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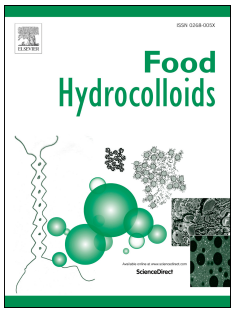

Novel “gel demineralizing” method for protein recovery from fat rendering waste stream based on its gelling properties

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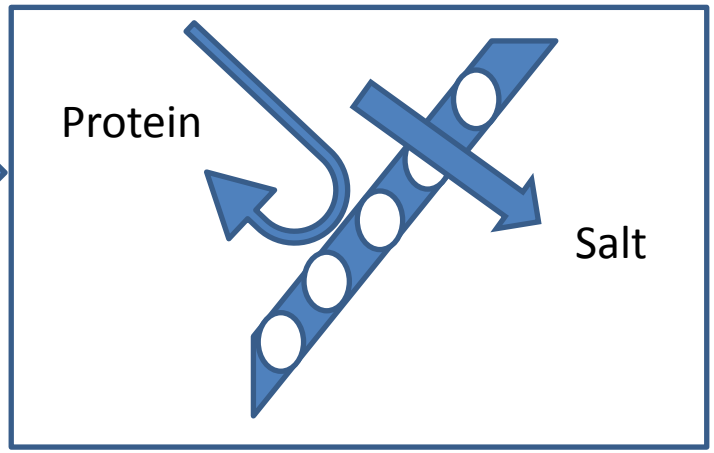
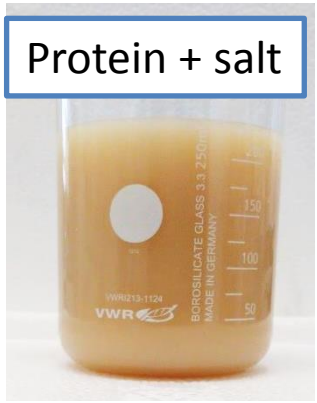
Membrane filtration

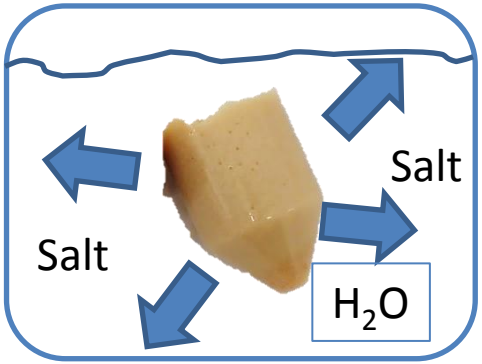


Protein + salt



Desalting by gel submersion

Gel



Protein recovered

Novel "gel demineralizing" method for protein recovery from fat rendering waste stream based on its gelling properties.

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Abstract

Fat rendering is a common process in the meat industry, whereby fatty or oily materials are melted away or cooked from the solid portion of the animal tissue. Once the fat, and more solid protein in the form of greaves, has been removed a co-product called glue water or stick water is produced which in generally considered a waste product. This study was established to investigate ways to revalorise this product and reduce the economic and environmental impact of this waste material. Proximate characterisation shows it contains 1.1-1.3% w/w of protein along with similar concentration of ashes (1.3% w/w). While low in protein this is a key pollutant if the product is disposed of, and could also represent an interesting protein source for downstream applications. In order to recover these proteins the salt has to be removed. Therefore, after the techno-functional properties of the raw material and of the recovered proteins were evaluated, especially those related to gelling formation, a new demineralizing method based on the excellent gelling properties of these proteins was developed and results compared with those obtained from three different ultrafiltration membranes (10, 3 and 1 kDa MWCO). Protein recovery was greater for the new method (79

to 90%) (50 to 77%); however, the amount of salt removed was higher when ultrafiltration was employed (90% compared to 81%).

Keywords: functional properties; protein; gelation; demineralizing; recovery; waste-streams

1. Introduction

According to the report of the Food and Agriculture Organization, waste generation is one of the main issues facing the food industry today (Gustavsson, Cederberg, Sonesson, Van Otterdijk, & Meybeck, 2011). One strategy aimed at reducing the impact of waste on the environment is to recover valuable biomolecules such as proteins, fatty acids, minerals or carbohydrates present in these streams (Mullen, Álvarez, Pojić, Hadnadev, & Papageorgiou, 2015; Otles, Despoudi, Bucatariu, & Kartal, 2015). In particular, protein recovery has targeted a number of secondary product streams, for example hair (Coward-Kelly, Agbogbo, & Holtzapple, 2006; Esteban, Garcia, Ramos, & Marquez, 2010), feather (Kota, Shaik, Kota, & Karlapudi, 2014; Lasekan, Abu Bakar, & Hashim, 2013), blood (D. Parés, Saguer, & Carretero, 2011) or different kinds of offal (Darine, Christophe, & Gholamreza, 2010; Dewitt, Gomez, & James, 2002; Lynch, Álvarez, O'Neill, Keenan, & Mullen, 2017).

Protein recovery steps need to be tailored taking cognisance of the composition and characteristics of the raw material: liquid or solid tissue, protein concentration, protein properties, protein stability or presence of undesired compounds (Galanakis, 2015). High amounts of minerals, frequently found in food wastes, have to be removed in order to obtain high protein concentrates (Gringer, et al., 2015). Membrane filtration, electro-dialysis, dialysis, diafiltration, reverse osmosis or forward osmosis are the preferential approaches for protein demineralizing (Dolors Parés, Toldrà, Saguer, & Carretero, 2014; Simon, Vandanjon, Levesque, & Bourseau, 2002; Xue, Yamamoto, & Tobino, 2016; Zhou, Baker, Grimsley, & Husson, 2016). However, all these methods present key disadvantages associated with membrane filtration, such as membrane fouling and large amounts of water required. Gel formation, typical of protein solutions, cause accumulation of molecules at the film layer of the membrane, leading to a loss of efficacy. Pre-treatment of the feed-stock, cleaning process and ultimately membrane replacement, incur significant economical investment (Arnal,

García-Fayos, & Sancho, 2011). For this reason, a need for greener, more economical novel demineralizing technologies still exists.

