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Ciclo XXX

"Recovery of valuable protein co-products from meat industry by enzymatic hydrolysis: bovine fleshing and hides"

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"May your joys be as deep as the oceans
Your troubles as light as its foam
And may you find sweet peace of your mind
Whenever you may roam"
Irish Blessing

Abstract

Animal products are not only important in terms of taste and tradition, but they also provide essential nutrients. Presently, around 16% of the global meat consumption takes place in EU, and it increased strongly over the last 50 years. The demand for animal-based food is further expected to increase during the coming decades, due to a growing world population and rising prosperity. In particular, meat consumption in developed countries is expected to increase of about 10% by 2030, but a much larger growth is expected in developing regions.

Meat, dairy and fish are all important sources of protein, and they are partly interchangeable and cannot be entirely replaced with vegetable protein sources. Nevertheless, their production is accompanied by huge amount of waste and loss of biomaterial. For example, beef meat specifically accounts for only 44% of total live animal weight, with the highest production of low value products and waste material being generated in the abattoir. This waste is composed of edible (e.g. offal) and non-edible (e.g. Specified Risk Materials) parts. Edible animal co-products are almost completely used in other foods, such as broths, jellies, emulsifiers, fat matter, extracts and concentrates. Beside this, other parts (fat tissue, some internal organs and blood), being protein-rich, are also used in pet food, feed, organic fertilisers and technical applications.

Despite all these applications, because of the high production of low value or waste material generated during slaughtering, meat industries anyway keep trying to add value to co-products through the generation of innovative food and non-food products. Then, there is increasing interest in studying these non-meat products with the aim to extract or recover additional value from the meat processing chain. Both reduction and recovery of the food waste have become hot topics and a challenge for the modern society.

One possible way to process the waste material is using non-thermal innovative methodologies, which do not affect its quality and the properties. Enzymatic hydrolysis offers indeed a fast and gentle alternative to other mechanical or chemical treatments. The ability of the enzyme to hydrolyse proteins to produce free amino acids and short peptides allows the nitrogen to be more soluble and easily recovered and purified.

This PhD project explored different ways to recover two meat industry co-products, bovine hides and bovine fleshing meat, through enzymatic hydrolysis. The first section is focused on the recovery of bovine fleshing, a low quality meat found immediately under the skin rich of collagen fibres and connective tissue, as possible food or feed ingredient. Fleshing is usually discarded by meat industry, even if having the potential to become a new source of proteins. In the present

project, different proteolytic enzymes and conditions were tested, demonstrating the feasibility for fleshing recovery in a high quality soluble protein hydrolysate. The most efficient enzyme (Alcalase) was then selected to a further optimisation of the process, in order to scale-up the reaction to a semi-industrial level. In the second section, the efficiency of the enzymatic unhairing methods for hides was studied as alternative to the presently used chemical methods, in order to produce raw material for the leather industry. The enzymatic reaction of unhairing was developed at a pilot scale level in semi-industrial reactors and compared with the chemical-based one. Furthermore the wastewaters obtained from both methods were characterized for a future possible valorisation in feed formulations or as food ingredients. The full recovery of bovine hides through total hydrolysis was then studied in the last section, using and optimizing proteolytic hydrolysis for obtaining high quality hydrolysates. Finally, the techno-functional properties of the hydrolysates were studied as a function of the hydrolysis conditions. The effect of the replacement of the meat part with the bovine hides' hydrolysates in Irish sausages was also evaluated.

In conclusion the enzymatic hydrolysis may be a useful method to obtain hydrolysates with a considerable potential in order to recover meat industry co-products as a source of proteins for food or feed industry**.**

Riassunto

L'importanza dei prodotti di origine animale non è solo una questione di gusto e di tradizioni alimentari, ma è strettamente correlata al fatto che sono fonte di nutrienti essenziali. Al presente in Europa si realizza il 16% del consumo mondiale di carne e il dato ha registrato un significativo aumento negli ultimi cinquant'anni. La domanda di cibo è destinata ad incrementare nei decenni futuri in correlazione con la crescita della popolazione mondiale e della prosperità economica. In particolare, il consumo di carne nelle regioni sviluppate salirà del 10% entro il 2030, con un incremento stimato maggiore nelle regioni in via di sviluppo.

Carne, latticini e pesce sono le fonti proteiche fondamentali e sono in parte interscambiabili, né possono essere interamente sostituite da fonti proteiche di origine vegetale. Ciò nonostante la loro produzione è accompagnata da un'enorme quantità di rifiuti e dalla perdita di biomateriali.

Per esempio, la carne di manzo, nello specifico, rappresenta solo il 44% del peso vivo dell'animale, con la più alta produzione di parti di scarso valore e di materiali di scarto che vengono generati nei mattatoi. Lo scarto si compone di parti edibili come le frattaglie e di parti non edibili quali i materiali a rischio specifico (SRM). Sottoprodotti animali sono già del tutto utilizzati in alimenti, come brodi, gelatine, emulsionanti, grassi, estratti e concentrati. Inoltre altre parti (il grasso, alcuni organi interni e il sangue) vengono utilizzati, essendo ricchi in proteine, come alimenti per animali, mangimi, fertilizzanti organici e per peculiari applicazioni tecnologiche.

Dal momento che durante la macellazione si ottiene un elevato quantitativo di prodotti di basso valore o di scarto, le industrie della carne cercano in ogni modo di valorizzare i sottoprodotti grazie alla creazione di prodotti innovativi alimentari e non. Per questo motivo è crescente l'interesse per questi prodotti non carnei, con l'intento di recuperare valore aggiunto nella filiera di trasformazione della carne. Sia la riduzione che il recupero dei rifiuti derivanti dagli alimenti sono diventati una controversa questione, e nel contempo una vera sfida, per la società moderna.

Una possibile modalità di trattamento del materiale originario prevede il ricorso a metodologie innovative non termiche, che non alterano la qualità e le proprietà del materiale. L'idrolisi enzimatica offre una via alternativa davvero veloce e delicata rispetto ad altri trattamenti meccanici o chimici. La capacità dell'enzima di idrolizzare le proteine, producendo aminoacidi liberi e corti peptidi, fornisce azoto solubile più facile da recuperare e purificare.

In particolare questo progetto di ricerca è volto ad individuare diversi metodi finalizzati al recupero ad uso alimentare di due sottoprodotti dell'industria della carne (le pelli bovine e il carniccio bovino) attraverso l'idrolisi enzimatica. La prima sezione dello studio mette a

fuoco il recupero, come possibile ingrediente di cibi e di mangimi, del carniccio che si trova immediatamente sotto la pelle, di basso livello qualitativo, ma ricco di fibre collagene e tessuto connettivo. Il carniccio viene solitamente scartato dall'industria della carne, nonostante sia potenzialmente una buona fonte proteica. Nel presente progetto si sono sperimentati differenti enzimi proteolitici in diverse condizioni per dimostrare la fattibilità del recupero del carniccio in forma di idrolizzati proteici solubili di alta qualità.

L' enzima più efficace (Alcalasi) è stato selezionato per un'ulteriore ottimizzazione del processo al fine di realizzare la reazione su scala semi-industriale.

Nella seconda sezione si è comparata l'efficienza dei metodi di epilazione enzimatica delle pelli, in alternativa ai metodi chimici, con la finalità di produrre materiali grezzi per l'industria conciaria. La reazione enzimatica di epilazione è stata condotta a livello di impianto pilota in reattori semi-industriali a confronto con i metodi chimici tradizionali.

Si sono, inoltre, caratterizzate le acque reflue ottenute con entrambi i metodi per una possibile valorizzazione futura nella formulazione di mangimi o in qualità di ingredienti alimentari.

Nell'ultima sezione è stato analizzato il completo recupero delle pelli bovine attraverso l'idrolisi totale, utilizzando e ottimizzando l'idrolisi proteolitica per ottenere idrolizzati di alta qualità.

Infine si è verificata l'influenza delle diverse condizioni di idrolisi sulle proprietà tecno-funzionali degli idrolizzati ottenuti. Si è valutato inoltre l'effetto della sostituzione di una parte della carne con gli idrolizzati delle pelli bovine nelle preparazione delle salsicce irlandesi.

In conclusione si potrebbe considerare l'idrolisi enzimatica come un metodo utile per ottenere idrolizzati con un significativo potenziale per il recupero dei sottoprodotti dell'industria della carne come fonte proteica per l'industria alimentare e dei mangimi.

Table of contents

1. INTRODUCTION

1. General introduction

The reduction and the recovery of the food waste have become a hot topic and a challenge for the modern society. Food waste is generated in the agriculture phase, in the industrial processing and manufacturing, during the retail phase and in the household. Mirabella et al (2014) estimated that up to 42% of food waste is created by household, 39% is originated by the food industry, 14% belongs to the food production and 5% occurs during the distribution chain. Particularly during the production chain in food industry several amounts of by-products are discarded and not recovered, products which might still have a high nutritional value as a source of protein, fibres, flavours and other components.

A traditional way to utilise these co-products is in the agricultural field, as fertilizer, but this application could lead to serious environmental problems, such as increased nutrient loss through leaching and erosion from agricultural fields. Whereas most of the production of food waste is generated in the developed countries, it has also to be considered that 15% of population in developing countries suffers of undernutrition and malnutrition (FAO, 2012). It seems quite obvious then trying to develop technologies so that this high quantity of waste and co-products can be recovered and reutilised in several sectors, aiming to decrease economic and environmental problems.

Furthermore, according to the idea of circular economy emerged in EU in the last few years (Mirabella, 2014), food waste could be used as a new raw material for the development of new products or additives not only for food or feed industry but also for other industrial fields.

1.1 Meat industry waste

Meat consumption in Europe is twice the world average; taking into account proteins from dairy sources, the amount of animal proteins consumed is even three times higher. Average EU consumption of meat, dairy and fish has increased strongly over the last 50 years following the increasing standards of living. Presently, around 16% of the global meat consumption takes place in the EU. Anyway, the demand for proteins from animal products is expected to increase more than 50% in 2030 in the world, compared to that of 2000, due to population growth and increasing wealth also in developing countries. Indeed, while meat consumption in rich regions is expected to increase by about 10% by 2030 (Westhoek et al., 2011), a much larger growth is expected in developing regions, such as Asia and Africa. The total global consumption of meat is expected to increase by almost 70% between 2000 and 2030 (Westhoek et al., 2011) and by another 20% between 2030 and 2050. It is therefore clear that an adequate supply of proteins having high nutritional value is to be provided in order to meet this demand.

