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Development of a method to detect gelatine in vegetarian products.

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FINAL REPORT

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Authors:	H. Grundy, P. Reece
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Principal Workers:	H. Grundy, G. Rees, P. Reece
Contract manager:	P. Reece
Distribution:	1. Dr. M. Woolfe (FSA) 2. Ms. C. Tsampazi (FSA) 3. Prof. J. Gilbert (CSL) 4. Dr. P. Robertson (CSL) 5. Information Centre (CSL) 6. Authors and Principal Workers

DEFRA Central Science Laboratory
Sand Hutton
York
YO41 1LZ

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EXECUTIVE SUMMARY.

1. The presence of gelatine (an animal protein) in vegetarian meals has routinely been detected by analysis for hydroxyproline, an amino acid thought to be unique to animal collagen and thus gelatine. However hydroxyproline has been shown to also be present in some plant materials.
2. A new method for gelatine extraction from vegetarian meals based on dilute acid extraction and tannic acid precipitation of the gelatine has been developed which

enables the selective detection of gelatine in the presence of plant proteoglycans containing hydroxyproline.

3. The new procedure provides advantages over the incumbent AOAC method in terms of selectivity. The new procedure does not suffer from interference from hydroxyproline-containing proteoglycans from gum arabic or, when precipitated with tannic acid (0.11% final concentration, w/v), from other ingredients typically found in common vegetarian meals.

1. INTRODUCTION.

Gelatine is an extremely versatile low cost food protein used as a thickener, stabilizer, texturizer, gelling and water binding agent in foods [1].

The protein is derived principally from porcine skin and bovine bone collagen (and occasionally hides) by either acid treatment (porcine gelatine) or alkali and acid treatment (bovine gelatine). There is also some production of fish and chicken gelatine.

The Food Standards Agency guidance on the use of the terms ‘vegetarian’ and ‘vegan’ in food labelling specifies that protein of animal origin must not be found in, or used to prepare, foods described as suitable for vegetarians or vegans. The current method in place for the enforcement of this description is the detection of hydroxyproline, an amino acid originally believed to be only present in collagen and gelatine. However plant cell walls also contain hydroxyproline in a number of the structural glycoproteins. As a result the direct analysis for hydroxyproline in vegetarian foods is an unreliable indicator of the presence of gelatine so a more appropriate approach is required to detect gelatine at low levels in vegetarian foods and beverages.

1.1 THE TECHNICAL PROBLEM BEING ADDRESSED.

Apart from its presence in some glycoproteins in plant cell walls, current knowledge is that hydroxyproline remains unique to gelatine and collagen so the colorimetric method for hydroxyproline [2] that has been extensively validated remains the most promising approach for the confirmation of gelatine in food products.

Gelatine is produced from porcine skin collagen by soaking the defatted skin at pH 1-4 in mineral acid for 8-30 hrs [3]. The skins are then washed and gelatine extracted in hot water and concentrated by vacuum drying or ultra-filtration.

In contrast, bovine gelatine is generally produced from bone after cooking and demineralising in 4-6% hydrochloric acid for 3-7 days followed by liming in calcium hydroxide at pH 12 for 30-70 days to remove non-collagenous protein before the gelatine is again extracted in hot water.

Both processes involve extraction of gelatine into hot water as a means of recovering almost pure gelatine. Incorporation of a hot water extraction procedure in a new method would therefore seem to offer a simple means to separate gelatine from insoluble plant-based hydroxyproline.

The hydroxyproline-rich glycoprotein superfamily of proteins in plant cell walls includes:

-Extensins (Serine-(Proline)4 repeats) which are covalently bound to the cell wall matrix and so are difficult to solubilise

-Repetitive proline-rich proteins which are covalently linked with the extensions and are also difficult to solubilise

-Arabinogalactans-proteins which are soluble e.g. gum arabic.

This latter group will prove difficult to separate from the gelatines. However they can be precipitated by ß-glucosyl Yariv reagent with overnight storage at 4°C [4,5]. This would suggest that extraction of food matrices with water, possibly including a ß-glucosyl Yariv precipitation step, would recover only hydroxyproline of a soluble gelatine origin.

An alternative approach could be through the treatment of the extracted protein with collagenase which would produce low molecular mass peptides containing hydroxyproline only from the gelatine substrate. These peptides could be recovered by centrifugal filtration prior to analysis for hydroxyproline.

Gelatine is used in the wine and brewing industry to precipitate haze and tannins from the final product. The converse of this is that tannins in the form of commercial tannic acid could be used to selectively precipitate gelatine in the presence of soluble arabinogalactan proteins. This is a speculative approach since polyphenols such as tannic acid are capable of precipitating a number of different proteins. However, if successful, the procedure would provide a very rapid and cost effective approach to separating gelatines from other plant-derived hydroxyproline-containing proteins.

1.2 OBJECTIVES OF THE PROJECT.

Number	Objective title	Achieved
01/01	Obtain authentic (commercial) samples of bovine, porcine, fish and avian gelatine	Yes
01/02	In-house validation of the AOAC determination of hydroxyproline	Yes
01/03	Optimise water extraction and ultra-filtration	Yes
01/04	Evaluate collagenase and ß-glucosyl Yariv reagent to eliminate interferences	Yes, tannic acid used as an alternative to ß-glucosyl Yariv reagent
01/05	Project progress meeting	Yes
02/01	Validation of modified procedure to detect gelatine	Yes
02/02	Analysis of commercial food samples	Yes
02/03	Preparation of final report	Yes

2. EXPERIMENTAL.

2.1 MATERIALS.

2.1.1 Food matrix samples.

2.1.1.1. Hydroxyproline analysis reference materials
FAPAS test materials T0147 and T0151 (meats containing hydroxyproline) were used as in-house reference materials during analyses to confirm complete digestion and release of hydroxyproline.

2.1.1.2.Vegetarian meal matrix
An in-house vegetarian risotto meal was prepared for use in spiking studies during the method development studies. The ingredients are listed in Table 1.

2.1.1.3 Commercial chilled and frozen vegetarian ready meals were purchased for investigation as sample matrices (Table 2). These were selected on the basis of the complexity of their ingredients to determine whether commercial food ingredients interfered with the analysis. The meals were stored at –20°C until use.

2.1.2 Reagents

Hydroxyproline (Fluka)
Gum Arabic (Sigma-Aldrich)
Chloramine-T (Fluka)
Sulphuric acid, 18M (Fisher)
Citric acid monohydrate (Sigma-Aldrich)
Sodium acetate trihydrate (Sigma-Aldrich)
Acid-washed filter paper (Whatman)
4-Dimethylaminobenzaldehyde (Alfa Aesar)
Perchloric acid (Alfa Aesar)
1-Propanol (Fisher)
2 Propanol (Sigma Aldrich)
VIVASPIN 20 ultrafilters (10kDa MWCO) (Sartorius).
Collagenase type VII (1359 collagen units per mg solid) (Sigma Chemical Co).
Tannic acid ACR reagent (Sigma Aldrich).

2.2 METHODS.

2.2.1 AOAC Method 990.26. Hydroxyproline determination in meat and meat products.

Preparation of reagents

A buffer solution was prepared by mixing 30 g citric acid monohydrate, 15 g sodium hydroxide and 90 g sodium acetate trihydrate in 500 mL water and the pH was adjusted to 6.0 with some of the acid or base components as necessary. 290 mL of 1-propanol was added and the volume was made up to 1 L with water. This solution was stored in an amber bottle at 4ºC and was stable for 60 days. Chloramine-T solution was prepared by dissolving 1.41 g of Chloramine-T into 100mL of the buffer solution and was stored in an amber bottle. This solution was stable at 4ºC for one week.

Colour reagent was prepared by dissolving 2.5 g of 4-dimethylaminobenzaldehyde in 8.75 mL perchloric acid (60% w/w). 16.25 mL of 2-propanol was then added slowly and mixed. The solution was stored at room temperature and used within 5 hours of preparation.