Rendering is a process which uses heat/pressure treatment to convert many animal co- and by-products into higher value edible or inedible products (Prokop, 1985). Many slaughter plants have integrated rendering operations which takes co-products from the slaughter hall. After processing such raw materials edible tallow or lard is obtained along with the solid protein product called greaves, with the concomitant generation of a product called sticky water or glue water; depending if bones or tissues are rendered; which represents a waste product for the industry, with the associated disposal cost. The three fractions abovementioned, keep the status of edible and can be used as food ingredient if processed properly under hygienically conditions.

In this study we have chosen to explore the recovery of protein from this waste stream and to investigate the characteristics of the recovered protein. Despite its low (≈1.1-1.3% w/v) protein content, given the quantities of raw material rendered, 72,000 tonnes of tallow was estimated to be produced in 2010 in the eight rendering plants in the Republic of Ireland, (A Resource Study on Recovered Vegetable Oil and Animal Fats, 2003); the amount of water consumed (0.5-1 m^3/ton) and the difficulties in disposing the product when not used for land-filling (Sustainable Practices in Irish Beef Processing, 2009), it is worthwhile to both examine recovery methods and assess protein functionality. In a time of increasing global protein demand and pressure for more sustainable processing it is important to examine this waste stream as a potential source of valuable functional protein. Therefore, the specific objectives of this work included recovery and characterisation of the protein fraction from glue water obtained from an integrated fat rendering system; design of a simple demineralizing method; and comparison of this demineralizing method to a diafiltration process.

2. Material and methods

2.1 Sample preparation

Fresh glue water was supplied on three occasions from an EU approved commercial abattoir. On each occasion 15 litres were collected and kept chilled at 4 °C at Teagasc Food Research Centre (Dublin, Ireland). Each batch was homogenised using a blender (Robot Coupe, R 201, Ultra) until a homogenous liquid was obtained. From this, the sample was divided into 2 litres aliquots and freeze dried (FD 80 model, Cuddon Engineering, Marlborough, New Zealand), until further use.

2.2 Proximate analysis

Protein content was analysed according to AOAC method 992.15 (1990), using a LECO FP628 protein analyser (LECO Corp, Michigan, USA), calibrated with EDTA. Raw data from each sample was adjusted using the protein correction factor of 6.25. Each sample was analysed in duplicate.

Moisture and mineral content was measured by placing 2 g of the sample in a ceramic furnace at 105 °C for 24 hours. Resulting dried products were placed at 600 °C for another 24 hours. Weight of dried material and minerals were recorded. The analysis was done in triplicate.

2.3 Amino acid profile

Proteins were hydrolysed for 23 h using 6 M HCl at 110 °C for total amino acid profile (TAA). For free amino acid profile (FAA) the hydrolysis step was not carried out. The amino acid profile of the resulting hydrolysates was determined as described by (Hill, 1965). Briefly, extraction of protein was carried out by mixing (1:1) 24% (w/v) trichloroacetic acid (TCA) with each sample. Mixtures were allowed to stand for 10 min followed by centrifugation at 14,400 g (Micro Centaur, MSE, UK) for 10 min. Samples were diluted with

the internal standard, norleucine (C6H13NO2), to a final concentration of 125 nmol/ ml. Amino acids were quantified using a JEOL JLC-500/V AminoTac™ amino acid analyser (JEOL Ltd., Herts, UK) combined with a JOEL Na+ high performance cation exchange column.

2.4 Molecular weight and protein profile analysis

SDS-PAGE gels were performed following Laemmli method (Laemmli, 1970). Freeze dried protein was diluted in a total volume of 2 mL of ultrapure water to a final protein concentration of 2 g/L. Laemmli buffer was then added to the samples and boiled for 10 minutes at 95 ºC in the presence of β-mercaeptanol (except for those when non-reducing conditions were employed). Pre-casted gels of 4-20% gradient from Bio-Rad were employed and a volume of 20 µL of sample was loaded. Finally, Coomassie staining was carried out. Bio-Rad broad range marker or Precision Plus Protein™ Dual Xtra Prestained Protein Standards marker was employed as molecular weight marker.

Size exclusion analysis was carried out in a Waters Alliance 2795 Chromatography Separations Module (Waters Corp., Milford, USA) coupled to a Waters 2996 PDA detector at a wavelength of 280 nm as reported in Ojha, Alvarez, Kumar, O'Donnell, and Tiwari (2016).

2.5 Fat content

Fatty acids were extracted by mixing 10 mL of the sample with 10 mL of hexane. The mixture was stirred for 1 hour; after a centrifugation step (2000g for 10 minutes) a clear supernatant was collected. This step was repeated three times. The hexane from the three extractions was pooled then evaporated under nitrogen, following which the weight of remaining fat was measured.

2.6 Functional properties

Functional properties were measured in both freeze-dried glue water and in the demineralized extract after freeze drying. Both powders were homogenised prior to analysis.

Solubility was determined by the method reported by De Vouno, Penteado, Lajolo Franco, and Pereira Dos Santos (1975) where 0.5 g of the protein sample was first dissolved in 10 mL of distilled water. The solution was centrifuged at 2400g for 30 min in a Lynx 6000 centrifuge (Thermo Scientific, Germany). The pH was varied between 3 and 8, using either 1 mol/L NaOH or HCl solutions, to test its effect on solubility. Protein concentration before and after centrifugation was measured using a wavelength of 280 nm (NanoDrop 1000 Spectrophotometer V3.7, Thermo Scientific, Germany). The percentage of protein detected in the supernatant relative to the total protein was calculated as solubility.