Meat, dairy and fish are all important sources of high nutritional value proteins, and they are partly interchangeable and cannot be entirely replaced with vegetable protein sources. The meat that can be obtained from a cow is approximately 44% of its total weight, meaning that most of the mass of the animal is wasted. Waste generation during cow meat production is not unique, since all meat production suffers from the same phenomenon. In particular the highest production of waste in meat industry is originated during slaughtering. The amount of the waste produced depends on the animal type, as it is shown in table 1.

Table 1 The specific waste index for slaughtered houses with respect to the type of animal

Animal	Specific waste index[a]
Cow	0.56
Calf	0.87
Pig	0.2
Sheep	0.1

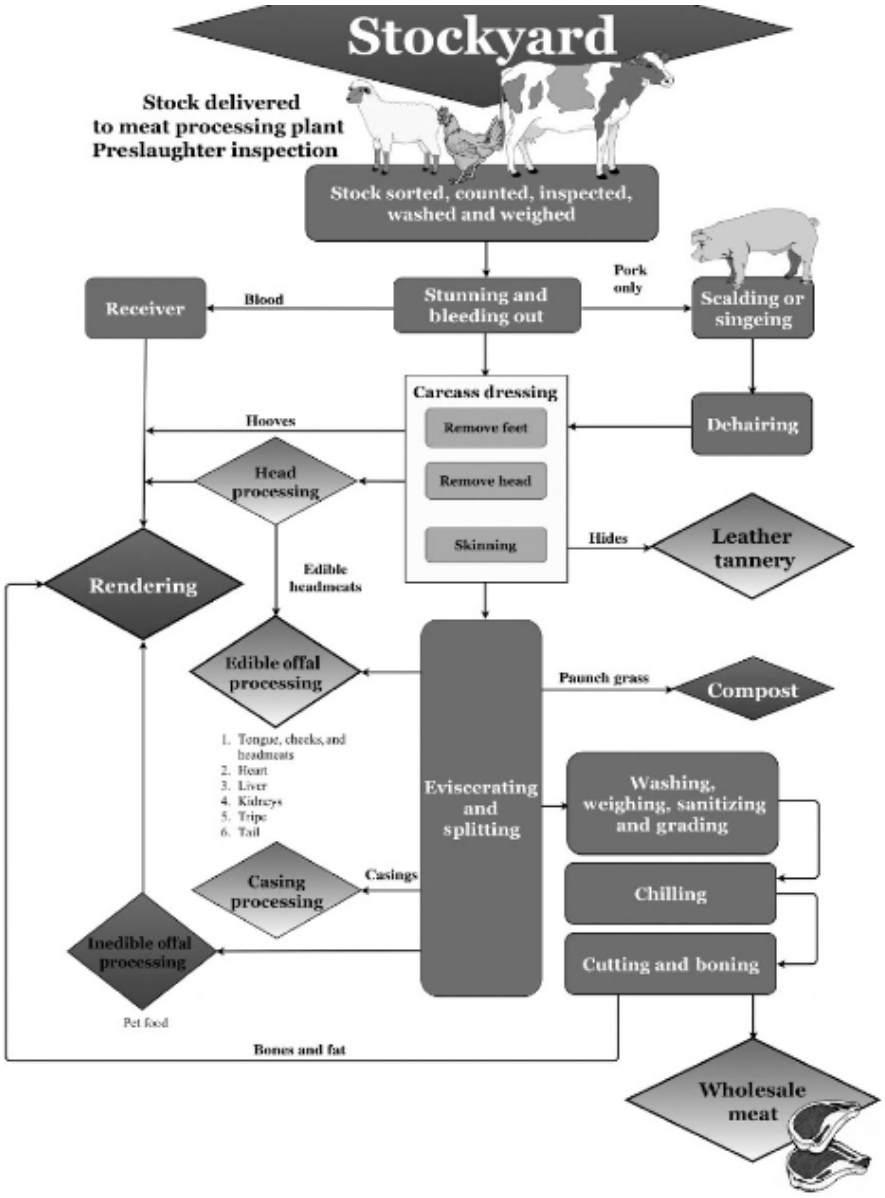
[a] Mass of accumulated waste divided by the mass of saleable product (Russ and Pittroff, 2004).

The correct definition of animal co-products was stated by the Commission of the European Communities Regulation (EC) No.1069/2009. It was defined that an animal by-product, as whole body of an animal or products, is derived from animals which are not meant for human

consumption (European commission, 2009). Animal by-products regulation can differ in other countries in the world. For example in the U.S.A the animal by-products are divided in two different classes: edible and inedible. The term offal is used, which means slaughter by-products, and includes the entire animal which is not part of the carcass. (Jayathilakan et al, 2011). As an example the meat trimmed from the head is considered as edible by-product by the US, otherwise in the UK the part derived from spinal cord and brain are banned for food because of BSE (Bovine Spongiformis Encephalopathy, known as Mad Cow Disease)(Schriber and Seybold, 1993). Beside this fact in the UK, the offal is classified as red offal (head, liver, lungs, tongue, tail, etc…) and white offal (fat), plus the guts and bladder, the tripe (rumen) and the four feet and trimmings (Jayathilakan et al, 2011). The list also includes poultry part such as the heart and the liver. During slaughtering, edible fat it is obtained, such as the cowl fat surrounding the rumen or stomach, or the cutting fat which is back fat, pork leaf fat or rumen fat (Jayathilakan et al, 2011). Finally blood is considered as a by-product, but for other countries in the world it is used as an edible product for human beings.

During the industrial process, raw materials undergo several steps. At each step or steps, there is an output of by-products, resulting in a multitude of wastes. These wastes could be defined, in a generic way, as: a desired product, a non-product-specific waste or a product-specific-waste (Russ and Pittroff, 2004). The quantity or the quality of the non-product-specific waste does not depend on the starting raw material. On the other hand the product-specific waste is generated during the production chain; the techniques used in the processing of the raw material influence the quantity but also the quality. Because of specificity and the quite high number of steps in the process, several by-products with different types and composition are created (figure 1). The slaughterhouse waste from meat production is an example of product-specific waste (Russ and Pittroff, 2004).

Figure 1 Process flow diagram for general meat processing (Hicks and Verbeek, 2016)



The production of waste during food production is a general problem of all industry, and not only of the meat production. It is in general difficult to manage this waste due to the following reasons:

- Biological stability and the potential growth of pathogens: many by-products could be altered by the microbiological activity; another possibility is that they already contain bacteria or microbes. For this reason the regulations concerning infectious disease must be followed. Moreover, also the hygienic conditions need to be taken under control because of the possible rise of maggots and mould.

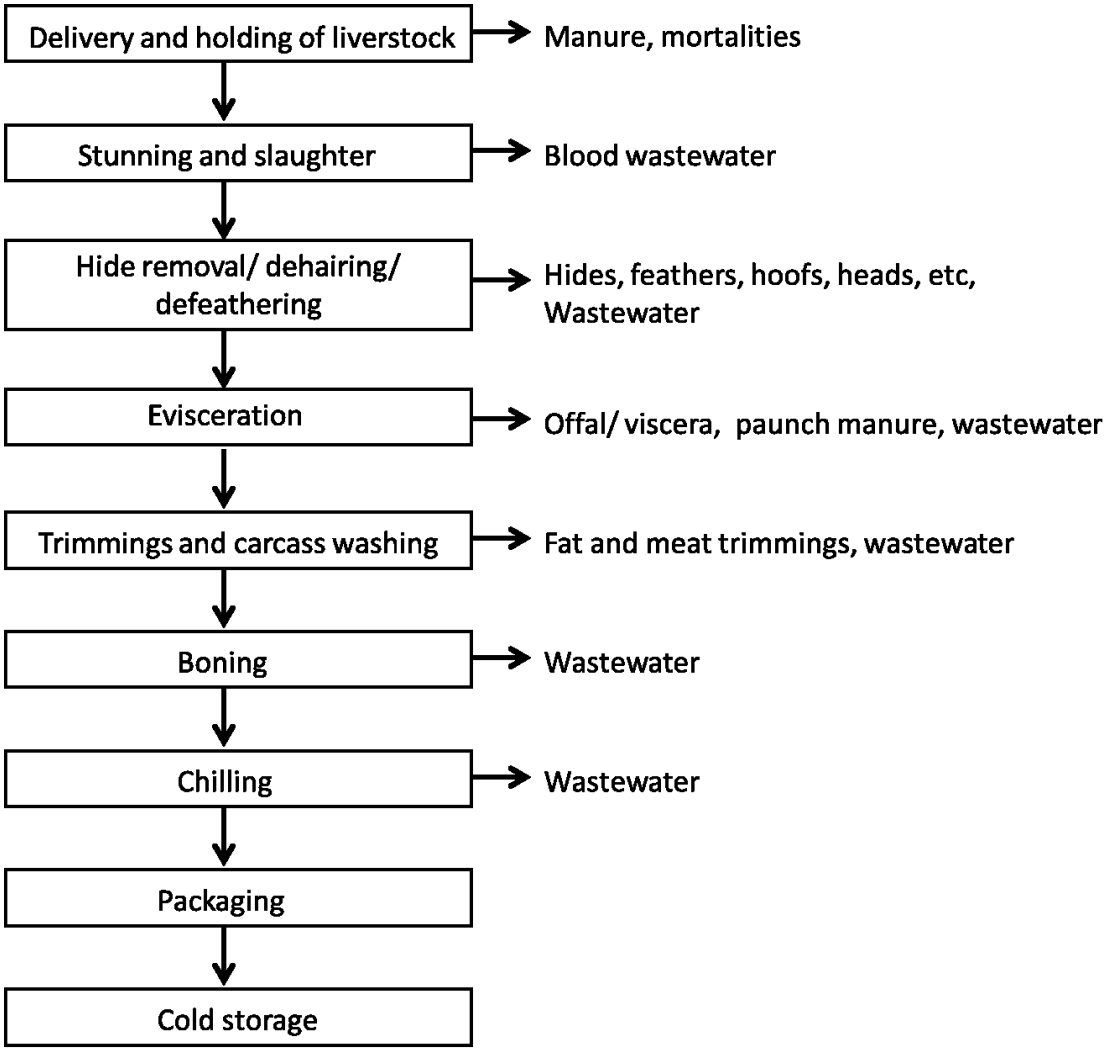
- High water content: the water content of meat and vegetables waste lies between 70 and 95% by mass. Not only the high quantity of water affects the transport cost of these wastes, but also the mechanically removing of water through different techniques leads to high costs. There are also problems related to the water disposal because the organic fraction contained in the by-products.

- Rapid oxidation: the high percentage of fat in the waste can undergo oxidation, with the consecutive release of foul-smelling fatty acids and the generation of peroxides and other obnoxious compounds.

- Changes due to enzymatic activity: enzymes, present in vegetables and fruits waste, can affect the acceleration and the intensity of spoilage because of their activity (Russ et al, 1998; Werschnitzky et al 1985).