Preparation of samples and standards

4.0 g of a representative sample of homogenised analytical matrix was placed in a conical flask, sealed with a watch glass and digested for 16 hours with 7N (3.5M) H_2SO_4 at 105°C. Following digestion, the sample was added to 250 mL of water and the volume made up to 500 mL with water. The sample was mixed by hand and filtered through acid-washed filter paper. The filtrate was analysed according to the following procedure:

The filtrate was diluted with water so that the hydroxyproline concentration of the final dilution was in the range of 0.5-2.4 µg/mL. Dilution of 5 mL filtrate to 100 mL was usually suitable*.

Standards were prepared from pure hydroxyproline solid dissolved in water with concentrations ranging from 0 to 2.4 µg/mL (= 0 to 4.8 ug in the final volume of 2 mL). 2 mL of each standard was included in the analysis.

Assay

2.0 mL of diluted filtrate or standard was mixed (by vortex mixer) with 1 mL Chloramine-T solution and incubated at room temperature for 20 minutes. 1 mL of colour reagent was added and the samples and standards were mixed by vortex mixer and incubated at 60°C for 15 minutes. The samples were then allowed to cool for 10 minutes in iced water (4°C). The absorbance of each sample was then measured against the reagent blank at 558 nm. A calibration curve of the standards was prepared and samples were diluted so that values fell within the calibration curve.

The hydroxyproline content of the samples (H) was calculated as follows:

H, g/100g = (h x 2.5)/(m x V)

Where h = µg/2mL filtrate, taken from the standard curve
m = weight of sample in g (4 g)
V = volume, mL of filtrate taken for dilution to 100 mL*

2.2.2 Optimised method for extraction of gelatine from a vegetarian matrix

4.0 g of a representative sample of homogenised analytical matrix was placed in a 100 mL sealed Duran bottle. 30 mL of 20mM H_2SO_4, preheated to 60°C, was added and the sample shaken vigorously on a vortex for 30 seconds. The sample was incubated in a water bath at 60°C for 20 minutes with vigorous agitation by vortex after 7 and 14 minutes for 30 seconds. Samples were then centrifuged at 3220 x *g* for 10 minutes at 30°C and the supernatant decanted and stored. The remaining pellet was transferred to the original 100mL Duran bottle and the extraction procedure was repeated twice, for 10 minutes each time. The combined extract was made up to 100 mL in a graduated flask using 20mM H_2SO_4. A 28 mL aliquot was removed, mixed with 6 mL of concentrated (18M) sulphuric acid and the sample digested overnight.

After digestion, the sample was diluted to 500 mL with water and analysed as described in section 2.2.1.

2.2.3 Optimised method for determination of gelatine in vegetarian meals

For SOP see APPENDIX 1

2.2.4 Preparation of in-house spiked samples.

Gelatines were ground with a pestle and mortar to reduce particle size. In-house risotto samples (2.1.1.2) were spiked by adding gelatine powder to final concentrations of 0, 1, 2, 5 and 10% (w/w) and were homogenised with a food mixer. If appropriate, 10% (w/w) gum arabic powder was also added to samples followed by homogenisation.

Samples were frozen to -20°C and thawed before analysis. All samples were used within 3 weeks of spiking.

2.2.5 Preparation of commercial spiked samples

Commercial meals were first homogenised and analysed for hydroxyproline content before spiking with gelatine. Meals were spiked with gelatine in solution to a final concentration of 2% gelatine as follows. Meals were homogenised in a household food blender and a 100 g aliquot was taken and incubated in a sealed container until the matrix reached 50°C. A gelatine sample was prepared by dissolving 25.16 g of gelatine in 150 mL water and incubating at 80°C. 30 mL of this solution was blended into 100 g of meal matrix in a household blender.

Eight meal samples were spiked with porcine gelatine (CSL sample no. 542). One sample was spiked with bovine gelatine (CSL sample no. 538), one with piscine gelatine (CSL sample no. 531) and one with avian (chicken) gelatine (CSL sample no. 530) as detailed in Table 2.

2.2.6 Collagenase digestion of extracted gelatine

8 mL of gelatine extract in 20mM sulphuric acid (method 2.2.2) was neutralised with 1.0 mL of 200 mM HEPES buffer pH 7.5 containing 50 mM calcium chloride. 50 µL of collagenase type VII (1.0 mg/mL in water) was added and the sample incubated at 37°C for up to 24 hr. The digested sample was then transferred to a Vivaspin-20™ 10kDa MWCO ultra-filter and centrifuged at 4000 x *g*, 30°C for 25 min. The filter was washed with 2 mL of deionised water and re-centrifuged for 10 min at 30°C. The filtrate was collected and sulphuric acid added to a final concentration of 7N (3.5 M H_2SO_4) before the sample was digested at 105ºC for 16 hours and analysed as described in section 2.2.1.

3. RESULTS AND DISCUSSION.

3.1 OBJECTIVE 01/01 OBTAIN AUTHENTIC (COMMERCIAL) SAMPLES OF BOVINE, PORCINE, PISCINE AND AVIAN GELATINE.

European and Asian providers of gelatines to the food industry were contacted. Twenty three samples of gelatine from seven European suppliers were obtained,

although one of the gelatines was of South American origin. These included ten bovine, eight porcine, four piscine and one avian gelatine, as shown in Table 3. No Asian samples were obtained. The hydroxyproline content of each gelatine was determined in duplicate according to method 2.2.1 and the results also are shown in Table 3. All of the gelatines examined contain between 8.9 and 12.7% hydroxyproline (w/w) with the piscine gelatines containing slightly less hydroxyproline (mean 9.9% (w/w)) than the mammalian gelatines (mean 11.9%(w/w)). Only one commercial avian gelatine could be sourced despite a high number of gelatine suppliers being contacted.

3.2 OBJECTIVE 01/02 IN-HOUSE VALIDATION OF THE AOAC DETERMINATION OF HYDROXYPROLINE

The AOAC method (2.2.1) was carried out four times by each of two analysts on FAPAS test materials T0147 and T0151 to test reproducibility. The results were submitted to statistical analysis. Analysis of variation (ANOVA) was performed and estimates of between analyst, between calibration within analyst and repeatability variation were extracted from the ANOVA table. A relative standard uncertainty was then calculated for a measurement using a single analyst and a single calibration. The associated expanded uncertainty was then calculated using the appropriate degrees of freedom and t-value. A linear regression was performed on the concentrations using an interaction effect by calibration to assess any difference in standard curves produced. Reproducibility was compared to the Horwitz equation and an assessment of bias was made [6]. The results of the statistical analysis showed that the reproducibility of the data fell within the tolerances for the method. The statistical analysis is shown in Appendix 2.

3.3 OBJECTIVE 01/03 OPTIMISATION OF WATER EXTRACTION AND ULTRAFILTRATION STEP

Initial studies centred on the use of hot water (65°C) to extract bovine gelatine spiked into the in-house vegetarian matrix. The results obtained suggested that all of the spiked gelatine (0.4 g) could be extracted from 4g of sample matrix by extracting with two washes of 30 mL of water at 60°C. However, when this method was applied to the range of gelatines collected (Objective 01/01), it was quickly found that recovery of gelatine as low as 55% was observed for some of the gelatines. The method was therefore modified and a third wash step was incorporated and the extraction medium was changed to 20mM sulphuric acid to aid recovery of the gelatine. The volume of the final extract was then made up to 100 mL with 20 mM sulphuric acid. Using this modified extraction procedure a mean recovery of 101.7% (+/- 5.9%) was observed when eighteen of the commercial gelatines were spiked at the 2% and 5% level into the in-house vegetarian matrix (Table 4). These extraction studies were carried out by both CSL and its subcontractor (BioArCh at The University of York) and recoveries from both groups were similar. No significant difference in recovery was observed between the 2% and 5% spiked samples (mean recoveries observed 100.1% +/- 5.0% and 101.9% +/- 6.2%) for the 2% spikes and 100.9% +/- 5.5% and 100.9% +/- 5.9% for the 5% spikes).