Water holding capacity (WHC) and oil holding capacity (OHC) were measured following M. J. Y. Lin, Humbert, and Sosulski (1974) method. Emulsifying ability was measured following the method reported by Inklaar and Fortuin (1969). Emulsions for this study were formed in a homogeniser (Omni Prep Multi-Sample Homogenizer) set at 10.000 rpm for two minutes. Amount of oil released was monitored for 120 hours.

Least gel concentration (LGC) was determined following the method reported by Coffmann and Garciaj (1977). Gelation temperature (Tg), melting temperature (Tm), elastic modulus (G') and viscous modulus (G''), were measured following the method reported by Lamsal, Jung, and Johnson (2007) with some modifications. Freeze dried samples were diluted to final concentrations from 6 to 16% (w/v) and then submerged in a water bath at 45 °C to prevent gel formation. While the sample was still in a liquid state the plates were moved to a distance of 1 mm apart. Immediately the rheometer temperature was set to 5 °C for 15 minutes to generate a gel. A ramp temperature from 5 to 45 °C to determine the melting temperature was performed; followed by a ramp from 45 to 5 °C to determine Tgel. Ramp temperature was 1.2 °C/min; the deformation (γ) was adjusted to 1% and the oscillation frequency was 1 rad/s. It was considered that the gel was formed or melted when G' and G'' crossed their values or alternatively when G' reached a value of 1Pa.

176 **2.7 Demineralizing by diafiltration.**

177 Fresh glue water was diluted to obtain a final protein concentration of 1 g/100 mL.
178 Aliquots of 10 mL of this sample were poured into centrifuge filter units equipped with three
179 different MWCO (molecular weight cut off): 10kDa, 3 kDa (Amicon Ultra, Millipore) and 1
180 kDa (Macrosep, Pall Life Sciences Ireland, Cork, Ireland). After that the samples were
181 centrifuged at 4000 g. for 3 hours (Lynx 6000, Thermo Scientific). The volume of liquid lost
182 in permeate was replaced by distilled water to keep the sample volume constant at the end of
183 each centrifugation step. After three centrifugations steps the volume of the retentate was
184 adjusted to 10 mL, and permeate was concentrated under a nitrogen stream until a final
185 volume of 10 mL. Finally, protein and mineral contents, and SDS-PAGE profile of both
186 permeate and retentate were determined.

187 **2.8 Gel demineralizing process**

188 Gels from 8 to 16% w/v were created by dissolving the freeze dried glue water in
189 distilled water then chilling at 5 °C. Samples of 15 grams were sliced and submerged in 150
190 mL of demineralizing buffer (0.1% of citric acid). A calibrated conductivity meter (Hanna
191 Edge EC, HI 2003) with temperature compensation was used to measure and monitor the
192 conductivity (mS/cm) of the buffer solution at 5 ºC; water changes were carried out when
193 conductivity remained constant for 30 minutes. Three water changes were carried out.
194 Finally, the demineralized gel was patted dry with absorbent paper and its proximate
195 composition (protein and mineral contents) and SDS-PAGE profile determined.

196

197 **2.9 Statistical analysis**

198 All experiments were carried out in duplicate and all samples were analysed by duplicate as
199 well. In order to see differences between the groups an ANOVA test was conducted followed
200 by a Duncan test to identify the treatments statistically different. Values of $p<0.05$ were

considered statistically significant. The Software employed was SPSS version 17.0.

3. Results and discussion

3.1 Waste stream characterisation.

Proximate composition of the samples showed that this waste stream is mainly composed of water with small amounts of proteins (1.14-1.34%) and minerals (1.18-1.22%) (Table 1). This protein content is quite low when compared with other meat co-products or processing streams such as blood (18%) (Moure, Rendueles, & Díaz, 2003), offal (from 10% up to 22%) (A. M. Mullen & Álvarez, 2016); or with brine solutions from fish processing (7.5%) (Gringer, et al., 2015). However, it does represent a significant pollutant and also holds potential for recovery of additional protein. After freeze drying, the obtained powder had a moisture content in the range of 8-11%, and high protein content of 30 to 40% along with a high mineral content (40-45%). Such high salt content is due to the addition of salt during the rendering process, to increase the density difference between fat and water, thus improving the separation process by centrifugation. The amino acid profile (Table 2), showed that the content of collagen related amino acids such as proline (12.12%), alanine (10.65%) or glycine (15.42%) were clearly higher than other amino acids. It has been reported that in mammal connective tissues, the percentage of proline is 12%, alanine is 11% and 33% for glycine (Szpak, 2011). Taking into account that the main raw material employed for rendering is bovine connective tissues, composed mainly by collagen, it is reasonable to conclude that collagen and collagen fragments are the main proteins present in this co-product. The non-reducing SDS-PAGE profile of raw material (Figure 1) was carried out and it was possible to identify both α-chains (120 kDa) and both β-chains (≈240 kDa) from collagen, same profile has been previously reported (Y. K. Lin & Liu, 2006). Additionally, other proteins and collagen fragments, with a molecular weight ranging from 75 kDa to 10 kDa could be

observed as well. Such fragments are originated because of the hydrolysis process promoted by the combination of time and high temperature in the rendering process; process which transformed the native collagen into gelatine. Further analysis employing SEC-HPLC, revealed that around a 10-15% of total signal observed at 280 nm was composed by peptides smaller than 5 kDa; peptides were found to be present from 0.5 kDa.

Total free amino acids (TFAA) analysis indicated 23.34±3.2% of total protein content was composed by free amino acids (FAA). This fact, together the relative high content in small peptides, is an indication of protein hydrolysis due to the aggressive temperature, pressure and alkalinity conditions employed during the fat rendering process.