As mention before, in meat industry slaughtering is the step producing the highest amount of waste, thus, from an economic point of view, it would be convenient to recover these by-products. Jayathilakan et al (2011) evaluated that the 11.4 % of the gross income in beef industry and 7.5 % of gross income in pork industry is generated by the re-utilization of meat by-products. Beside the waste produced during the slaughtering, more waste derives from meat processors, wholesalers and rendering plants. In figure 2 is reported a general process flow diagram of wastewaters and by-products generated in a non-specific meat industry. In solid waste blood, hides, skin, feathers, hoofs, heads, visceral, fat and meat trimmings are included. The quantity of wastewaters discarded from meat plants is variable, but they are extremely difficult to purify, because of their considerable content of organic, mineral and biogenic matter (Bohodziewicz and Sroka, 2005).

Figure 2 General process flow diagram for meat processing operations



Adapted from http://www.gpa.uq.edu.au/cleanprod/res/facts/fact7.htm

The differentiation among the quantity of by-products obtained from sheep, cattle and pigs is reported in table 2.

Table 2 By-products as a percentage of market live weight

Item	Pigs		Cattle		Sheep	
	%	Kg	%	Kg	%	Kg
Market live weight		100		600.		60
Whole carcass	77.5	77.5	63.0	378.0	62.5	37.5
Blood	3.0	3.0	18.0	4.0	2.4	
Fatty tissue	3.0	3.0	4.0	24.0	3.0	1.8
Hide or skins	6.0	6.0	6.0	36.0	15.0	9.0
Organs	7.0	7.0	16.0	96.0	10.0	6.0
Head	5.9	5.9				
Viscera (chest and abdomen)	10.0	10.0	16.0	96.0	11.0	6.6
Feet	2.0	2.0	2.0	12.0	2.0	1.2
Tail	0.1	0.1	0.1	6.0		
Brain	0.1	0.1	0.1	6.0	2.6	0.156

U.S. Department of Agriculture (2001) Livestock slaughter 2000 summary. National Agricultural Statistics Service. Mt An 1-2-1 (01a), Washington, DC. (DCN 00183)

Bhaskar et 2007 all reported that generally meat waste by-products could constitute from 60 to 70 % of the whole animal carcass; in particular 40% is considered in edible form and the remaining 20% is inedible. Due to this reason meat industry has been developing several new strategies to recycle these by-products. Recovery of meat waste requires a severe hygiene and health limitations in order to protect human health (Mirabella et al, 2014). For example, poultry by-products may contain up to 100 different species of microorganisms in contaminated feathers, intestinal contents and processing plants, for example *Salmonella* sp., *Staphylococcus* sp. and *Clostridium* sp. (Chen, 1992; Salmine and Rintala, 2002). BSE emerged as a new most hazardous disease, for which the European Union promulgate legislative measures (Regulation 999/2001 and 853/2004). Despite this negative aspect, meat by-products have good nutritional values, as it can be seen in table 3.

Table 3 Composition of meat waste (Cunningham, 1976; Connor et al, 1983; Nambi et al, 1992)

Composition (%)	Values
Crude protein	15-36
True protein	8.8-12.9
Fibre	8.38-24.6
Dry matter	89.94-97.2
Ash	6.23-40.8
Calcium	0.6-12.5
Phosphorus	0.93-2.8

As a comparison, muscles freed from adhering fat contain on the average 76% moisture, 21.5% N-substances, 1.5% lipids and 1% of minerals. Edible meat waste contains essential nutrients, such as amino acids, hormones, minerals, vitamins and fatty acids. Some wastes are somehow different: blood and other meat by-products - lung, kidney, brains, spleen and tripe - have a higher level of moisture than meat. On the other hand some organs, including kidney and liver, include a higher amount of carbohydrate than other meat materials (Devatkal et al 2004). It is of interest to note that pork tail has the highest fat content and the lowest moisture content of all meat by-products. Among the cattle by-products, liver, tail, ears and feet own the protein content close to lean meat tissue one, but a large level of collagen is found in ears and feet (Unsal and Aktas, 2003). On the other hand the lowest protein content is found in the fat-rich tissue, such as brain, chitterlings and fatty tissue. Many organs have a great amount of polyunsaturated fatty acids such as brain, chitterlings, heart, kidney, liver and lungs (Liu 2002). Cholesterol is five times higher in organ meats than in lean meat; brain has the highest level of cholesterol and large quantities of phospholipids compared to other meat by-products (Jayathilakan et al, 2011). Because of the variable quantity of connective tissue in the animals, the amino acids composition of meat by-products is very different among them and in comparison with the level in lean tissue. As an example ears, feet, lungs, stomach, tripe, hides and skins are composed by a high level of glycine, proline and hydroxyproline, due to their high collagen content. Otherwise they are lacking of tryptophan and tyrosine. As far as vitamins are concerned, liver is one of the best sources of niacin, vitamin B12, B6, folacin, ascorbic acids, vitamin A and riboflavin. Also kidneys are rich of riboflavin, but also vitamin B6, B12 and folacin (Jayathilakan et al, 2011). Meat by-products are a good source of metals too: iron (kidneys, liver, lungs and spleen), copper (liver of beef, lamb and veal), phosphorous and potassium (thymus and sweetbreads) (Devatkal et al, 2004).

Given their nutritional value, the recovery of meat waste, beside the nutritional importance, is also significant for preventing the need to use additional primary natural resources (Toolkit, reducing the food wastage footprint, FAO, 2013).

Nowadays there are several strategies under study, and some are already utilised in meat industry to recover the meat by-products. There are different possible solutions to treat these biomasses, going from composting to feed preparations. Anyway, the proposed solutions should take in consideration also the legislative issues. The European legislation (Article 3 of Regulation (EC) 1069/2009) splits by-products into three categories on the level of risk of the by-products for the transmission of pathogens and toxic substance (Lasekan et all, 2013). Category 3 is considered as

the low risk, while the categories 1 and 2 are considered the highest risk materials. Category 1 by-products should be transferred, under approved agency, in the specific sites to be buried or incinerated. On the other the 2nd category by-products must be firstly pre-treated at high temperature and pressure, in order to avoid their pathogenic and toxic risk, and then they could be used as feedstock for composting, biogas production and energy generation (Lasekan et all, 2013). Category 3 materials include parts of animals that are not considered as suitable for human consumption and because they are not normally eaten for commercial reasons or because of they are not processed in that way. Several by-products derived from healthy animal could be employed in some application falling in the third category, such as feathers, hoof, skin, hides, horn, feet, heads. Beside this fact they can be used in feed or pet food under several restrictions according to Transmissible Spongiform Encephalopathies Regulations. Nevertheless they can be utilized for the manufacture of derived products (e.g. medical devices), as specified by the Regulations.

Table 4 shows the chicken by-products and their corresponding categories according to the EU legislation (European Commission, 2009). Obviously this classification may be appropriate not only for poultry by-products, but also for meat by-products in general.

Table 4 Methods of utilizing chicken by-products and their corresponding categories according to the UE legislation.

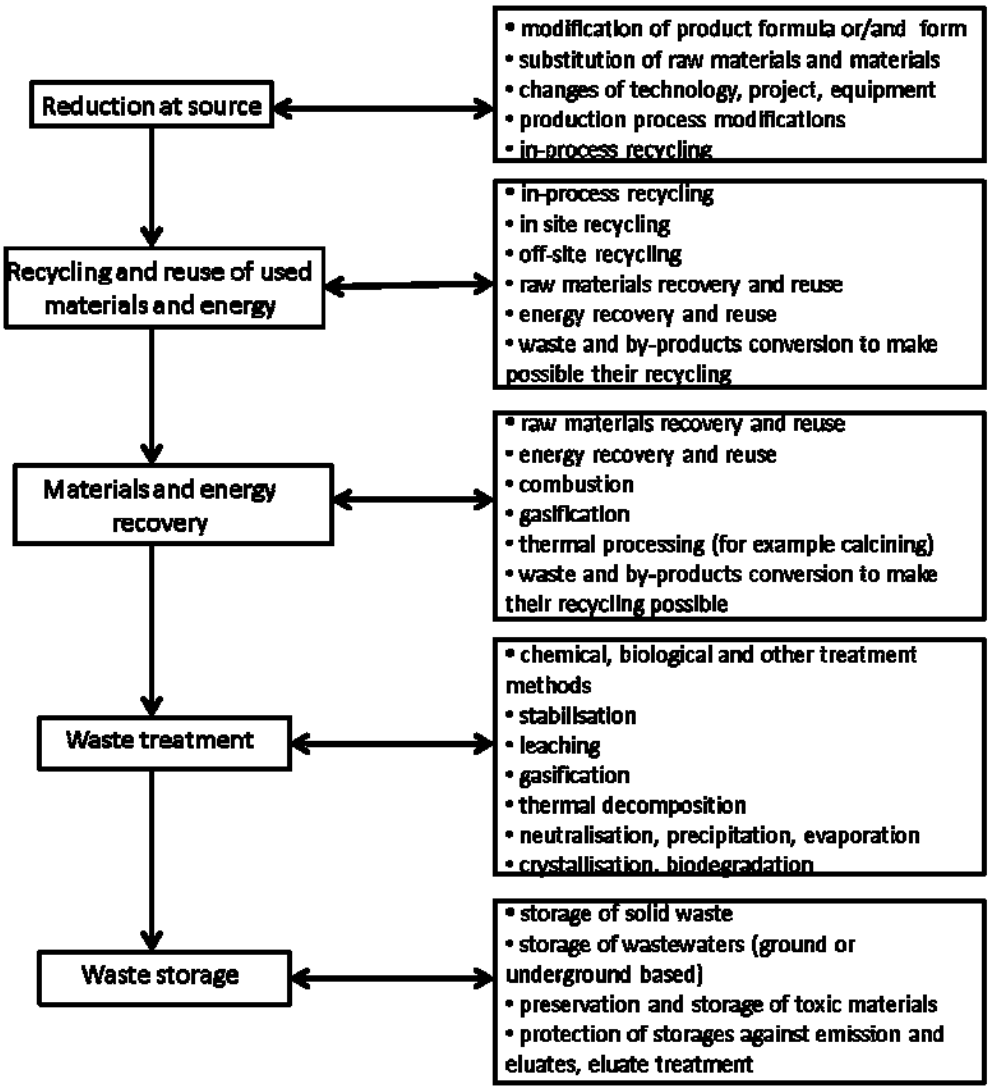
Uses of chicken by products	EU classification of the by-products[a]
Livestock feed	Category 3
Pet food	Category 3
Aqua feed	Category 3
Cosmetic products	Category 3
Compost	Category 2 and 3
Products of biogas	Category 2 and 3
Products of thermal and electrical energy	Category 1, 2 and 3
Production of biofuel	Category 1, 2 and 3

[a]The current European Union document on the utilization of animal by-products should be consulted for guidelines on handling and processing of these by-products.