This optimised gelatine extraction procedure formed the basis on which the further studies were carried out to develop a technique to differentiate between solubilised

hydroxyproline derived from gelatine and solubilised hydroxyproline derived from plant material such as gum arabic. Two procedures were initially proposed, one involving the use of collagenase in combination with ultra-filtration to break down the relatively large gelatine polypeptides containing hydroxyproline to smaller peptides which could pass through a 10kDa ultrafilter. Since collagenase would not hydrolyse the plant arabinogalactans that contain hydroxyproline, these would be retained by the filter after collagenase treatment. The second approach planned was the use of β-glucosyl Yariv reagent, a specific arabinogalactan precipitant, which would theoretically separate plant-derived hydroxyproline from gelatine. A third method involving the use of tannic acid precipitation of gelatine was introduced while research on the first two methods was underway.

3.4 OBJECTIVE 01/04 EVALUATION OF COLLAGENASE OR ß-GLUCOSYL YARIV REAGENT TO ELIMINATE INTERFERENCE

Collagenase.

0.08 g of gelatine was dissolved in 100 mL of 20 mM sulphuric acid. This was equivalent to the concentration obtained by extracting a 4 g sample containing 2% gelatine with 100 mL of 20 mM sulphuric acid according to the modified extraction procedure described above (method 2.2.2). 8.0 mL aliquots of this solution were digested with collagenase (method 2.2.6) over 24 hr to determine the timescale necessary to hydrolyse the gelatine to peptides sufficiently small to pass through a 10 kDa ultra-filter. The results in Figure 1 show that after a 5 hr digestion, 93.5% of the hydroxyproline applied to the filter was found in the filtrate after centrifugation. However a further 19 hr digestion was required to reduce to 0.8% the proportion of hydroxyproline still remaining with the retentate after centrifugal filtration. Due to the relatively high cost of collagenase it is recommended that an overnight incubation of 8 ml of gelatine extract with 68 units of collagenase (50 µL of a 1 mg/mL solution of 1159 units/mg) be used as a standard treatment rather than employing an increased concentration of collagenase for a shorter incubation period. This treatment recovered 96% of the gelatine hydroxyproline in the filtrate from the 2% (w/v) gelatine extract.

In order to determine whether this standard treatment would release hydroxyproline from gum arabic, 12.0 mL of a 1% gum arabic solution was treated with collagenase in the standard manner (gum arabic was shown to contain 400 µg/g hydroxyproline). No hydroxyproline was detected in the filtrate after 16 hr or after a prolonged incubation of 30 hr (45.8 µg hydroxyproline detected in the retentate, equivalent to 95% of the hydroxyproline applied to the filter).

The collagenase/ultra-filtration procedure therefore appeared to discriminate between plant-derived and gelatine-derived hydroxyproline. However the method was time consuming, with a reduced sensitivity since only 8 ml of the 100 ml gelatine extract from a 4g sample was analysed in this procedure (volume limit set by the size of the ultrafilter unit). Increasing the size or number of filters could raise this to 28 ml of the gelatine extracted however no more than 28 ml of an aqueous sample could be digested with sulphuric acid to retain the same digest sample volume as used in the AOAC method for hydroxyproline (method 2.2.1). Work on this method was therefore suspended while the use of alternative precipitants was pursued.

Glucosyl Yariv reagent.

Due to the cost of the ß-glucosyl Yariv reagent (£237 for 10 mg supplied by Biosupplies, Australia Pty Limited), it was decided to investigate the use of tannic acid as a gelatine-specific precipitant before investigating the ß-glucosyl Yariv reagent. As well as being a more cost effective approach, the tannic acid precipitation of gelatine also offered the potential of a significant increase in sensitivity of the method since gelatine from much larger volumes of extract could be digested if it was precipitated first.

Tannic acid

An initial investigation into the effect of pH on the precipitation of gelatine with tannic acid was carried out. 8.0 ml aliquots of gelatine solution in 20mM sulphuric acid were adjusted to pH values of 3, 6 and 9 with 1.0M sodium hydroxide. 1% tannic acid was added as a 10% aqueous solution and the samples held at 4°C for 1 hr before centrifugation. The precipitates were washed with 10 mL of deionised water before analysing the supernatants and precipitates for hydroxyproline (method 2.2.1). The results (Figure 2) showed that all of the gelatine was precipitated under acidic conditions. High pH conditions resulted in incomplete precipitation of the gelatine and poor recovery of hydroxyproline.

Similar treatment of a range of concentrations of gelatine showed that tannic acid precipitation gave quantitative recovery of the gelatine over a wide concentration range from 17 µg/mL to 5.0 mg/mL hydroxyproline.

The addition of gum arabic at 18 mg/ml to the 8 mL gelatine sample prior to precipitation with tannic acid did not interfere with the precipitation of gelatine or the recovery of hydroxyproline from the gelatine, suggesting that the arabinogalactans from the gum arabic were not precipitated by tannic acid. 104% mean recovery of hydroxyproline was observed after precipitation of 8.0 mL of extract containing 4 mg/mL of gelatine in the presence of 18 mg/mL of gum arabic.

As a result of the successful evaluation of tannic acid as a gelatine specific precipitant, the validation of a method using tannic acid to separate gelatine from gum arabic was undertaken in the remainder of the project. Since separation of precipitated proteins by centrifugation was proving difficult due to the low density of the precipitate, filtration through glass fibre filters was used to recover the solids. The method is described in full in Appendix 1. Commercial tannic acid available from all suppliers we contacted (approximately five suppliers) is extracted from Chinese natural grass gall nuts.

The optimum precipitation time was determined by comparing recoveries of gelatine from risotto spiked with 2% porcine gelatine (CSL sample no. 540). Samples were extracted according to the method described in Appendix 1 with 10 mL of 12.5% tannic acid added to the whole (100mL) extract and stored for 1, 4 and 24 hours at 4°C or room temperature in duplicate. The data is shown in Table 5. As expected, precipitations performed at 4°C gave the highest yield in all cases compared to precipitations performed at room temperature (20ºC ±4ºC). The highest yield was observed in the samples precipitated at 4°C for 24 hours. However, a 1 hour precipitation at 4ºC gave a yield of only 3.7% less than that of precipitation at 4°C for 24 hours. Precipitating for one hour rather than for 24 hours would make the final test considerably more user-friendly for Public Analysts and a correction factor could be

applied to the final yield if necessary. This 1 hour precipitation step was therefore incorporated into the modified gelatine extraction method.

3.5 OBJECTIVE 02/01 VALIDATION OF MODIFIED PROCEDURE TO DETECT GELATINE.

Commercial food grade porcine gelatine (CSL sample no. 542) was used to prepare solutions of a series of gelatine concentrations in 20mM sulphuric acid for analysis to provide an estimate of between run variation and bias of the modified extraction method.

Porcine gelatine solutions of 0, 0.07, 0.35, 0.70 1.40, 3.51 and 7.01% gelatine were prepared. Six 100mL aliquots of each solution were analysed in duplicate on three different days by precipitating each chilled (4°C) solution with tannic acid for 1 hour prior to digestion. The results were submitted for statistical analysis as described in Appendix 3. The fitness for purpose of the method was assessed by comparing the size of the estimated standard uncertainty to the equivalent Horwitz standard deviation. HORRAT ratios of less than 2 were observed for all concentrations examined in this study. Hence, this initial assessment of the results suggests that the method was fit for purpose when the results were corrected for the bias associated with the method. A correction factor (multiplication by 1.196) must be applied to the results to account for the 83.58% recovery measured. The statistical analyses are detailed in Appendix 3.