According to the SDS-PAGE, the molecular weight protein of protein/peptides ranged from 3 kDa up to 100 kDa; with most within the range from 20 to 65 kDa. SDS profiles of purified bovine collagen show three main bands which correspond to collagen subunits of 114 and 130 kDa molecular weight (Savvateeva, et al., 2015). The absence of such larger proteins supports the fact that proteins were hydrolysed in the rendering process. Besides, this protein size distribution match those described for gelatin extracted under intense conditions of pH, temperature and time (Johnston-Banks, 1990) in order to increase yield extraction. Regarding fat content, an insignificant amount was detected, with all below 0.1% w/w of the sample, demonstrating the effectiveness of the rendering process for fat recovery. Thus further downstream fat recovery is unnecessary. For this reason, glue water can be considered mainly as a source of proteins, peptides and amino acids.

3.2 Functional properties of raw material

Functional properties results obtained for the freeze dried glue water are presented in Figure 2 and Table 3.

Solubility profile varied remarkably depending on the batch analysed. However, a maximum of solubility was found at pH 3 for all samples. For batch 3, solubility at pH = 8 was found to be as high as for pH =3. It has been reported that gelatine, composed mainly of collagen, is an amphoteric protein with isoelectric point between 5.0 and 9.0, depending on raw material and method of manufacture (Johnston-Banks, 1990), which is in agreement with our results. No major differences in the solubility were observed when the pH was modified within range values of 4 to 7; however, a significant increase in solubility at pH 3 was observed within each one of the batches. Additionally in Batch 3, a remarkable higher solubility was found at pH 8. The WHC (ranging from 12.10 to 14.55 g of water/ g protein) of glue water powder was four times higher to those reported for pepsin extracted bovine gelatine, in the same way, the OHC was twice higher than the collagen extracted by pepsin. (Lassoued, et al., 2014). This might be because enzymatic hydrolysates are composed of significantly shorter peptides than the protein fragments found in the glue water. Finally, it was found that WHC and OHC of protein from glue water were slightly higher, when compared to commercial porcine skin gelatin, which yielded values of 7.20 g/g and 1.05 g/g respectively (Coorey, Tjoe, & Jayasena, 2014)

Regarding emulsifying capacity, proteins from glue water performed significantly better than proteins recovered from other co-products such as blood (Álvarez, Bances, Rendueles, & Díaz, 2009). A concentration of 0.8% w/v of dried glue water (0.3% of protein) was able to emulsify more than 95% of oil; while 1% of dried glue water (0.38% of protein) was able to emulsify 100% of added oil. Besides, the emulsion created was stable for 120 hours at room temperature, when both 0.8% and 1% concentrations were employed. The stability of the emulsions formed with less than 0.8% of protein was poor; in the first 24 hours more than 50% of the emulsified oil was released. No significant difference ($p<0.05$) in the emulsifying ability or stability was found in the three batches assayed, in the range of

concentrations studied. This high emulsifying capacity is typical of type-A gelatins, which have a relatively high isoelectric point (pH ≥ 7-9) (Gómez-Guillén, Giménez, López-Caballero, & Montero, 2011)

Finally, least gel concentration (LGC) for gel generation ranged from 2.25 to 2.75% protein. These values are in the same range of serum albumin, frequently used by the food industry as gelling agent (Polo, Rodríguez, Ródenas, Morera, & Saborido, 2009). As reviewed by Gómez-Guillén, et al. (2011), the gelling mechanism for collagen and gelatin is completely different, although both of them are thermo-reversible. Gelatin gelation is based on the coil-to-helix transition, triggered by lowering solution temperature (usually lower than 30 ºC). Besides, collagen gels melt by decreasing the temperature, while gelatin gels melt by temperature increasing. The fact that proteins extracted from glue water showed thermo-reversibility and that generated gel melted with increasing temperature supports the assumption that the sample was composed mainly of gelatine-type proteins.

Four different rheological parameters (G', G'', Tg and Tm) were also characterised at different protein concentrations. It was observed that higher protein concentrations lead to higher G' and G'', which means stronger gels were formed. The temperature required for gel formation and gel melting also increased. For instance, a gel composed by 2% of protein shows a G' of 7.1±0.2 Pa, G'' of 0.55±0.3 Pa, Tg of 5 °C and Tm of 25.03 °C, while a gel composed by 5.7% of protein showed values of 626.0±5.2 Pa (G'), 18.9±0.4 Pa (G''), 24.7 °C (Tg) and 30.9 °C (Tm).

3.3 Demineralization by diafiltration

In order to remove the excess of minerals, to obtain a final product with higher protein content, a demineralization step is required. Multiple approaches are possible; however, membrane filtration is commonly used in food industry with, despite issues with fouling,

300 excellent results for protein concentration and salt removal (Gringer, et al., 2015; Undeland,
301 Kelleher, & Hultin, 2002). Prior to filtration the protein content was adjusted to 1 g/100 mL,
302 by dissolving the freeze dried samples in ultra-pure water, in order to standardize all batches.
303 The SDS profile of all retentate and permeates can be observed in Figure 3 after diafiltration
304 with 3 kDa MWCO membranes. At the end of the process, the protein content in supernatant
305 was 0.50±0.03; 0.65±0.04 and 0.77±0.03 g/100 mL after using 10, 3 and 1 kDa MWCO
306 respectively. Protein content in permeate was 0.43±0.01; 0.29±0.06 and 0.16±0.02 g/100 mL
307 respectively. As expected, when 10 kDa MWCO was employed, higher protein loss was
308 observed, nearly 50% of initial protein content. On the other hand, when 1 kDa MWCO was
309 employed, nearly 80% of the protein content was retained.