The main idea is to create a meat production which is as much “waste-free” as possible, with the use of a cleaner production method. The definition of “cleaner production” is based on the idea of preventing effects on the natural environment in all phases of the production cycle, starting from the raw materials to the final storage of the product (Kowalski and Krupa-Zuczek, 2007). Among

the cleaner productions, the protection of natural resources of the starting material, the reduction of the quantity and toxicity of all type of waste are the most considered factors. A particular scheme is reported in figure 3 which underlines the options in the prevention of waste and pollutions production.

Figure 3 The hierarchy of pollution prevention options and the waste management techniques



Kowalski and Krupa-Zuczek, 2007

1.2 Recovery and valorisation of meat by-products

Food wastes are generated in different forms, compositions, also according to regional and seasonal characteristics. The need to find an economical feasible, sustainable and safe recovery and valorisation of high added-value compounds requires following a holistic approach (Galanakis, 2015). A lot of aspects are to be taken into account:

- Waste minimisation prior to the recovery process
- The abundance and the distribution of meat wastes
- Proper mixing and of meat wastes in order to minimise variations in the components
- The development of a production line near but not inside the food industries in order to ensure minimum transportation
- The development of a method which provides the highest recovery yields and discharges minimum quantity of by-products
- Nondestructive separation of valuable compounds
- The addition of food grade materials and the utilisation of green solvents
- The proper management of the selected stages and technologies
- The prevention of the compounds' functional properties
- The development of qualitative products with a constant concentration of the target compounds and stable at sensory characteristics

The current recovery and disposal practice and requirements for solid and liquid wastes, generated in meat industry and farming, are listed down here.

1. The burial of dead animals or by-products is strictly controlled by severe regulation under directive 1991/21/EC (Commission of the European Communities, 1999). These rules are mandatory because of the problems which can affect the local environment, in particular it can generate pollution not only in the surface water, groundwater, soil and air, but also it can influence the global greenhouse effect. There are also restrictions on the land filling of organic waste (Commission of the European Communities, 1999).

2. Composting is a method used for organic waste disposal and a treatment for waste before land application (Cambardella et al, 2003). It is an aerobic biological process to decompose organic material by microorganism naturally present (Arvanitoyannis and Ladas, 2008). The microorganisms which are involved in composting are mesophilic and thermophilic (Goyal et al, 2005). The advantage to use this methodology is that this process reduces pathogens and converts nitrogen from unstable ammonia to stable organic forms. Another benefit is the conversion of a by-product, containing organisms which can cause potential disease, into a valuable and pathogen free fertiliser. By contrast the emission to air, water and land could be a problem in addition with the possible reduction of nitrogen content in the compost (Salminem and Rintala, 2002).

3. Anaerobic digestion could be a promising alternative for the treatment of meat by-products, because it is a biological process where the organic matter is degraded to methane under anaerobic conditions (Salminem and Rintala, 2002). It can be a possible solution for the energy conservation because methane can be well controlled and can be used as fuel. Also the pollution is taken under control due to the elimination of pathogens and the absence of odours. Most nutrients remain in the treated material, so they can be recycled for agriculture or feed use (Salminen et al, 2001). A negative aspect is the generation of high quantity of ammonium produced from the protein breakdown, occurred in protein rich by-products (Arvanitoyannis and Ladas, 2008).

4. Aerobic digestion is used in the treatments of wastewater, in particular for the removal of suspended material and soluble organic contaminants (Arvanitoyannis and Ladas, 2008). A positive aspect of aerobic digestion is the high effectiveness in the degradation of organic pollutants, odours and pathogens (Skjelhaugen and Donantoni, 1998). Nevertheless it is an exothermic process and the utilisation of heat can help support the treatment cost, it is an expensive process. Another problematic arises from the uncontrollable releasing of high amount of nitrogen compounds derived from animal manures which is hazardous for the environment and the human health (Arvanitoyannis and Ladas, 2008).

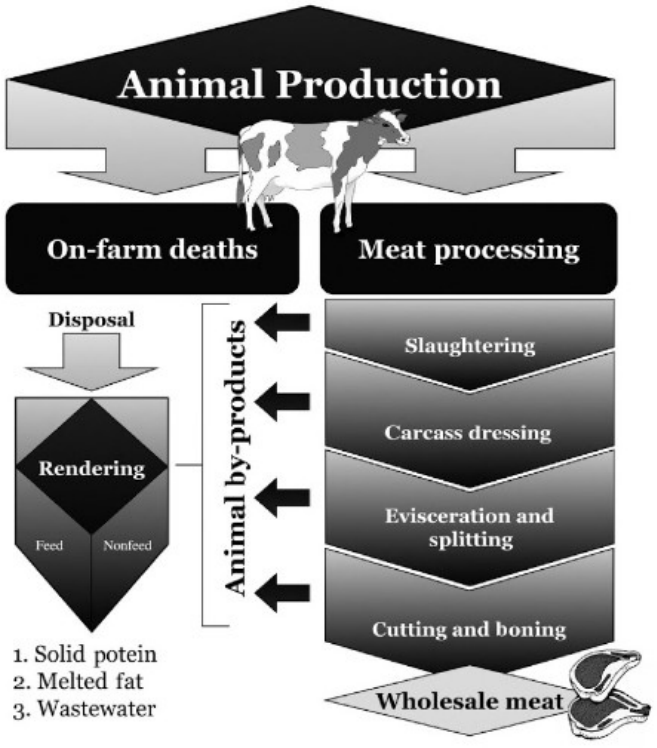
5. Incineration seems to be the most effective process for the thermal destruction of waste because of its effectiveness in the destroying of potential infectious agents (Ritter and Chinside, 1995). The energy (gross calorific value) released depends on the intrinsic composition of the waste, therefore materials with a high level of moisture have little or no energy value. This process is strictly controlled due to the possible emission in the air of dioxins, CO, particles, SO_2, NO_x, HCl and HF (Bernstad and Jansen, 2012). On the other hand also the disposal of solid and liquid residue must be taken under control.

6. The utilization of waste as biofuel through thermal recycling of residues increase the interest for power plant operations. The energy recovery from the direct combustion of waste in this process, as biogas, could be reuse for the generation of electricity, heat and vehicle fuel (Bernstad and Jansen, 2012). This method seems to be a viable alternative for the production of high heating value and it derives from a renewable energy source (Jayathilakan et al, 2011). Despite this positive aspect during the combustion NO_2 and SO_2 can be generated, for this reason their emission must be taken below the local and/or international limits.

7. The rendering process (figure 4) is an effective method for ensuring bio-security because processing conditions and volumes, raw material characteristics and drying create an unfavourable environment for viruses, bacteria and other micro-organisms to survive and grow (Salmien and Rintala, 2002). This methodology is used for the conversion of the food by-products, in particular meat wastes, into marketable products, including edible and inedible fats and proteins for agricultural and industrial use (Jayathilakan et al, 2011). Meat by-products normally rendered are: fat, bone, blood, feathers, hatchery by-products (infertile eggs, dead embryos, etc.), and dead animals. As an example lard and food grade tallow are considered as edible rendering product. They are obtained after low or high temperature separating fat from proteins. For the inedible rendering there are two possible processes: the wet and the dry rendering; because of the high costs of dry rendering usually the wet one is used (Jayathilakan et al, 2011). Beside this considerations, rendering is the most logical

method for collecting and processing animal by-products because it possesses the infrastructure to safely and responsibly recycle these products, to allow traceability and to produce safe finished products (Hamilton., 2004). On the other hand most studies indicate that meat protein is not severely damage when it is processed. The nutritional quality of proteins can be changed as a result of oxidation during processing and storage. Oxidative compounds, such as peroxides from the oxidation of unsaturated fatty acids, may occur naturally or be formed in food during processing and storage.

Figure 4 Sources of raw material for rendering from animal production (Hicks and Verbeek, 2016)



8. Another possible way to reduce the effect of accumulation of waste is the incorporation into productive processes. The recycle of food by-products in animal feed is an accepted practice due to the need to find alternative feed sources (Arvanitoyannis and Ladas, 2008). Therefore the incorporation of raw and rendered animal by-products as ingredient in pet food of in feed is established because of it provide adequate

nutrients and good digestibility (Toldrà et al, 2012). In fact the composition of these by-products results rich in proteins, fats, minerals and vitamins, which are required in animal nutrition. There are several regulations about the conditions of disinfection and the removal of pathogens of meat waste to be biologically degraded. In EU there are also regulations regarding the addition of meat and bone meal (MBM) in feed for cattle, pig, poultry, fish or animal: EC 1774/2002 and the amendment No 808/2003 of the European Parliament and Council.

9. As mention before, by-products are the part of the carcass which is not considered in strict sense as “meat”. Despite this fact some by-products which are judged as inedible in a country, can be considered as precious products in other countries. Furthermore some by-products own good nutritive value and have an important role in the diet of different countries of the world (Nollet and Toldrà, 2011). Table 5 shows the possible applications, storage and preparation of meat by-products reuse. In addition animal glands and organs could be exploited for the extraction of particular molecules with a functional role in the organism. For example the anticoagulant ability to prolong the clotting time of blood is obtained by heparin, which is extracted from liver, lungs and the lining of the small intestines. Sexual hormones (progesterone and oestrogen) are extracted from pig ovaries; also relaxin is taken from saw pregnant ovaries and used during childbirth (Jayathilakan et al, 2011). From pancreas derives insulin, which regulates sugar metabolism and is employed in the diabetes treatment. In diabetes it also consumed glucagon to increase sugar in blood, to treat insulin overdose or boots the sugar level because of alcoholism (Jayathilakan et al, 2011). Also some enzymes are obtained, chymotrypsin and trypsin to improve healing after surgery or injury. Finally the internal surgical sutures are acquired from the intestine of sheep and calves (Jayathilakan et al, 2011).