In order to assess transferability of the method, ten commercial food grade gelatines were spiked into the in-house risotto matrix at the 2% (w/w) level, with and without 10% (w/w) gum arabic. 4.0 g of each sample was extracted and precipitated either by CSL or by its subcontractor, BioArCh at The University of York, and the recoveries of hydroxyproline were compared. Samples were extracted by the modified procedure (Appendix 1) and the tannic acid precipitate digested and analysed according to the AOAC method by CSL. The results (Table 6) showed no difference in recoveries between CSL and its subcontractor since the sample means overlap by less than one standard deviation. Further, the results confirmed separation of the gelatine from gum arabic by the tannic acid precipitation method (2.2.3) since the mean value for the gelatine and gum arabic spiked sample fell within the standard deviation of the gelatine-only spiked sample (Table 6).

3.6 OBJECTIVE 02/03 ANALYSIS OF COMMERCIAL SAMPLES

Commercial vegetarian meal samples (Table 2) were spiked with gelatine (as described in 2.2.5) and analysed by the tannic acid precipitation method (Appendix 1) to confirm whether commercial food ingredients interfere with the method. Eleven commercial food products were analysed using the tannic acid precipitation method both before and after spiking with 2% gelatine. Eight of the retail samples were spiked with porcine gelatine (CSL sample no. 542) and three samples with an avian, piscine or bovine gelatine (CSL sample no. 530, 531 or 538 respectively). The results are shown in Table 7. The unspiked samples showed hydroxyproline levels ranging from 0.0014 to 0.0059 g hydroxyproline per 100g sample (estimated content as this is below the limit of detection as discussed in Appendix 4), suggesting either the

modified method was not removing all of the plant derived hydroxyproline from the sample or all of the samples contained a small amount of gelatine equivalent to a range of 0.01 to 0.05% gelatine.

Recovery was 0.1748 g hydroxyproline per 100g sample for the sample spiked with bovine gelatine (69.3% recovery of the gelatine added when taking into account the hydroxyproline content of the blank material), 0.1757 g hydroxyproline per 100g sample for the sample spiked with avian gelatine (68.5% recovery), and 0.2057 g hydroxyproline per 100g sample for the sample spiked with piscine gelatine (80.1% recovery).

The recovery of hydroxyproline from the samples spiked with 2% porcine gelatine ranged from 56-77%. These data suggested that applying a single correction factor was not sufficient to account for all of the gelatine in every matrix. Possible solutions to this variability in recovery could be to use a buffer to control the pH of the extract from each matrix or to increase precipitation time, decrease precipitation temperature or precipitate gelatine at higher concentrations of tannic acid from complex commercial matrices. As a preliminary investigation into this scenario, experiments were conducted to determine whether increasing the amount of tannic acid used for precipitation would increase the yield in commercial food matrices. Combined supernatants from extracts of three gelatines (CSL sample nos. 530, 546 and 548) mixed into in-house risotto were aliquoted and precipitated with different tannic acid loadings. The results (Table 8) suggested that increasing the amount of tannic acid did improve the yield of gelatine extracted from commercial matrices.

As described in Appendix 4, the limit of detection of the AOAC Method 990.26 was determined as 0.25% gelatine. The limit of detection of the current method (Appendix 1) involving pre-analysis extraction of the sample in 20mM sulphuric acid followed by precipitation with tannic acid was determined to be 0.19% gelatine and thus was slightly more sensitive than the AOAC method.

4. CONCLUSIONS.

A method to selectively extract gelatine from other hydroxyproline-containing compounds has been developed by combining the extraction of gelatine from the food matrices in 20mM sulphuric acid at 65ºC followed by precipitation of gelatine using tannic acid. The limit of detection of this method was determined as 0.19% gelatine which was shown to be a slightly more sensitive LOD than the AOAC method.

The recovery of hydroxyproline was 83.6% and therefore there was the possibility of applying a correction factor to the results to account for this recovery. The recovery of hydroxyproline from commercial foods matrices spiked with gelatine was 56-77%. Preliminary analyses suggested that increasing the amount of tannic acid used during the precipitation step should improve this gelatine recovery.

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7. GLOSSARY.

HEPES	N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid
kDa	kilo Dalton
MWCO	Molecular Weight Cut Off
std. dev.	Standard deviation
var	Variance
LOD	Limit of Detection

8. TABLES.

Table 1. Contents of in-house vegetarian meal matrix (risotto)

Ingredient	**Mass/Volume**
mushroom	170 g
courgette	225 g
tomato	165 g
celery	475 g
red pepper	80 g
long grain rice	1000 g
organic sunflower oil	20 mL
water	1000 mL

Table 2. Retail vegetarian ready meal samples spiked with gelatines

Commercial Sample number	Product name	Lot number	Species of gelatine used for spiking
1	Asda meat free Two red onion and goats cheese parcels	8029A	Porcine
2	Asda meat free Six Glamorgan sausages	2019 L80GS	Porcine
3	Asda Four spring rolls	WT025577/001 3015AN2 200866	Porcine
4	Goodfellas Delicia thin classic Mediterranean pizza	8024 K 13:17	Porcine
5	Cauldron Two Mediterranean vegetable bistro bakes	448024 04:45	Porcine
6	Asda meat free Two garlic kievs	ERM 2687 15:24	Porcine
7	Asda two cheese omelettes	L8025 1238	Porcine
8	Asda meat free creamy leek and gruyere cheese tarts	L8036EWP	Porcine
9	Asda meat free Six Glamorgan sausages	2019 L80GS	Bovine
10	Asda meat free Six Glamorgan sausages	2019 L80GS	Avian
11	Goodfellas Delicia thin classic Mediterranean pizza	8024 K 13:17	Fish

Table 3. Hydroxyproline content of commercial gelatine samples collected.

Species and CSL Sample Number (Prefix SO7-021)	Country of origin of gelatine	Hydroxyproline content (mg/g)
Avian		
530	UK	114.2
Bovine		
543	Italy	121.8
544	Italy	127.3
545	Italy	112
538	Belgium	125.1
539	Belgium	110.9
535	France	111
528	Italy	114.5
547	S.America	121.2
532	UK	116.6
550	Germany	112.9
Fish		
536	France	104.7
529	Italy	102.8
548	Italy	88.7
531	UK	92.4
Porcine		
537	Belgium	111.5
534	France	111
549	France	115.6
540	Italy	125.5
541	Italy	113.9
542	Italy	120
546	Sweden	116.9
533	UK	116.2

Table 4. Recovery of added gelatine from spiked in-house matrix using the modified extraction procedure.

	2% gelatine spike			5% gelatine spike	
Gelatine species	Identifier	% Recovery		Identifier	% Recovery
Avian	530	106.5		530	109.8
Bovine	532	90.2		532	106.6
	535	106.1		535	106.2
	538	100.2		538	96.6
	544	95.2		539	103.4
	545	95.1		544	105.1
	547	97.4		547	95.4
Piscine	529	108.7		531	105.8
	531	107.1		536	104.3
	536	99.6			
	548	102.1			
Porcine	533	109.7		534	90.0
	534	99.4		537	99.6
	537	104.3		542	92.2
	540	96.2		546	103.3
	542	97.9			
	546	100.6			
	549	99.4			

Table 5. Optimisation of Tannic Acid Precipitation of 100ml of 2% gelatine solution

Length of precipitation (hours)	**Temperature of precipitation**	**Hydroxyproline detected (g/100g)**	**% recovery**
1	**4ºC**	**0.2310**	**92.4**
4	**4ºC**	**0.2265**	**90.6**
24	**4ºC**	**0.2398**	**95.9**
1	**ambient**	**0.2260**	**90.4**
4	**ambient**	**0.2084**	**83.4**
24	**ambient**	**0.2140**	**85.6**

Table 6. Yield of hydroxyproline from different commercial gelatines in the presence and absence of gum arabic and in-house vegetarian matrix using the tannic acid precipitation step.