310 It was observed that only a 26.76±2.15% of TFAA were present in permeate when 1
311 kDa MWCO membrane was employed. When using 10 and 3 kDa membranes 36.98±3.56%
312 and 27.11±2.85% of TFAA of the free amino acids permeated. It has been reported that
313 charged free amino acids cannot permeate through organic ultrafiltration membranes, and this
314 is highly dependent on the pH (Tsuru, Shutou, Nakao, & Kimura, 1994). In this experiment
315 the original pH of the glue water was 7 and some amino acids can be charged at this pH. For
316 example, it was found that more than 95% of glycine was detected in the retentate (pKa: 2.34
317 and 9.60); while more than 75% of histidine (pK_a lateral chain is 6.10) permeated, regardless
318 of the MWCO employed. It was found that FAA composed 18.7% (10 kDa), 22.9% (3 kDa)
319 and 42.9% (1 kDa) of total protein loss; meaning that some other small peptides can permeate
320 through these membranes. It is likely that small peptides, >2-3 kDa, could account for some
321 of the loss. Such peptides cannot be detected following the SDS-PAGE protocol used and for
322 this reason there were no obvious differences in the protein size profile across the UF
323 membrane sizes (Figure 3).

Finally, mineral content was determined in the final supernatants obtained and no significant differences ($p<0.05$) were observed between the different membranes employed. A reduction of 90% in ash content was achieved.

3.4 Demineralization by gel soaking

Based on observations from preliminary experiments, namely the ability of glue water to form a gel and the gel stability when immersed in water, a new demineralizing method was developed and assessed against the UF method performance. The powder employed to generate the gels contained 35.5% protein and 60% of non-protein solids. A mass balance regarding the protein and total solids in the gel before and after demineralizing was carried out. Results are presented in Table 4.

The conductivity of the demineralizing buffer, parameter that is directly related with the amount of minerals diluted in a solution (Rosborg, Nihlgård, & Ferrante, 2015), was monitored and the curve showed the typical saturation curve, reaching a stable plateau after 4 hours of processing. At this time the equilibrium between mineral content in gel and demineralizing buffer was reached and the salt diffusion was stopped. Final conductivity of demineralizing buffer, when the two first buffer changes were completed, was proportional to the total solids employed to generate the gel. The conductivity differences were lower in the second buffer change. Finally, the conductivity in the third change was not significantly different regardless the initial solid content of the gels employed.

Within the range of gel concentration studied, the percentage of minerals removed ranged from 81.72 to 84.49%; however no significant differences were found when different gel solid concentrations were employed at $p<0.05$.

The final weight of the gel increased remarkably once the demineralizing process was completed. The increase ranged from 118% (8% gel) to 142% (16% gel) of the original

weight. It has been reported how high salt content in gels can prevent the water absorption (Richardson & Jones, 1987), which may explain this observation. Swelling capacity or ability to adsorb liquid while keeping its shape of this kind of hydrogel is becoming more relevant in industries such as agriculture, food-packaging or pharmacy. The high swelling and water binding capacity of collagen and gelatine type products makes them suitable materials for reducing drip loss and impairing juiciness in frozen fish or meat products (Gómez-Guillén, et al., 2011)

Gels ranging from 8% to 12% of total solids showed no statistical differences ($p<0.05$) regarding the percentage of protein recovered neither the dried gel protein concentration achieved. However, for higher concentration of total solids (14% and 16%), both protein recovery and gel protein concentration were significantly lower ($p<0.05$). Ofstad, Kidman, Myklebust, Olsen, and Hermansson (1995) reported how increasing concentration of salt can generate larger pores in a protein matrix. Larger pore sizes in the matrix can enhance the migration of protein to the demineralizing buffer, and may explain why a lower protein recovery was observed in the highest concentrated gels which would have both higher protein and salt.

SDS-PAGE profile of the gel before and after demineralizing is shown in Figure 4. After the demineralizing by gel immersion process, proteins in the range of 15 to 30 kDa were not detected in the SDS-PAGE gel, which could partially explain the protein loss observed. In addition, free amino acids and certain proteins can easily diffuse out of the gel to the demineralizing buffer, as they were not integrated into the gel matrix.

3.5 Diafiltration compared with gel submersion method.

Significantly higher protein recovery (86.56% for 10% solid content gels) was obtained using the new demineralizing method compared to diafiltration (77% when 1 kDa

MWCO membranes were used). However, the amount of minerals removed was higher when UF was employed. It is possible that a better degree of demineralization can be achieved in the new gel immersion method reported here by controlling some important parameters: gel/ buffer ratio, initial solid content or pH of the buffer. Additionally, current experiments were performed in batch mode, but the process could easily be scaled-up and adapted to operate in a continuous mode, with potentially better yields. Furthermore, this method would be more advantageous for protein solutions, which are particularly prone to fouling using membrane systems. UF systems require a high initial investment which combined with membrane fouling (Arnal, et al., 2011) has a big impact on the economics of this process.

The water consumption for both methods was estimated. In the diafiltration process 3.3 mg of protein were demineralized for each mL of water employed. In the gel immersion method values ranged from 6.2mg/mL to 12.5 mg/mL of water when 8% gels and 16% were employed, respectively. However, glue water had to be concentrated to reach total solids content of 10% to form a gel able to be demineralised. It means that 90% of the water has been removed. In order to develop a greener process, this evaporated water could be used for the demineralizing buffer, as gel/buffer ratio employed is 1:10. So while an energy input would be necessary for dewatering potential exists for re-using this recovered water negating the need for additional water use.