Table 5 Potential uses and preparation of edible meat by-products

Kind of meat	Storage and preparation	Way in which it is used
Liver	Frozen, fresh or refrigerated	Braised, broiled, fried, in loaf, patty and sausage
Kidney	Whole, sliced or ground Fresh or refrigerated	Broiled, cooked in liquid, braised, in soup, grilled in stew
Heart	Whole or sliced Frozen, fresh or refrigerated	Broiled, cooked in liquid, luncheon meat, patty, loaf
Brain	Whole or sliced	Sausage ingredient, broiled, braised and cooked in liquid, poached, scrambled
Tongue	Frozen, fresh or refrigerated Whole	Cooked in liquid, cured, sausage casing, sausage ingredient
Stomach	Fresh, refrigerated, smoked or pickled	Broiled and cooked in liquid, sausage casings, sausage ingredient
Spleen	Fresh, refrigerated and pre-cooked	Fried, in pies, in blood sausage
Tail	Frozen, fresh or refrigerated	Cooked in salty liquid
Intestines (small and large)	Whole, frozen, fresh or refrigerated Remove faeces, soak, wash, add salt before use	Sausage casing
Cheek and head trimmings	Frozen, fresh or refrigerated	Cooked sausage
Ear	Frozen, fresh or refrigerated	Smoked and salted, stewed with feet
Skin	Fresh or refrigerated	Gelatine
Feet	Frozen, fresh or refrigerated	Jelly, pickled, cooked in liquid, boiled
Fat	Frozen, fresh or refrigerated	Shortening, lard
Blood	Fresh or refrigerated	Black pudding, sausage, blood and barley loaf
Bone	Frozen, fresh or refrigerated	Gelatine, soup, jellied products, rendered shortening, mechanically deboned tissue
Lung	Frozen, fresh or refrigerated	Blood preparation, pet food

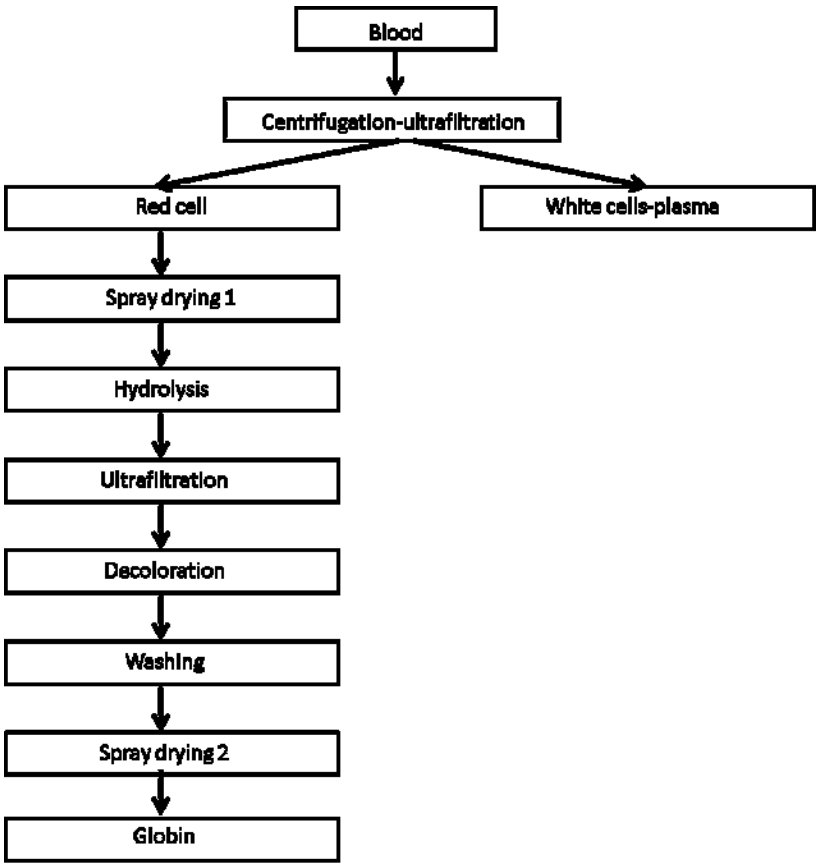
Ockerman and Hansen, 2000

The case of the recovery of animal blood is a particularly interesting case. Blood is constituted by 80.9% of water, 17.3% of protein, 0.23% of lipid, 0.07% of carbohydrate and 0.62% of minerals (Toldrà et al, 2012). It derives mainly from bovine and porcine species and it consists of cellular fraction (red blood cells, white blood cells and platelets) and plasma fraction (Ofori and Hsieh, 2011). Despite the protein content, blood is also rich of heme iron. Some components of blood have an effective application in food industry: serum (blood without red cells), plasma (serum without fibrinogen), globin (the uncoloured part of haemoglobin) and heme (Kowalski and Krupa-Zuczek, 2007). It is then a useful edible by-product, and then it is widely used in Europe for making blood sausages, blood pudding, biscuits and bread. In Asia is employed in blood curd, blood cake and blood pudding. Because of its use in food, there are regulations about its acquisition, in particular it is necessary that the blood is taken from an animal that has been inspected and passed for use in meat food products (Jayathilakan et al, 2011). Plasma also plays an important role in food production; it can be obtained as liquid plasma (up to 91 % of water), plasma concentrate (up to 79% of water) and dried powdered plasma (up to 5-7% of water).

The percentage of blood in slaughtered animals can vary from 3 % in pigs to 18% in cattle, as reported in table 2. The employment of blood in food and feed is anyway limited because of the aesthetic concerns. This problem is related to the brown colour which affects food and feed products formulated with blood (Gomez-Juarez et al, 1999). In order to solve this problem several techniques were set up for the discoloration of bovine blood.

Blood is a protein-rich source. The figure 5 illustrates a typical methodology for producing a protein concentrate from blood (Arvanitoyannis and Ladas, 2008).

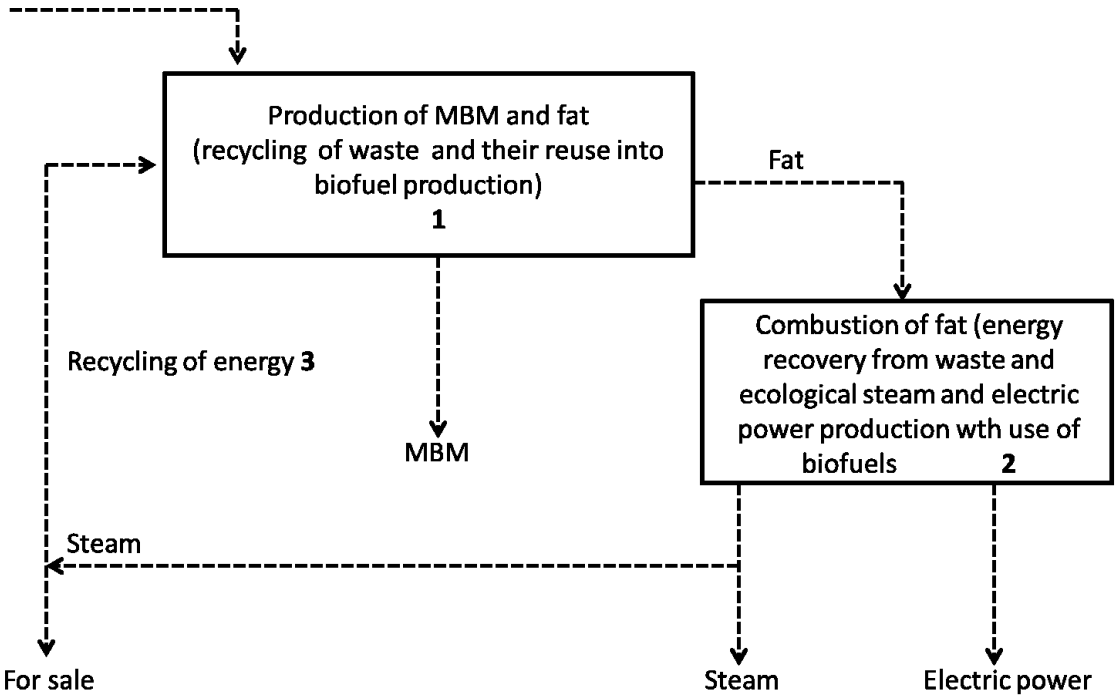
Figure 5 Flow diagram of a typical process for isolating a protein concentrate from blood. (Fallows and Wheelock, 1982; Gomez-Juarez et al, 1999).



Blood proteins have showed relevant technological properties like gelification, foaming and emulsifying ability.

Meat-bone meal is another interesting case. It is a process which is normally used for the recovery of animal waste (Kowalski and Krupa-Zuczek, 2007). This kind of process is generally applied for the recovery of high amount of meat from bones by pork, beef or lamb. It is then added to food products but its characteristics are totally different from the high quality meat: the colour is darker and the softness is higher because of the high content of water; beside this, the quality and the flavour of the end product are reduced (Jayathilakan et al, 2011). Because of the spread of BSE, there are several regulations about the content of MBM for the human and animal consumption. It was suggested a level of 5.0-20.0% of MBM in hamburger and ground beef and from 10.0-40.0% in sausage (Jayathilakan et al, 2011). In the U.S.A MBM cannot be used for hamburgers, baby food, ground beef and meat pie. Figure 6 reports the diagram for the production of MBM with the simultaneous origination of inedible fat, which can be used as fuel substitute of natural gas (Kowalski and Krupa-Zuczek, 2007).

Figure 6 The diagram of materials and energy streams connections for the existing production lines. Cleaner production elements: 1 – meat waste off-process recycling, processing, and the reuse of waste, biofuel (fat) recovery from meat waste; 2 – energy recovery from the waste (biofuel); 3 – in-process recycling of heat energy. (Kowalski and Krupa-Zuczek, 2007).



A further interesting case is gelatine application in food industry. The food protein products obtained with collagen derived from animal (bones and skins), from cold-blooded animals (fish) and insects is considered gelatine (Mariod & Adam, 2013). Collagen is a structural protein which constitutes about 25% to 35% of the mammals whole-body protein content. It is mainly found in the connective and fibrous tissues, such as tendons, ligaments and skin; however it is also abundant in corneas, cartilages, bones, blood vessels and intervertebral discs (Brikmann et al., 2005). From the structural point of view, collagen owns a rod-shape molecule made up of three polypeptide chains with left-handed helices conformation. These three chains are twisted together into a right-handed coiled coil, a triple helix which represents a quaternary structure of collagen. This structure is stabilized by a high amount of hydrogen bonds and intra-molecular Wan deer Waals interactions, giving an unusual strength and stability (Brikmann et al., 2005). The primary structure of collagen shows a regular arrangement of amino acids: the sequence is a repetitive unit of glycine (Gly)-prolyne (Pro)-X or Gly-Y-hydroxyproline (Hyp), where Gly is the 1/3 of the sequence, while X and Y may be other amino acids residues. Table 6 illustrates a simple

classification of collagen types, but it has been taken into account that nowadays 27 different types of collagen have been identified.

Table 6 Classification of collagen (Gorgieva and Kokol, 2016)

Type	Description
Type I	the most abundant genetic type, it could be found in tendons, bones and ligaments, composed of two α1 (I) and α2 (I) chains
Type II	The main component of nose cartilage, the outside of the ears, the knees and parts of larynx and trachea, composed of three α1 (II) chain
Type III	Present in skin and blood vessels, composed of three α1 (III)
Type IV	Present in basement membrane, with a triple helical conformation interrupted with non helical domains

Other types such as IX, XI, XII and XIV show short chains with a fibril conformation, which contain some non-helical domain. On the other hand type VI is microfibrillar collagen and type VII is anchoring fibril collagen (Samuel et al, 1998).