Gelatine Sample (CSL sample no.)	Hydroxyproline, (g/100g) no gum arabic	Hydroxyproline, (g/100g) with gum arabic
529	0.1632	0.1638
530	0.1980	0.2008
531	0.1483	0.1920
532	0.1841	0.1651
533	0.1880	0.1971
534	0.1805	0.1746
536	0.1709	0.1373
538	0.2195	0.2164
539	0.1862	0.2113
540	0.1971	0.2017
541	0.1578	0.1955
542	0.2060	0.1200
544	0.2006	0.2054
545	0.1797	0.1850
546	0.1832	0.1923
547	0.1825	0.1971
548	0.1524	0.1578
549	0.1694	0.2078
CSL mean	0.1863	0.1834
CSL std. dev.	0.022	0.027
Subcontractor mean	0.1756	0.1832
Subcontractor st. dev.	0.012	0.099

Table 7. Overview of hydroxyproline yield from spiked (2% w/w) and unspiked (blank) retail samples.

Species of gelatine	Retail Sample No. (see Table 2)	Total Hydroxyproline (g/100 g) recovered from spiked sample	Blank Sample No.	Hydroxyproline (g/100 g) recovered from unspiked sample
Porcine	Sample 1	0.1980	Blank 1	0.0059*
Porcine	Sample 2	0.1610	Blank 4	0.0044*
Porcine	Sample 3	0.1782	Blank 5	0.0015*
Porcine	Sample 4	0.1615	Blank 6	0.0054*
Porcine	Sample 5	0.1567	Blank 7	0.0014*
Porcine	Sample 6	0.1438	Blank 8	0.0031*
Porcine	Sample 7	0.1696	Blank 9	0.0059*
Porcine	Sample 8	0.1828	Blank 10	0.0050*
Bovine	Sample 9	0.1748	Blank 11	0.0015*
Avian	Sample 10	0.1757	Blank 12	0.0044*
Fish	Sample 11	0.2057	Blank 13	0.0054*
	Mean	**0.1734**	**Mean**	**0.0035***
	St. dev.	**0.018**	**St. dev.**	**0.002**

*denotes values that are below the limit of detection

Table 8. Comparison of yields of hydroxyproline when precipitated from in-house risotto with increased concentrations of tannic acid.

Volume of 12.5% tannic acid added to the 100 mL extract	Final % tannic acid (w/v)	Yield, g hydroxyproline per 100g matrix	% recovery
10.0	1.136	0.3109	77.3
30.0	2.88	0.3287	81.7
60.0	4.69	0.3394	84.4

9. FIGURES.

Figure 1. Effect of incubation time on release of hydroxyproline by collagenase

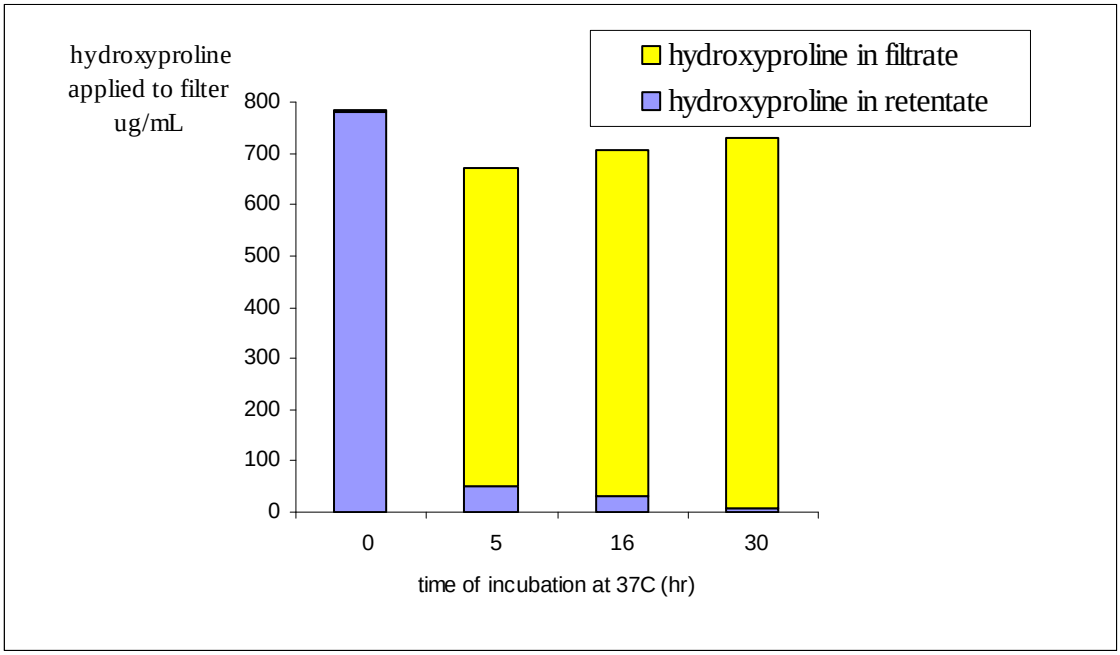


Figure 2. Effect of pH on the precipitation of gelatine with tannic acid.

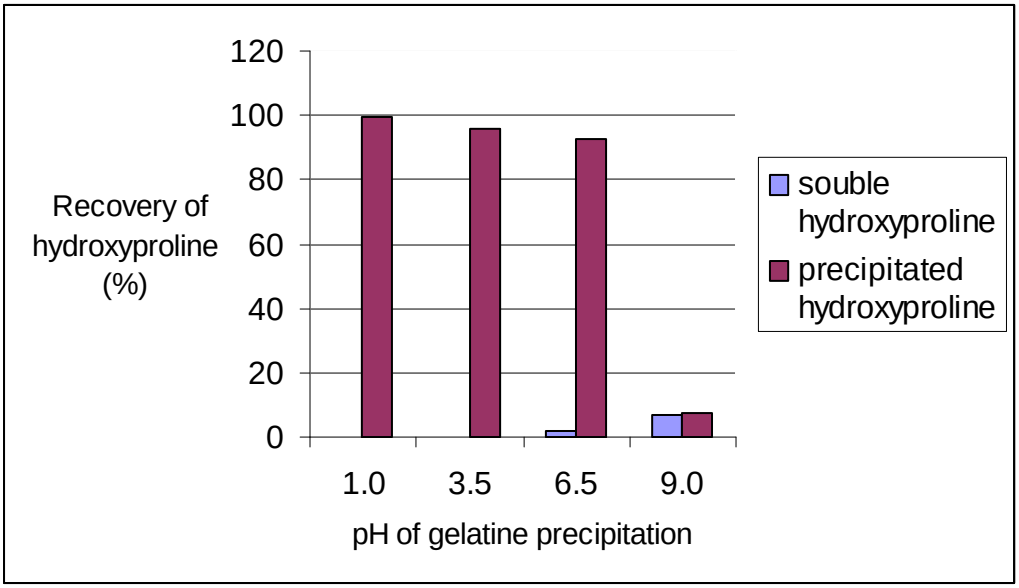
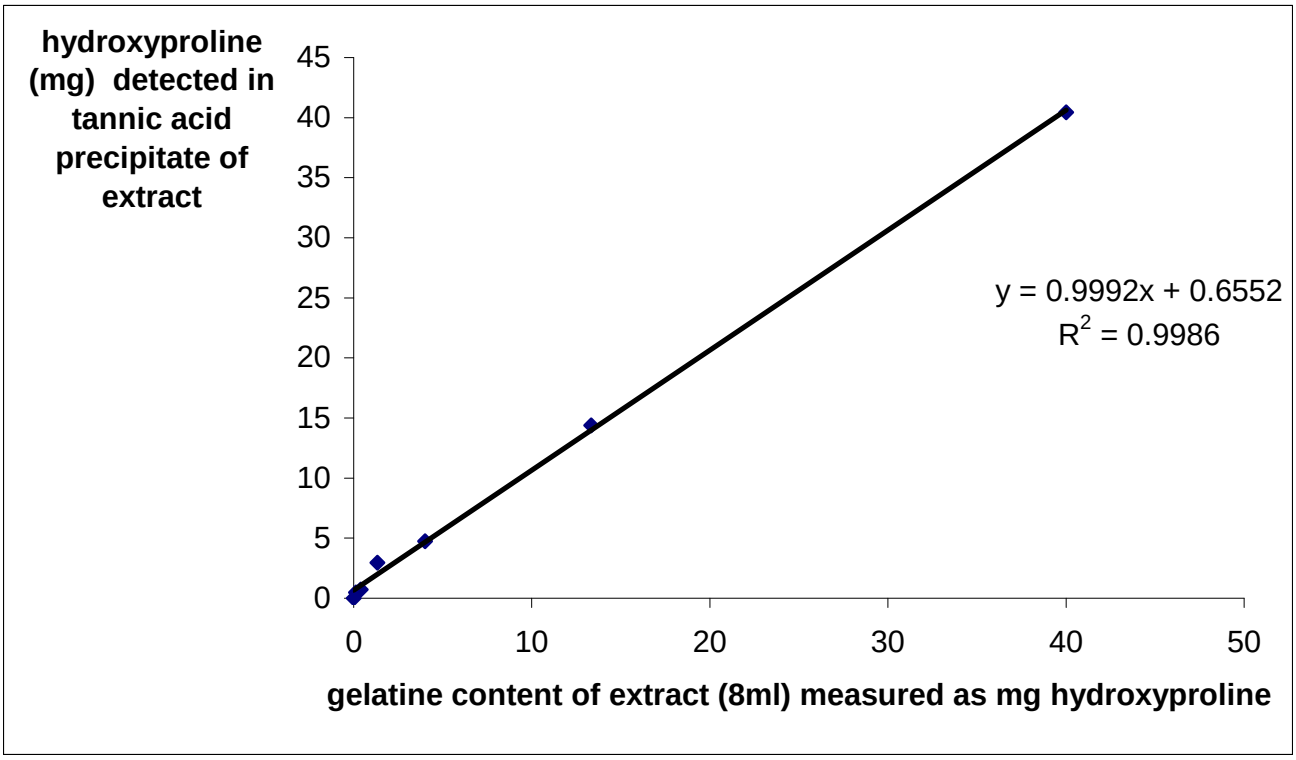


