	•	•	•				
	1.1e-04		•		2.6e-04		•	
	8.3e-04		•					
	2.7e-04		•	•				
	7.4e-11		•	•				
	7.3e-10		•					
	3.1e-05		•		1.8e-05		•	
	1.8e-05		•					
Type III								
	7.2e-05		•					
	4.7e-05		•					
	8.5e-04		•	•				
	1.8e-06		•					
Total precursor ions	398				415			
Gelatin precursor ions	29				18			
Non specific		4	4	4		2	2	2
Avian		0				0		
Mammal			10	10			9	9
Cow			15				7	
Pig				0				0

7. ACKNOWLEDGEMENTS

A number of collaborators were involved in this study:

Eurofins Laboratories Ltd, Manchester
Food and Environment Research Agency (FERA), York
Department of Archaeology, University of York
School of Biological Sciences, Royal Holloway University of London (RHUL)
School of Science and Technology, Nottingham Trent University

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8. ABBREVIATIONS

BS	British Standard
Ct	Crossing threshold
DNA	Deoxyribonucleic acid
ES-MS/MS	Electrospray – Tandem Mass Spectrometry
FERA	Food and Environment Research Agency
FSIS	Food Surveillance Information Sheet
GC-MS	Gas Chromatography - Mass Spectrometry
HCl	Hydrochloric Acid
HPLC	High Performance Liquid Chromatography
HyP	Hydroxyproline
ISO	International Standards Organisation
LC/MS/MS	Liquid Chromatography Mass Spectrometry
MS	Mass Spectrometry
M/z	Mass to charge ratio
MS/MS	Tandem Mass Spectrometry
MSTFA	N-methyl-N-trimethylsilyltrifluoroacetamide
NIST	National Institute of Science and Technology, U.S.A
OJ	Official Journal of the European Union
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
rtPCR	Real-time Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulphate
SI	Statutory Instrument

TMCS	Trimethylchlorosilane
RHUL	Royal Holloway University London
TIC	Total Ion Chromatogram
TOF-MS	Time of Flight Mass Spectrometry