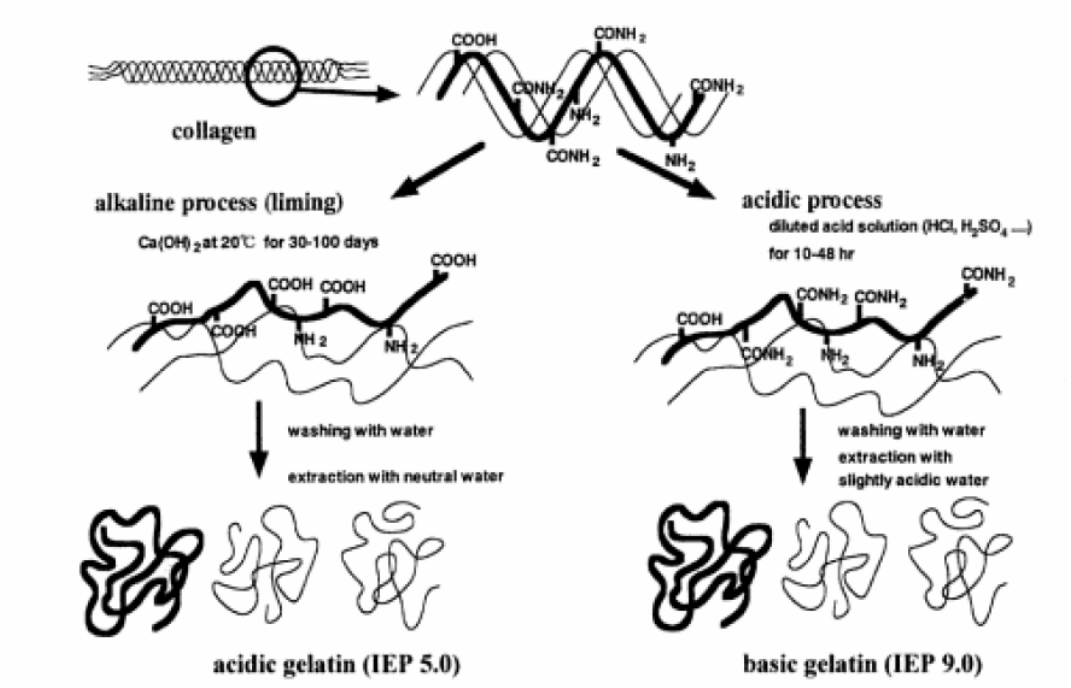
Collagen is biodegradable, biocompatible, non-cytotoxic, it has good ability to support cellular growth and it can be processed into a variety of forms such as: cross-linked films, steps, sheets, beads, meshes, fibres and sponge. Due to this reason collagen is widely used in food industry for the preparation of desserts, candies, bakery, jellied meat, ice cream and dairy products (Toldrà et al, 2012). Other collagen applications in food are as clarifying agent, stabilizer or protective coating material.

The product of thermal denaturation or disintegration of insoluble collagen is gelatine. The molecular weight (MW) and the isoelectric point (IEP) could vary between the type of process applied and the source of collagen used. The most common processes utilised for the production of gelatines could be divided in two methodologies: the acid process and the alkaline or liming process.

In the alkaline process type B gelatine is obtained. This method requires 30-100 days in order to hydrolyse bovine hides or bones. By contrast pig's skin is the raw material for the production of

gelatine type A through acid process, which needs 10-45 hours to be completed (Damrongsakkul et al, 2008). The denaturation process of collagen for both the methodology is reported in figure 7.

Figure 7 Two methods for gelatine extraction from tissues containing collagen (Ikada, 2002).



During the alkaline denaturation process the collagen triple-helix is hydrolysed at covalent site, which link together three peptides, generating a partial hydrolysis of collagen with the releasing of a polydisperse polypeptide mixture with average of MW of 40-90 KDa. On the other hand in the acid process the collagen denaturation is limited to the thermal hydrolysis of peptides with a small amount of α-chains. Type A gelatines (acid process) has an IEP around 7-9, otherwise the IEP of gelatines obtained with the alkaline process is between 4.6-5.4 (Gorgieva and Kokol, 2016). Gelatine could not be considered as a complete polydisperse mixture, because it has a definite molecular weight distribution pattern. In particular it is a mix of different polypeptide chains including α-chains, β (dimers of α-chains) and γ (trimers of α-chains).

Approximately 80 per cent of the edible gelatine produced in Europe is pure pigskin gelatine. 15 per cent comes from cattle hide split. This is the thin layer containing collagen between the upper skin and the subcutaneous layer. The remaining 5 per cent comes from pig and cattle bones and fish.

The application of gelatines in food industry could be divided into four main groups: confectionery (mainly for providing chewiness, texture and foam stabilization), jelly desserts (to provide

creaminess, fat reduction, and mouth feel), dairy products (to provide stabilization and texturization) and meat products (to provide water binding) (Nishimoto et al., 2005, Karim and Bhat, 2009). Table 7 shows the functional properties of gelatines and their application in food.

Table 7 Functional properties of gelatine in food (Turner, 1998)

Function	Type of food
Gel former	Gelled dessert, lunch meats, confectionery, pate, consommé, aspics
Whipping agent	Marshmallows, nougats, mousses, soufflés, chiffon, whipped cream
Protective colloid	Confectionary, icings, ice creams, frozen desserts and confections
Binding agent	Meat rolls, canned meats, confectionery, cheeses, dairy products
Clarifying agent	Beer, wine, fruit juices, vinegar
Film former	Coating for fruits, meats, deli items
Thickener	Powdered drink mixes, bouillon, gravies, sauces, soups, puddings, jellies, syrups, dairy products
Process aid	Microencapsulation of colours, flavours, oils, vitamins
Emulsifier	Cream soups, sauces, flavourings, meat pastes, whipped cream, confectionery, dairy products
Stabilizer	Cream cheese, chocolate milk, yogurt, icings, cream fillings, frozen desserts
Adhesive agent	To affix nonpareils, coconut and other items to confections, to bond layered confections together, to bind frosting to baked goods, to bind seasoning to meat products

Table 8 Advantages and disadvantages of gelatine in food applications (Schrieber and Gareis, 2007)

Advantages	Disadvantages
Multifactorial (texture, surface activity, emulsifier, stabilizer, film former)	Low stability to heat
Melts at body temperature with rapid and intense release of flavour	Low gelation temperature
Unique texture, elasticity and brilliance	Slow gelification
Easy to process	Soluble only at high temperatures (exceptions: instant gelatine and gelatine hydrolysates)
Preventive function in osteoarthritis and osteoporosis	BSE discussion
Protein enrichment	Animal source (vegetarian/vegans)
	Religious reservations

Gelatine production has increased over the years because of the wide range of application in food industry, but also the disadvantages need to be taken into account (table 8).
The biggest problem is related to religious concerns because the main source of gelatine derives from pigskins, cattle bones and hides. In particular both Judaism and Islam do not consume any pork-related products, while Hindus forbid the cattle-related products. Also, in the last decade, the spread of vegetarians/vegans eating habits outlined the need to find other sources to replace the mammalians gelatines in food applications. Also the risk of transmission of pathogenic diseases such as prions (BSE) needs to be taken under control in order to guarantee the safety for the consumer.

To conclude, there is a large variety of techniques and process for treating meat by-products: for the production of energy, for the recycle in human and animal food and for chemical and pharmaceutical use. As far as proteins are concerned, one of the main approaches to recover protein material, particularly if difficult to solubilize, is to produce protein hydrolysates.

1.3 Protein hydrolysates

The protein-rich by-products are used in pet food, feed, organic fertilisers and technical applications. A possible way to add value to proteinaceous meat waste is to convert them into hydrolysates for the production of food ingredients. Due to the broad range of application of protein hydrolysates, there are several methods for producing the hydrolysates, generating mixtures with specific functions. The choice of the method to use depends on the by-products nature, the functional properties for the applicability, the efficiency of the process and the equipment and materials related to the procedure (Lasekan et al, 2013). The amino acid composition (in free form or bound to peptide) is determined by the method and the conditions of the hydrolysis process (Kristinsson and Rasco, 2000). The peptide length is also influenced by the reaction conditions and by the specific protocol used for the hydrolysis; this aspect affects the techno-functional properties of the hydrolysates. In order to hydrolyse proteins, thermal, chemical, microbial and enzymatic methods or the combination of these methods can be employed, which also have an effect on the other components of the biomass matrix or on the integrity of the protein/amino acid themselves. As an example, acid ensilage is widely used to convert by-products derived from livestock/fish and fruit/vegetables but this method requires the use of mineral acids (H_2SO_4, HCl). Rai et al (2009) proposed to substitute these acids with milder organics acids (such as propionic and formic acid) in the hydrolysis of sheep and goat skin, as a better alternative to hazardous and strong mineral acids. Several studies demonstrated that acid treatments deteriorate lipid fraction with a high increase of free fatty acids. Not only acid hydrolysis but also other chemical treatments originated damages in the food components and change the nutritional quality. As an example, the treatment with hydrogen peroxide, which has been used to sterilize milk and decolorize fish protein concentrate, and processes such as hot-air drying, or light irradiation in the presence of air, or even lengthy storage may oxidize some amino acids residues (Cheftel, 1979). The methionine of intact proteins is susceptible to changes caused by degradation and oxidation by hydrogen peroxide and lipid peroxides (Strange *et al.,* 1980).

As an example, hydrogen peroxide is one of the most used chemicals for food treatment, because of its strong oxidising property: it is used as a bleaching agent in some foods such as wheat flour, edible oil, egg white etc. in countries like the US, Canada, Australia and New Zealand. A further application is the use as an antimicrobial agent in food, e.g. milk, and as a sterilizing agent for food

packaging materials. In processing food, the dosage of hydrogen peroxide should be limited to the amount sufficient for the purpose.

As an antimicrobial agent in the production of modified whey (including, but not limited to, whey protein concentrates and whey protein isolates) by ultrafiltration methods, it should be used at a level not to exceed 0.001 percent by weight of the whey, providing that residual hydrogen peroxide is removed by appropriate chemical or physical means during the processing of the modified whey.

Even at these doses and short treatments time, it induces a loss of amino acids and the origination of effluents harmful to the environment and the human beings.

The replacement of strong chemicals such as hydrogen peroxide or extreme pH, or harsh mechanical treatments to treat food products, is the reason why there is an increasing interest in the development of fast and gentle enzymatic methods, in order to improve recovery and keep nutritional quality.