Figure 3. Correlation between hydroxyproline as gelatine in 20mM sulphuric acid extracts and hydroxyproline in the tannic acid precipitate.



10. APPENDICES.

10.1 APPENDIX 1.

SOP is attached separately.

10.2 APPENDIX 2: AOAC method reproducibility.

Standard curves were produced for measuring hydroxyproline at levels of 0, 0.6, 1.2, 2.4, 3.6, 4.8µg in 2mL. Two analysts produced four calibrations each. For each calibration two 'unknown' samples were analysed and the calibration used to estimate the concentration. One replicate per sample per calibration was used.

Methods

Analysis of variance (ANOVA) was performed on the hydroxyproline concentrations (µg) for each 'unknown' sample. Estimates of between analyst, between calibration within analyst and repeatability variation were extracted from the ANOVA table. A relative standard uncertainty[1] was then calculated for a measurement using a single

[1] Relative Standard Uncertainty = sqrt((analyst SD)^2 + (analyst/calibration SD)^2 (within SD)^2/2) / 'mean concentration'

analyst and a single calibration. The associated expanded uncertainty[2] was then calculated, using the appropriate degrees of freedom and t-value[3].
In addition, a linear regression was performed onto the concentrations using an interaction effect by calibration, to assess any differences in the standard curves produced.

Results
The between analyst, between calibration within analyst and repeatability variation estimates are shown in Table A for each sample along with the relative standard uncertainty estimate (for a measurement using a single analyst and single calibration).
For sample 0147 all SD estimates are relatively larger than for sample 0151. For sample 0147 the between analyst variation is greater than the between variation and within, but the reverse is true for sample 0151.
The expanded uncertainty (equivalent to a 95% confidence interval) shows the true concentration to be between -0.15 to 0.30ug for sample 0147 and 0.86 to 0.95ug for sample 0151.

Table A. Between analyst and within analyst SD with relative standard uncertainty when using a single analyst for measurement of gelatine

Sample	Overall Mean (g/100 g)	Between Analyst SD	Between Calibration SD	Rep SD	Standard Uncertainty	%Relative Standard Uncertainty	Expanded Uncertainty	
							Lower	Upper
Sample 0147	**0.075**	**0.006**	**0.003**	**0.003**	**0.007**	**8.98**	**-0.132**	**0.282**
Sample 0151	**0.905**	**0.000**	**0.012**	**0.015**	**0.016**	**1.81**	**0.863**	**0.946**

Table B. Between analyst and within analyst SD with relative standard uncertainty when using a single analyst for measurement of Gelatine

Sample	Overall Mean (g/100g)	Between Analyst SD	Between Calibration SD	Standard Uncertainty	%Relative Standard Uncertainty	Expanded Uncertainty	
						Lower	Upper
Sample 0147	**0.075**	**0.006**	**0.003**	**0.007**	**8.98**	**-0.132**	**0.282**
Sample 0151	**0.905**	**0.000**	**0.012**	**0.016**	**1.81**	**0.863**	**0.946**

The reproducibility estimate can be compared to the Horwitz equation[6] for reproducibility. This models the predicted percentage relative standard deviation (%RSD_R) as a function of concentration:

$$\%RSD_R = 2^{1-0.5\log_{10} C}$$

where C is the concentration expressed as a dimensionless fraction. As a guide the estimated reproducibility should not be greater than two times the %RSD_R.
For sample 0147 we have a mean concentration of 0.075g/100g, therefore C=0.075/100=7.5*10-4, giving an Horwitz %RSD_R estimate of 5.91%. For sample

[2] Expanded uncertainty = 'Overall Mean' +/- (t * RSU)
[3] t(0.975;8df)=2.306

0151 we have a mean concentration of 0.905g/100g, therefore C= 0.905/100=9.05*10-3, giving an Horwitz $\%RSD_R$ estimate of 4.06%. Both samples have reproducibility estimate less than twice the predicted Horwitz.

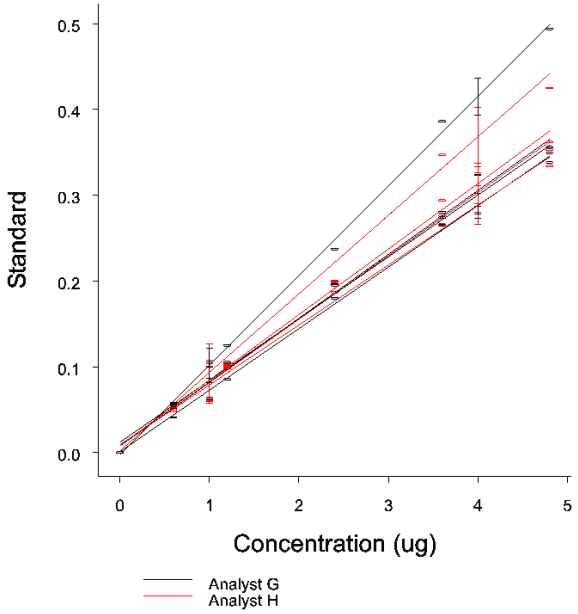
An assessment of bias can also be made by calculating a z-score using the assigned value and a target standard deviation for that round:

$$Z_L - score = \frac{result - assigned}{target_{SD}} = \frac{result - assigned}{rel.uncertainy \times conc}$$

The FEPAS assigned value was 0.075 and 0.827g/100g for each sample respectively. A target standard deviation, $Z_{L,}$ of 0.0082157 and 0.0537394 was used for each sample respectively during the round.
For sample 0147 we have a z-score of 0 and for sample 0151 we have a z-score of 1.45. Each of these are within +/- 2 suggesting there is no significant bias.