3.6 Gelling properties of demineralized proteins

After the gel soaking demineralization process, the rheological parameters of the gels generated using demineralised proteins were characterised and compared with those generated using raw glue water. The main difference between gels was the ratio of protein/minerals: 0.6 in the untreated gels and between 1.7 and 2.7 in the demineralized gel (Table 4). It was also found that the values of G', G'', Tg and Tm increased significantly

399 when the same protein concentration was employed to generate the gels (Figure 5). For
400 example, a protein concentration of 2.8% and protein/minerals ratio of 0.6 generated a gel
401 with the following characteristics: G' = 48 Pa; G'' = 1.44 Pa; Tg = 10.3 °C and Tm = 26.5
402 °C. After demineralization, a gel with the same protein concentration displayed the following
403 properties: G' = 142 Pa; G'' = 5.66 Pa; Tg = 15.71 °C and Tm = 27.2 °C. Higher sodium
404 chloride contents produce weaker gels (Kuhn & Foegeding, 1991; Mulvihill & Kinsella,
405 1988) as salt can compete with protein to form covalent bonds. Gels are stronger when more
406 interactions occur between proteins with the system becoming more complex as the number
407 of protein interactions increases. For the same reason, the higher the protein content
408 employed, the stronger the gel. When the values of G' and G'' are compared, a very strong
409 linear correlation between both parameters was found, in both types of gels (R^2 = 0.997,
410 $p<0.001$, before demineralizing (n=12); and R^2=0.979, $p<0.001$, after demineralizing (n=10)).
411 It means that the system has the same degree of viscoelasticity (Marcet, Paredes, & Díaz,
412 2015). However, after demineralizing, G' is 28 times higher than G'', in the range of protein
413 concentrations studied; while before demineralizing G' was 32 times higher than G''. This
414 shows that the system is still viscoelastic after demineralizing, but is becoming slightly
415 viscous when the salt is removed.
416
417 **4. Conclusions**
418 Glue water is in many cases considered by the meat industry as a waste product from
419 the fat rendering process. This work investigates the potential revalorization of this waste
420 stream by means of a conventional and a novel (gel submersion) demineralising technique.
421 Glue water is mainly composed of fragments of collagen and dissolved minerals. While the
422 protein content is very low the proteins tested demonstrated good gelation properties, forming
423 thermo-reversible gels without an additional heating step. Protein recovery was greater for

gel submersion method (79% to 90%) compared to diafiltration (50% to 77%); however, the amount of salt removed is still higher when diafiltration is employed (90% compared to 81%). Rheological properties of the gel generated after demineralising showed gels that were stronger, with higher transition temperatures. This data demonstrated that a current waste stream holds potential as a source of proteins with excellent gelling properties.

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559 Figure captions:

560 Figure 1: Non reducing (A) and reducing (B) SDS-PAGE electrophoresis of raw material.
561 Molecular weight markers are indicated in the lane on the left.

562 Figure 2: a) Emulsifying capacity and emulsion stability and b) Solubility of freeze dried
563 glue water.

564 Figure 3: Retentate (a) and permeate (b) obtained after 10 kDa UF (2,3); 3 kDa (4,5) and 1
565 kDa (6,7). First and last lane correspond to molecular weight markers: Aprotinin, (6,5 kDa);
566 α-Lactalbumin (14,2 kDa); trypsin inhibitor (20 kDa); trypsinogen (24 kDa); carbonic
567 anhydrase (29 kDa); Glyceraldehyde-3-phosphate dehydrogenase (36 kDa); ovalbumin (45
568 kDa); albumin (66 kDa).

569 Figure 4: SDS-PAGE obtained after (AD) and before (BD) gel demineralization of 14%
570 solid content gel. Proteins within the range of 15 to 30 kDa were solubilized into
571 demineralizing buffer. MW: molecular weight markers employed are the same than those in
572 Figure 3

573 Figure 5: Rheological properties of gels before demineralizing (left side) and after
574 demineralizing (right side). Parameters studied were G' (elastic modulus), G'' (viscous
575 modulus), gelling temperature (Tg) and melting temperature (Tm).

Table 1: Average proximate composition of analysed batches (n = 3).

Parameter	Average value of analysed batches
Moisture (%)	97.40±0.24
Total solids (%)	2.47±0.08
Protein (%)	1.15±0.14
Ash (%)	1.08±0.17
Fat (%)	<0.1
pH	7.06±0.15

Table 2: Average amino acid profile of glue water after freeze drying (n = 3)

Amino acid	% of analysed amino acid	Amino acid	% of analysed amino acid
Cys	0.99±0.01	**Ile**	2.27±0.11
Met	1.44±0.05	**Leu**	5.25±0.28
Asp	7.60±0.22	**Tyr**	1.83±0.08
Thre	2.68±0.05	**Phe**	2.80±0.13
Ser	3.18±0.03	**His**	2.43±0.07
Glu	13.04±0.39	**Lys**	6.62±0.32
Gly	15.42±0.61	**Arg**	5.38±0.22
Ala	10.65±0.08	**Pro**	12.12±0.39
Cys	1.91±0.03	**NH3**	5.55±0.21
Val	4.40±0.02		

Table 3: Average water holding capacity (WHC), oil holding capacity (OHC) and least gel concentration (LGC) in glue water (n=3).

Sample	**WHC** **(g H_2O/ g protein)**	**OHC** **(g oil/ g protein)**	**LGC** **(% protein)**
Glue water	13.24±1.01	4.71±0.52	2.50±0.20

Table 4: Percentage of protein recovered and percentage of minerals removed at different gel solid contents. Results expressed as the average value of two independent experiments. Standard deviation is presented also.