Compared to acidic or alkaline hydrolysis, enzymatic hydrolysis of proteins, using selective peptidase, provides milder conditions of the process and few or no undesirable side reactions of products. Enzymatic proteolysis and solubilisation of proteins from various sources has been studied extensively and described by several researchers (Kristinsson and Rasco, 2000 a,b; Liaset et al., 2000; Nilsang et al., 2005; Bhaskar et al., 2007b). Because of the raising interest in the extraction of new volatile flavour compounds, considered as “natural” products instead of their synthetic counterparts, the proteolytic enzymes strategies were evaluated by Simpson et al (1997). The aroma quality was analysed after optimization studies for the enzymatic hydrolysis of the shrimp meat. In particular the free amino acids distribution and the peptide content influence the taste and other properties of the hydrolysates. As an example the bitter taste derives from the interior hydrophobic amino acids side chains: valine, isoleucine, phenylalanine, tryptophan, leucine and tyrosine (Nilsang et al, 2005).

The hydrolysis reaction in general leads to a gradual cleavage of protein molecule into smaller peptide units, increasing the solubility of the hydrolysed protein (Rai et al, 2009). Furthermore enzymes hydrolyse proteins to free amino acids and short peptides, thereby allowing the nitrogen to be more soluble and making the hydrolyzed mass a more available amino acids source (Espe et al., 1989; Vidotti et al., 2003). As a matter of fact protein hydrolysates could be exploited in the nutritional field for the individuals who are not able to digest the whole/intact protein, for their

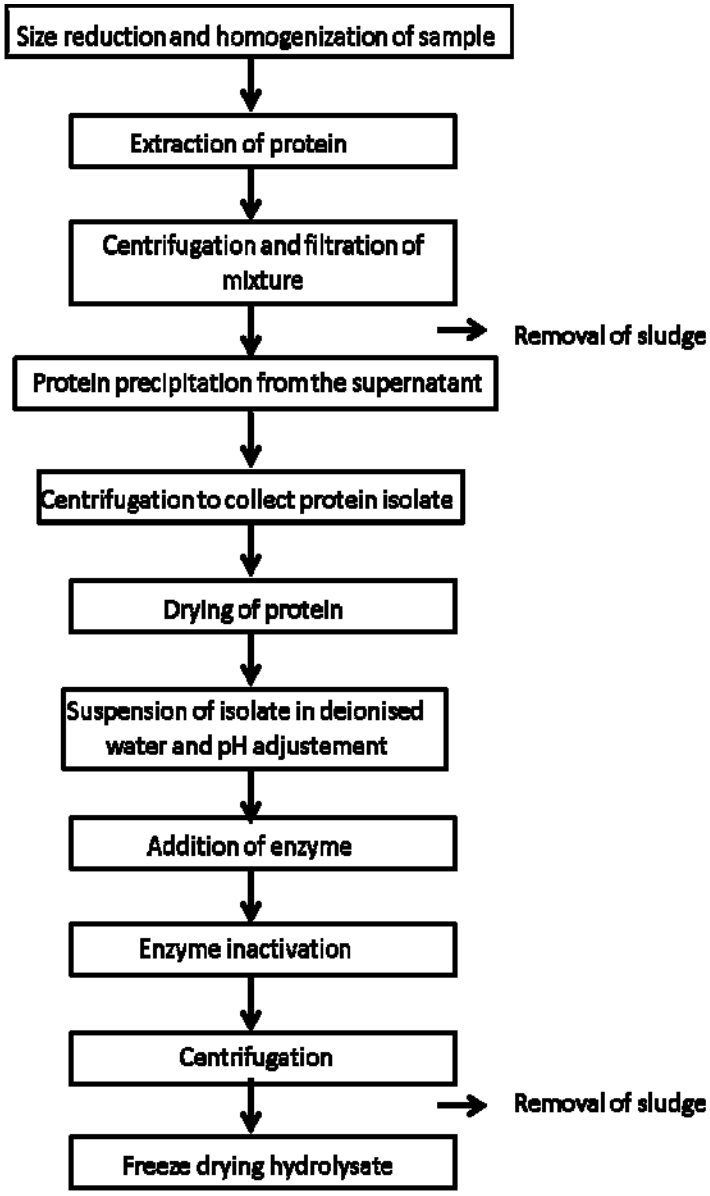
bioactivity and functional properties (Bhaskar et al, 2006). Traditional methods for the preparation of autolytic hydrolysates, like the fish silage, exploit the endogenous enzymes. Anyway, it is rather difficult to control the autolysis by endogenous enzymes due to several factors including the variability of the raw material and the amount of enzymes (Sikoroski and Naczk, 1981). Addition of exogenous enzymes makes the hydrolytic process more controllable, thereby making it reproducible. Several factors, like pH, time, enzyme to substrate ratio and temperature, influence the enzymatic activity co-operatively and thus offer possibilities to control the process (Viera et al., 1995; Liaset et al., 2000;). The most suitable enzymes are derived from plants and microorganism. The preferred commercial bacterial-derived enzymes used by several researchers are Alcalase, neutrase and protease, whereas the plant-derived enzymes are papain, bromelain and ficin (Haslaniza et al, 2013). Beside the differences in the cleavage site, all the enzymes, purchased to produce protein hydrolysates, have at least one common characteristic: they should be food grade and, if they are of microbial origin, the producing organism has to be non-pathogenic. The choice of substrate, the protease that is employed and the degree to which the protein gets hydrolysed generally affect the physiochemical properties of the resulting hydrolysates (Bhaskar et al., 2008). Table 9 summarises the main differences between chemical and enzymatic hydrolysis.

Table 9 Differences between enzymatic and chemical hydrolysis of proteins (Alvarez et al, 2012)

Hydrolysis by peptidases	**Chemical hydrolysis**
Protein is broken in targets: peptides produced are predictable	New peptides can be produced and new functions can be discovered
No desalting step is needed	A salt is produced after neutralisation
There is no loss of amino acids	Some amino acids are destroyed
Free amino acids are poorly produced	Appreciable amounts of free amino acids are produced
Peptidases are an expensive material	Acid and alkalis are cheap reactive
Environmental conditions of hydrolysis (pH, T, etc) must be carefully controlled	Easy control of process
Small amounts of protein can be hydrolysed	Large amounts of protein can be processed
Medium time of hydrolysis (5–6 h)	The hydrolysis time can be controlled through temperature control

Normally the industrial process for the production of protein hydrolysates takes place in a batch-fed reactor or in a continuous way using an ultrafiltration membrane reactor (Toldrà et al, 2012). Figure 8 below portrays a possible flow diagram of protein hydrolysates production.

Figure 8 Process diagram for the production of protein hydrolysates (Lasekan et al, 2013).



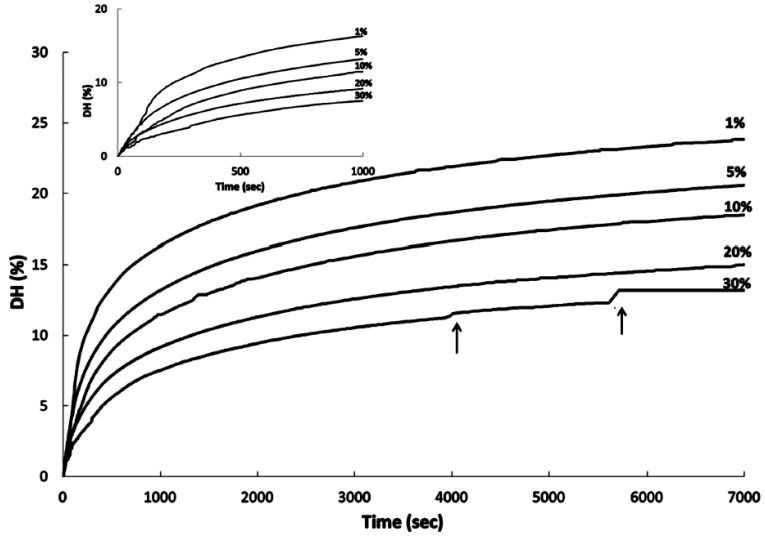
In the first step the protein fraction is obtained from the organic material using water, alkali or acid extraction, depending on the solubilisation pH of the proteins. Then the protein isolate is obtained after precipitation and desiccation (Lasekan et al, 2013). This product is hydrolysed with a partial enzymatic, chemical and chemical-enzymatic hydrolysis reaction. These protein hydrolysates are rich of free amino acids and low molecular weight peptides, especially di- and tri-peptides (Bhaskar et al, 2006).

A deeper analysis of the enzymatic process and mechanism is needed to understand the techno-functional and bioactive properties of these hydrolysates and their future field of use.

Generally the enzymatic hydrolysis mechanism starts after the enzyme binding to the substrate particles, realising peptides and free amino acids in solution. In the process there is an initial rapid phase, after the addition of the enzyme, where a large number of peptide linkage were cleaved per time (Guerad et al, 2001). By time passing, a rapid decrease in rate is observed and the curve reached a plateaux.

Typically this mechanism is studied following the Michaelis-Menten kinetics rules using k_{cat}/k_M to describe the enzyme efficiency. However this type of mechanism is not applicable because in the enzymatic protein hydrolysis there is not a single substrate but a large number of different cleavage sites that can be used as substrate. Beside this experimentally a decrease of the hydrolysis rate and the degree of hydrolysis was observed increasing the substrate concentration and consequently reducing the amount of water in order to reduce the energy consumption fig 9 (Butré et al, 2014).

Figure 9 Hydrolysis curves of whey protein suspensions hydrolysed by Alcalase at 40° for protein concentrations ranging from 1 to 30% (w/v); arrows indicate effect of gelification.