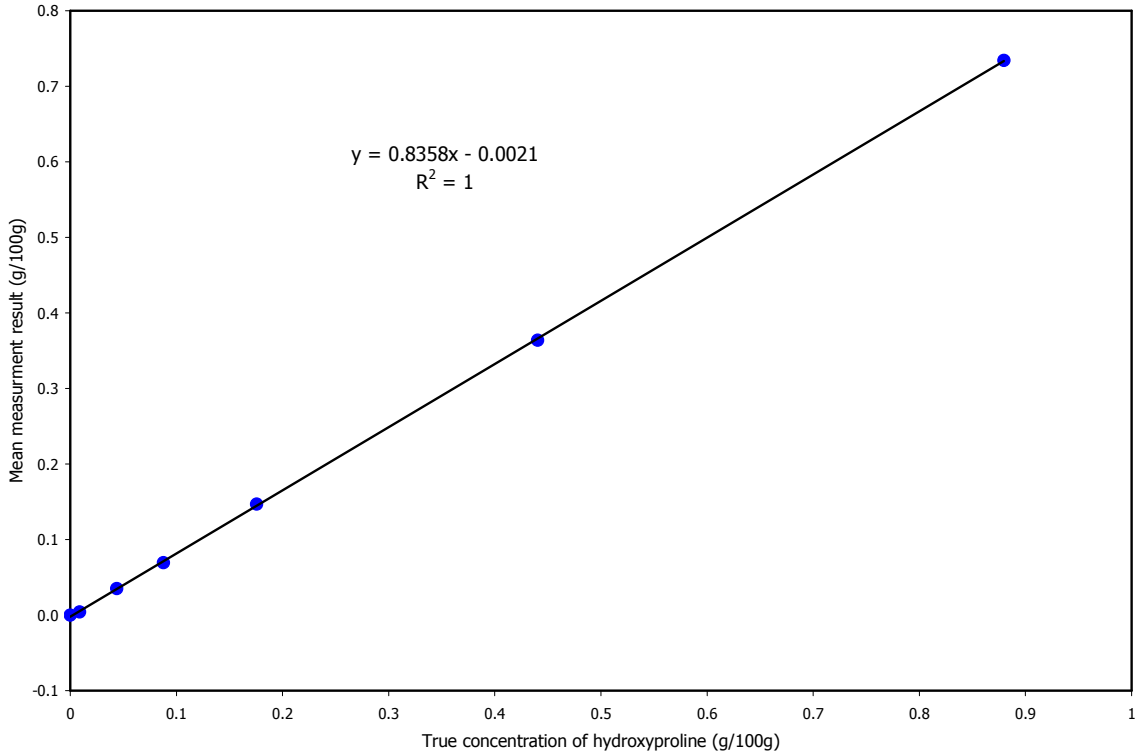
Figure A below shows the fitted standard curves for each of the 8 calibrations by analyst. The regression shows that there are two calibrations that have significantly larger slopes than the others (calibration one for Analyst H, four for Analyst G) and which are also significantly different from each other, $p<0.001$. Prediction intervals at 1 and 4ug have also been plotted for each individual calibration. This gives an indication as to the overlap of predicted results when using the different calibrations.

Figure A. Standard curves by analyst, with 95% prediction intervals at 1 and 4 g/100g



10.3 APPENDIX 3: Assessment of method for the measurement of hydroxyproline

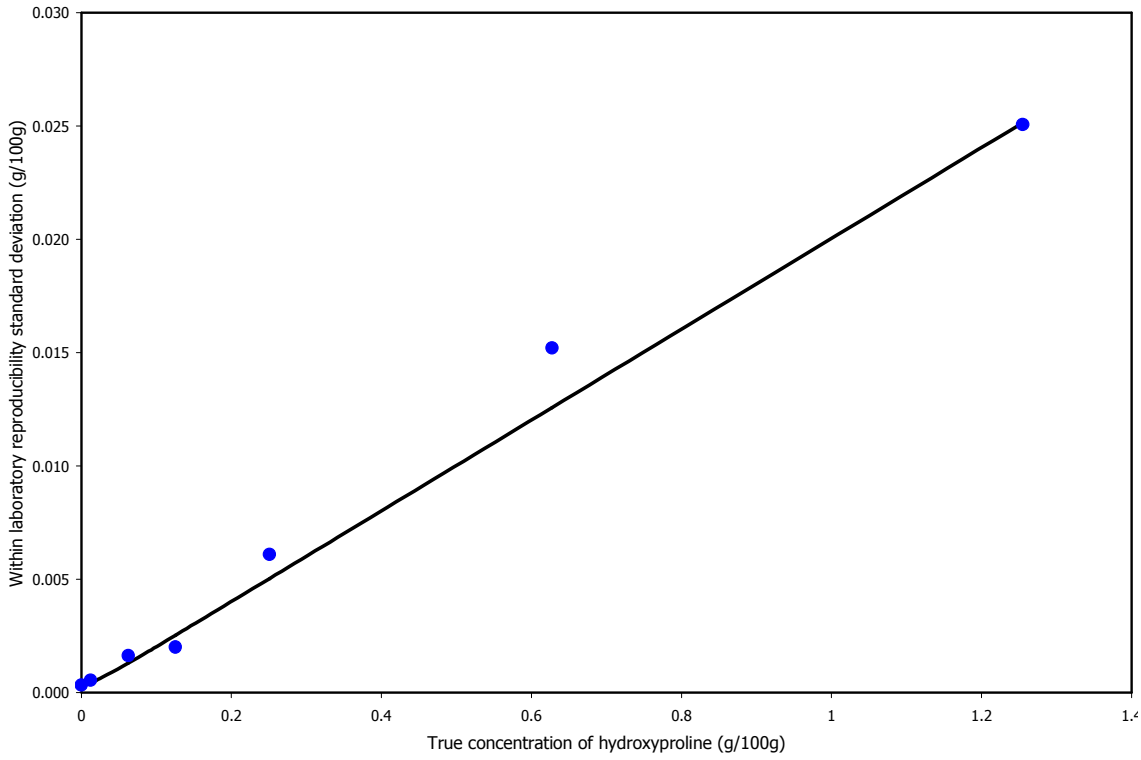
The bias associated with measurement results was assessed by a linear regression of mean measurement result against the concentration of hydroxyproline (as gelatine containing 12.55% hydroxyproline added to each sample).



Proportional bias= 0.8358 with standard uncertainty 0.0025
Fixed bias = -0.00215 with standard uncertainty 0.00096

Measurement variability
The size of within day and between day variation was estimated by Anova (Appendix 1) of duplicate measurements on each of three days at 7 concentrations. Table 1 gives estimates of measurements repeatability standard deviation and within-laboratory reproducibility.