Solid content % (protein %)	**Gel weight difference (%)**	**Final protein content (%)**	**Final solid content (%)**	**Protein recovery (%)**	**Protein concentration in dried gel (%)**	**% of minerals removed**
8% (2.13%)	117.76	1.96±0.09[a]	2.91±0.07[a]	90.32±2.3[a]	67.49±1.3[a]	81.82±0.29[a]
10% (3.55%)	123.61	2.40±0.20[b]	3.06±0.10[b]	86.56±0.31[a]	65.78±2.12[a]	82.48±2.9[a]
12% (4.27%)	131.66	2.55±0.35[b,c]	3.76±0.54[b,c]	85.94±0.11[a]	67.88±0.44[a]	84.49±2.43[a]
14% (4.98%)	144.86	2.45±0.13[b]	4.08±0.27[c]	79.12±1.07[b]	60.35±0.80[b]	82.19±1.54[a]
16% (5.69%)	141.86	2.90±0.01[c]	4.79±0.09[d]	80.35±0.16[b]	60.62±0.87[b]	81.87±0.72[a]

Figure 1

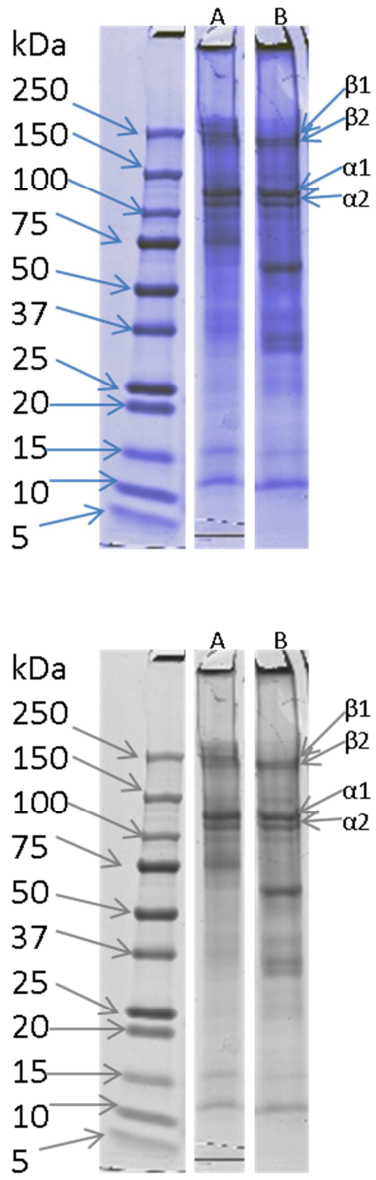


Figure 2

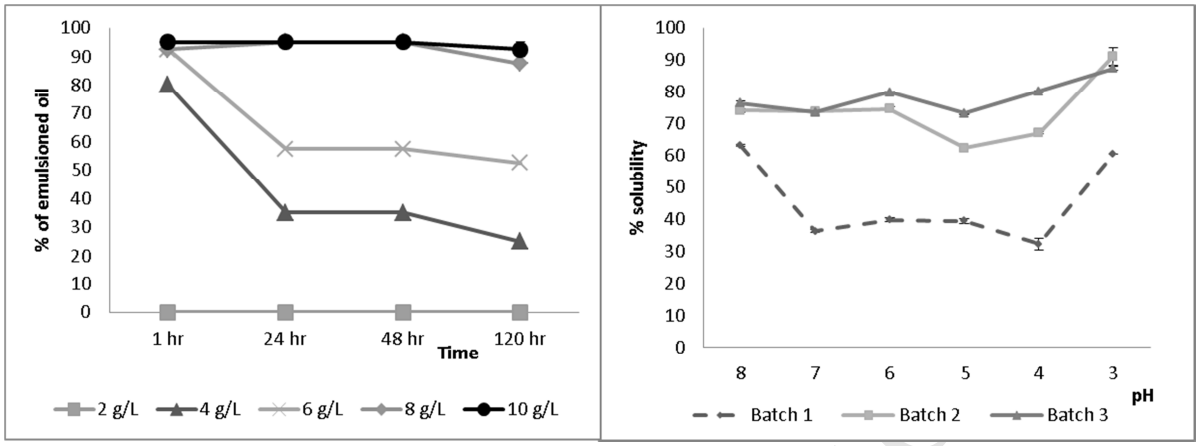


Figure 3

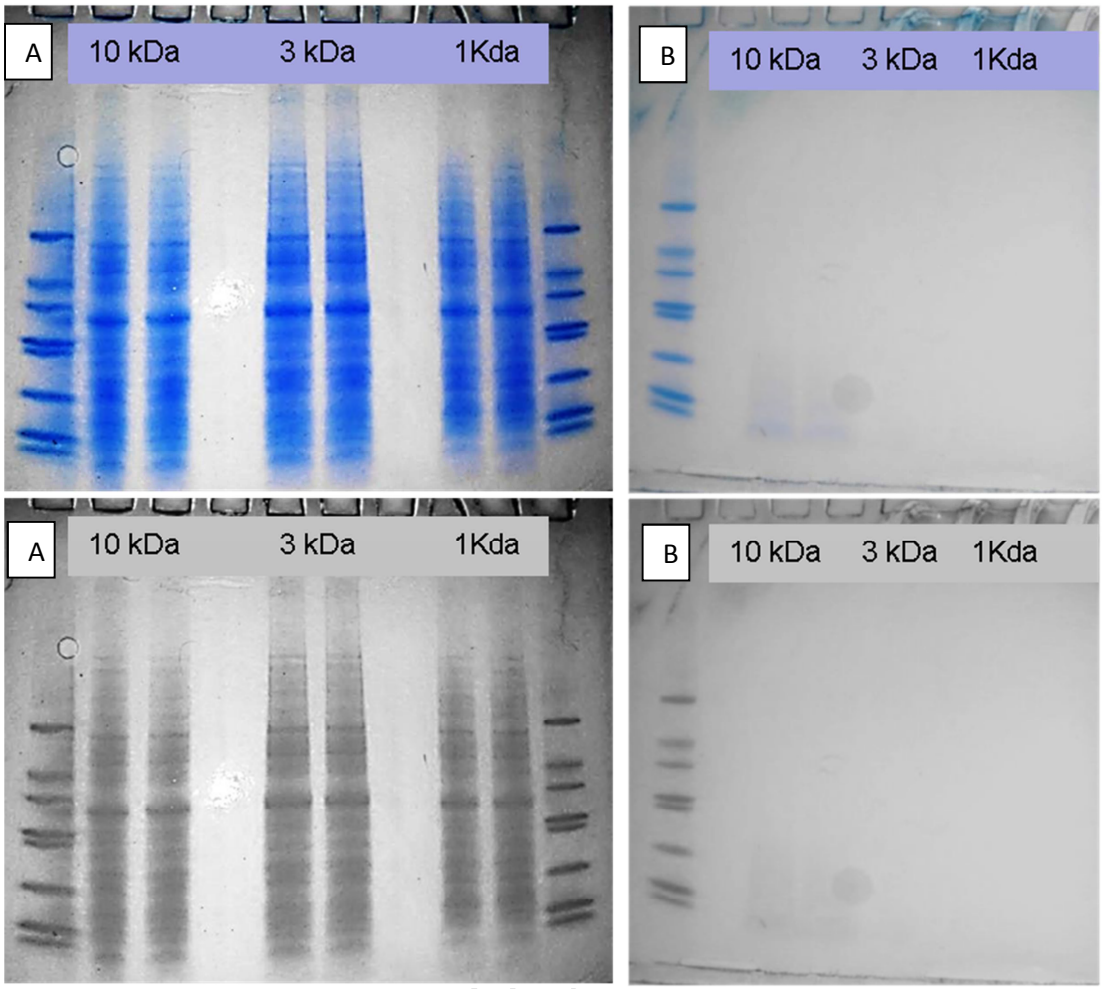


Figure 4

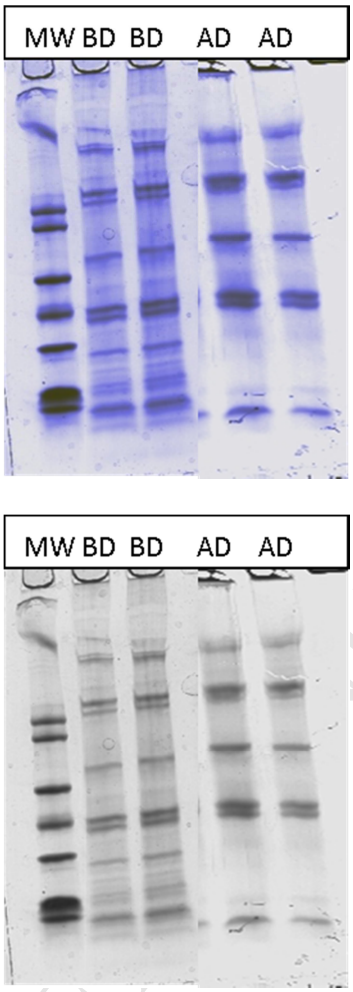
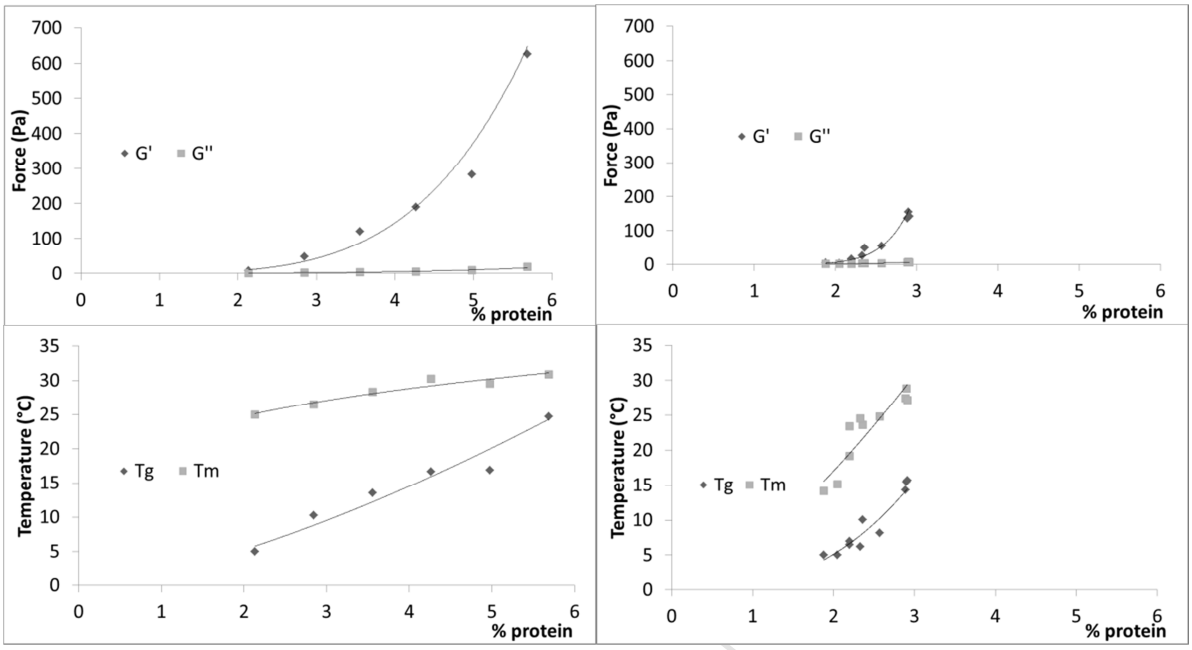


Figure 5



Highlights:

- A waste-water produced from rendering of meat processing products has been characterised

- Protein recovered showed excellent gelling and emulsifying properties

- A novel demineralising method based on protein gelling properties has been developed

- The new method is able to recover higher protein amounts than diafiltration