% Gelatine	Hydroxyproline g/100g	Mean result	Sr	Sb (between day)	Sw (within-lab reproducibility)
0	0	-0.00021	0.000335	0	0.000335
0.07	0.008785	0.00413	0.000101	0.000543	0.000552
0.35	0.043925	0.03512	0.001282	0.001001	0.001627
0.7	0.08785	0.06912	0.002011	0	0.002011
1.4	0.1757	0.14677	0.0056	0.002421	0.006101
3.51	0.440505	0.36374	0.008589	0.012547	0.015205
7.01	0.879755	0.73406	0.020896	0.013842	0.025065



The relation between true concentration of hydroxyproline (C) and within-laboratory reproducibility standard deviation S was estimated as:

$$S = \sqrt{0.00034^2 + 0.020^2 \times C^2}$$

Hence given a measurement result R g/100g, the best estimate of the concentration of hydroxyproline in a sample E is given by:

$$E = \frac{R + 0.00215}{0.8358}$$

The standard uncertainty associated with the estimated concentration was estimated by combining the relation between concentration and within laboratory reproducibility with the estimates of uncertainty associated with bias, giving:

$$u(E) = \frac{\sqrt{0.0010^2 + 0.020^2 \times R^2}}{0.8358}$$

The fitness for purpose of the method was assessed by comparing the size of the estimated standard uncertainty to the equivalent Horwitz standard deviation[6]. HORRAT ratios of less than 2 were observed for all concentrations examined in this study. Hence, this initial assessment of the method suggests that results are fit for purpose when corrected for the bias associated with the method:

Hydroxyproline (g/100g)	u(E)	Horwitz standard deviation	HORRAT
0	0.001001	NA	NA
0.008785	0.001013	0.0007	1.412888
0.043925	0.001414	0.0028	0.502784
0.08785	0.002282	0.0051	0.450253
0.1757	0.004272	0.0091	0.46784
0.440505	0.010537	0.0199	0.5286
0.879755	0.021025	0.0359	0.586048

Calculations: Results of analysis of variance

Nested ANOVA: c=0, c=0.07, c=0.35, c=0.7, c=1.4, c=3.51, c=7.01

Nested ANOVA: c=0 versus Day

Analysis of Variance for c=0

Source	DF	SS	MS	F	P
Day	2	0.0279	0.0140	0.124	0.888
Error	3	0.3372	0.1124		
Total	5	0.3651			

Variance Components

Source	Var Comp.	% of Total	Std.Dev.
Day	-0.049*	0.00	0.000
Error	0.112	100.00	0.335
Total	0.112		0.335

* Value is negative, and is estimated by zero.

Expected Mean Squares

1	Day	1.00(2) + 2.00(1)
2	Error	1.00(2)

Nested ANOVA: c=0.07 versus Day

Analysis of Variance for c=0.07

Source	DF	SS	MS	F	P
Day	2	1.1996	0.5998	58.871	0.004
Error	3	0.0306	0.0102		
Total	5	1.2302			

Variance Components

Source	Var Comp.	% of Total	Std. Dev.
Day	0.295	96.66	0.543
Error	0.010	3.34	0.101
Total	0.305		0.552

Expected Mean Squares

1 Day 1.00(2) + 2.00(1)
2 Error 1.00(2)

Nested ANOVA: c=0.35 versus Day

Analysis of Variance for c=0.35

Source	DF	SS	MS	F	P
Day	2	7.2937	3.6468	2.218	0.256
Error	3	4.9318	1.6439		
Total	5	12.2255			

Variance Components

Source	Var Comp.	% of Total	Sd.t Dev.
Day	1.001	37.86	1.001
Error	1.644	62.14	1.282
Total	2.645		1.626

Expected Mean Squares

1 Day 1.00(2) + 2.00(1)
2 Error 1.00(2)

Nested ANOVA: c=0.7 versus Day

Analysis of Variance for c=0.7

Source	DF	SS	MS	F	P
Day	2	3.6982	1.8491	0.457	0.671
Error	3	12.1313	4.0438		
Total	5	15.8295			

Variance Components

Source	Var Comp.	% of Total	Std. Dev.
Day	-1.097*	0.00	0.000
Error	4.044	100.00	2.011
Total	4.044		2.011

* Value is negative, and is estimated by zero.

Expected Mean Squares

1 Day 1.00(2) + 2.00(1)
2 Error 1.00(2)

Nested ANOVA: c=1.4 versus Day

Analysis of Variance for c=1.4

Source	DF	SS	MS	F	P
Day	2	86.1804	43.0902	1.374	0.377
Error	3	94.0961	31.3654		
Total	5	180.2765			

Variance Components

Source	Var Comp.	% of Total	Std. Dev.
Day	5.862	15.75	2.421
Error	31.365	84.25	5.600
Total	37.228		6.101

Expected Mean Squares

1 Day 1.00(2) + 2.00(1)
2 Error 1.00(2)

Nested ANOVA: c=3.51 versus Day

Analysis of Variance for c=3.51

Source	DF	SS	MS	F	P
Day	2	777.2367	388.6183	5.267	0.104
Error	3	221.3334	73.7778		
Total	5	998.5701			

Variance Components

Source	Var Comp.	% of Total	Std. Dev.
Day	157.420	68.09	12.547
Error	73.778	31.91	8.589
Total	231.198		15.205

Expected Mean Squares

1 Day 1.00(2) + 2.00(1)
2 Error 1.00(2)

Nested ANOVA: c=7.01 versus Day

Analysis of Variance for c=7.01

Source	DF	SS	MS	F	P
Day	2	1639.7188	819.8594	1.878	0.296
Error	3	1309.9857	436.6619		
Total	5	2949.7044			

Variance Components

Source	Var Comp.	% of Total	Std. Dev.
Day	191.599	30.50	13.842
Error	436.662	69.50	20.896
Total	628.261		25.065

Expected Mean Squares

1 Day 1.00(2) + 2.00(1)
2 Error 1.00(2)

10.4 APPENDIX 4: Determination of the limit of detection

In order to determine and compare the limit of detection (LOD) of both the AOAC method and of the developed method, laboratory grade porcine gelatine was spiked at a level of 0.2% gelatine into 500 g of cooked basmati rice which had been homogenized in a household blender at 50ºC by adding a gelatine solution (heated to 60ºC) with constant mixing.

Seven 4 g aliquots of this rice mixture were extracted and analysed according to AOAC Method 990.26 for the determination of hydroxyproline. Similarly, seven aliquots were extracted and analysed according to the SOP of the method developed during this project (Appendix 1).

The absorbance measurements of the samples were submitted for statistical analysis and the results for both analyses are tabulated below. The limit of detection for the AOAC method, taking two standard deviations of the mean, was determined as 0.031g of hydroxyproline (H) per 100 g of sample with a false negative probability of 2.5%. (Appendix 4 Table A). This is equivalent to a limit of detection of 0.250% gelatine. The critical level of the absorbance of the standard curve, taking one standard deviation of the mean, was determined as an absorbance of 0.204 with a probability of a false positive of 2.5%.

The LOD for samples extracted by the method developed during the current project was determined as 0.024g H per 100 g of sample with a false negative probability of 2.5% (Appendix 4 Table B). This is equivalent to a limit of detection of 0.192 % gelatine. The critical level of the absorbance of the standard curve, taking one standard deviation of the mean, was determined as an absorbance of 0.158 with a probability of a false positive of 2.5%.

Therefore, these results show the new developed method to be more sensitive than the AOAC method for hydroxyproline, and thus gelatine, determination.

Appendix 4 Table A showing Limit of Detection data for the AOAC Method 990.26

Absorbance for each sample	Mean Absorbance	Std. Dev. of Absorbance	2 std. dev. of Absorbance plus mean (critical level)	LOD of Absorbance	LOD of Hydroxyproline in 2 ml	LOD of Hydroxyproline, g/100g	Equivalent % gelatine (Hydroxyproline, g/100g x 8)
0.174	0.1753	0.0144	0.2040	0.2327	2.4948	0.0312	0.2495
0.178							
0.184			FP=2.5%			FN=2.5%	
0.186							
0.179							
0.144							
0.182							

Appendix 4 Table B showing Limit of Detection data for the method developed during this project.

Absorbance for each sample	Mean Absorbance	Std. dev. of Absorbance	2 Std. Dev. of Absorbance plus mean (critical level)	LOD of Absorbance	LOD of Hydroxyproline in 2 ml	LOD Hydroxyproline, g/100g	Equivalent % gelatine (Hydroxyproline, g/100g x 8)
0.112	0.1207	0.0185	0.1577	0.1947	1.9197	0.0240	0.1920
0.113							
0.099			FP=2.5%			FN=2.5%	
0.122							
0.113							
0.157							
0.129							

FP = False positive
FN = False negative