This affect could be explained by several factors: increase in the viscosity, structural changes or stability of the substrate or of the enzyme and finally the change of the selectivity of the enzyme (Butré et al, 2014; Butré et al, 2012). In a recent study the influence of the viscosity was shown not to be the reason of the decrease of the DH nor the presence of the non-dissolved proteins (Butré et al, 2012). The changes of the enzymes could affect the accessibility of the substrate; however Qi

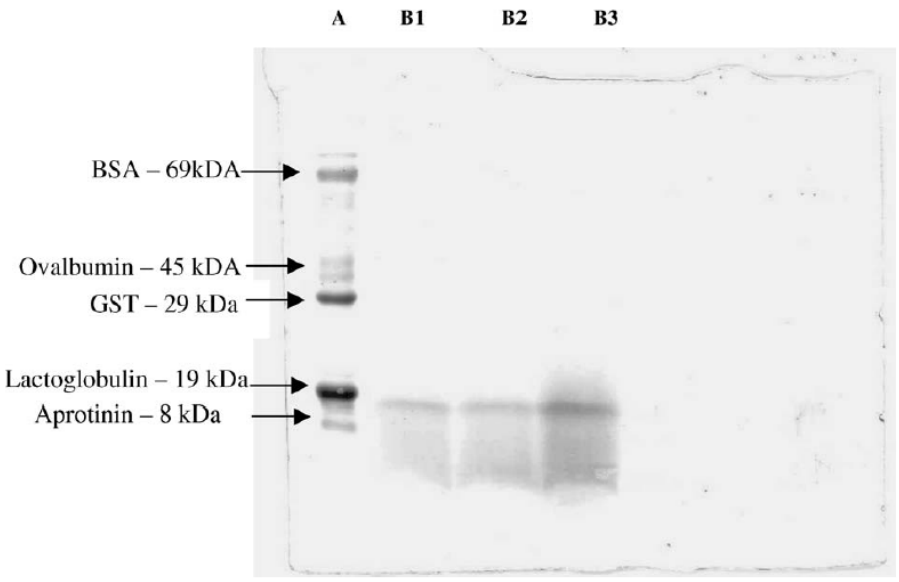
et al (1995) found out that the increase in the concentration showed a minor effect on the structural stability of intact β-lactoglobulin. Butré et al, 2014 results showed that the change in the selectivity can be identified as a function of substrate concentration. The differences of the selectivity could derive from the primary, secondary, tertiary and quaternary structures of the protein. Therefore at high concentration these differences happened because of peptide aggregation, or shuffling of disulphide bridges.

In particular the high concentration of soluble peptides in the solution decreased the degree of material released in the solution and the ratio of the reaction. Due to this reason the higher concentration of the substrate increases the inhibition of nitrogen recovery (Liaset et al, 2000). A possible solution could be the addition of water into the hydrolysis reaction to enhance the protein recovery. On the other hand the addition of water generates high cost for its removal through evaporation or spray-drying.
Also the lipid amount plays an important role in the hydrolysis reaction because Slizyte et al, 2005, observed that the high quantity of lipids in substrate inhibited the solubilisation of the protein fraction. Several protocols showed a pre-treatment step of heating in order to inactivate endogenous enzymes. The heating phase originated denaturised proteins which are highly resistant to enzymatic hydrolysis, reducing the solubilised products (Slizyte et al, 2005).

A useful method for appreciating the solubility of protein hydrolysates is the measure of the percentage of soluble nitrogen. This methodology is done after stirring and centrifugation. The nitrogen solubility value varies from 75% to 100% for different fish protein hydrolysates (Kristinsson and Rasco, 2000).

It is also important to investigate the peptide length and its distribution in the protein hydrolysates. In general the decrease of the large proteins is correlated to the progress of the hydrolysis reaction. This is due to the continuous cleavage of the enzyme, thereby the disappearance of the large proteins bands in the electrophoretic profile occurred. Bhaskar et al (2007) observed that protein hydrolysates from sheep visceral mass had an average weight of >10 KDa, as reported in figure 10.

Figure 10 Electrophoretic pattern of visceral protein hydrolysate: (A-standard marker proteins; B1-B3-sheep visceral protein hydrolysate)



In other cases, the presence of protein fractions with molecular weights ranging from 6500 KDa to free amino acids was observed by Guerard et al (2001), using Alcalase as enzyme with an aspecific site of cleavage.

For this reason there is the need to evaluate the degree of hydrolysis. The degree of hydrolysis is based on the number of peptide bonds which were cleaved and the amount of free amino acids present in solution (Haslaniza et al, 2013). Several methods are used for its detection such as OPA and TNBS methods. Usually by time passing the degree of hydrolysis increase in the hydrolysis reaction because of the quantity of free N-species in the solution increased. Therefore a positive correlation exists between time and degree of hydrolysis. The effect of higher lipid content in the raw material caused in the hydrolysis reaction a decrease in the degree of hydrolysis. This fact is due to emulsion capacity, emulsion stability and fat adsorption which generated low solubility as noticed by Gbogouri et al (2004). By contrast the hydrolysates with a high solubility show a higher degree of hydrolysis.

The degree of hydrolysis also gave an important impact in the taste of the hydrolysates; in particular the bitterness is less detected in protein hydrolysates with a higher degree of hydrolysis (Bashkar et al, 2008). It is remarkable to notice that the hydrolysates, obtained with Alcalase with higher degree of hydrolysis, showed low bitterness compared to the other protease (Hoyle and Merrit, 1994; Benjakul and Morrisey, 1997). It is also important to consider the aroma quality and intensity of these hydrolysates. Simpson at all 1997 stated that the aroma quality of enzyme

hydrolysates derived from the higher quantity of free amino acids in the preparation. During the enzymatic treatment a higher amount of volatile compounds are generated. These molecules derive from the reaction of Strecker and Maillard and also from thermal decarboxylation and deamination reaction of free amino acids in solution (Simpson at all 1997). As a matter of fact the amino acids composition of the protein hydrolysates is important, in particular as far as the essential ones are concerned (histidine, threonine, valine, cysteine + methionine, isoleucine, leucine, tyrosine + phenylalanine, lysine and tryptophane) (Bhaskar et al, 2007). From the analysis of amino acids the EAA (essential amino acids) and the PDCAAS (protein digestibility corrected amino acids score) could be calculated to determine the nutritional benefit of the hydrolysates.

To conclude it is important that a protein hydrolysate owns a higher recovery of soluble nitrogen and a good degree of hydrolysis. The reasonable amount of low weight peptides could lead to find bioactive properties of the hydrolysates. Finally to use these hydrolysates in food or feed as a nutritional supplement, a good PDCASS and in vitro digestibility need to be observed.

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2. AIMS OF THE THESIS

AIMS

The general aim of this thesis was to study routes for recovering and valorising two meat industry co-products thought enzymatic hydrolysis: bovine fleshing and bovine hides.

In particular, the following lines of research have been pursued:

1. Recover bovine fleshing meat by enzymatic hydrolysis, by testing the proteolytic activity of six different enzymes and characterising in molecular details the N-fraction of the hydrolysates in order to assess the best enzyme for the digestion in terms of nitrogen compound recovery and preservation of protein quality. As further step, optimise the conditions of the enzymatic hydrolysis with the most efficient enzyme for a scale up-reaction (Chapter I and II).

2. Compare, on bovine hides, the efficiency of two cleaner unhairing methods developed at a pilot scale level in semi-industrial reactors: one based on proteolytic enzymes and the other based on chemical agents (sodium hydroxide and hydrogen peroxide). The wastewaters obtained from both methods were characterized in order to assess their potential for future possible valorisation in feed formulations or as food ingredients (Chapter III).

3. Examine the use of enzyme hydrolysis as a mean to obtain protein hydrolysates from full pieces of bovine hide. The effect of hydrolysis time and water/substrate ratios on hydrolysis process was evaluated. In addition the techno-functional properties and film forming ability of the freeze-dried hydrolysates were evaluated and the glycation reaction was tested to enhance the techno-functional properties of the peptides-rich hydrolysates. Finally the effect of two levels of replacement (10% and 20%) of bovine hides hydrolysates was evaluated in a real food matrix (Irish breakfast sausages) (Chapter IV, V and VI).

3. EXPERIMENTAL STUDIES

Chapter I

Degradation of collagen increases nitrogen solubilisation during enzymatic hydrolysis of fleshing meat

Degradation of collagen increases nitrogen solubilisation during enzymatic hydrolysis of fleshing meat

Abstract

The meat portion directly attached to bovine hides (fleshing meat) is a co-product of leather industry that is a potential new source of proteins. In literature different enzymatic and chemical methods have been proposed to hydrolyse and solubilize fleshing meat. Enzyme hydrolysis is preferable for the mildness and the lower environmental impact. Enzymatic hydrolysates have never been deeply characterized, nor the specific enzyme efficiency deeply investigated. In this study, the activity of six proteolytic enzymes was tested in order to determine their efficiency in solubilizing fleshing meat and to characterize at the molecular level the composition of the nitrogen fraction of the obtained hydrolysates. Total nitrogen content and the degree of hydrolysis were determined by Kjeldhal and o-phthaldialdehyde (OPA) methods. In particular amino acids and peptides were analysed by high performance liquid chromatography (HPLC) and mass spectrometry techniques. The results showed that papain and Alcalase appear to be the most efficient ones, and the corresponding hydrolysates were rich in peptides and amino acids characteristic of collagen, notably absent in the hydrolysates obtained with other enzymes. Thus, the ability to efficiently hydrolyse collagen seems to be related to the efficiency in fleshing meat hydrolysis.

Keywords: fleshing meat; enzymatic hydrolysates; amino acid analysis; degree of hydrolysis; peptide analysis

1. Introduction

Hides form a significant part of the meat industry co-product mass, accounting for 5% to 8% in cattle [1]. The removed material, before using hides as raw material in leather industry, is called fleshing meat and constitutes about 15% of the hides mass [2]. In the case of salted raw hide used in leather processing the reported average composition of fleshing meat is 80% water, 8% proteins (mostly collagen), 8% salts and 4% fats [3]. Differently the composition of fleshing meat before tanning was 87% water, 4-6% proteins, 1-2% fat and carbohydrates [4].

The reuse of this fleshing meat usually is done through a solubilisation with thermal processes and the use of chemicals such as strong alkali that allow the efficient solubilisation of collagen, but they also require high amounts of energy, are time consuming, not environmentally friendly. By contrast the use of proteolytic enzymes is a more favourable alternative to solubilize fleshing meat and requires low energy, low temperatures and no use of chemicals. In literature, recently Sundar et al. [4] reviewed different studies where several approaches were tested for the hydrolysis of fleshing meat .

In the study of Kumaraguru et al. [5], the hydrolysates, that were obtained from the pancreatic enzymes after a total solubilization, were analysed in terms of total protein, total collagen and total fat content.

Bajza and Vrcek [6] used an Alcalase preparation to solubilize lime treated fleshing meat, obtaining a 80-90% solubilisation of the starting material at very high enzyme concentrations (500 units per gram of fleshing meat), providing free amino acid content measured by thin layer chromatography (TLC). Indeed, in a recent paper by Zhang et al. [7], Alcalase activity on collagen was reported to be very high, leading to high degree of hydrolysis. The proteolytic activity of trypsin was tested by Fernandez-Hervas et al. [8] to solubilize collagen from sheepskin trimmings, but only a 5.8 percent of collagen was extracted.

The effect of papain and neutrase to extract gelatine from lime treated fleshing meat was studied by Damrongsakkul et al. [9]. The product from papain hydrolysis showed a solubilisation percentage around 70-80%. Particularly it was a gelatine solution of low viscosity and forming gels with low strength, while that from neutrase hydrolysis is collagen hydrolysate with viscosity as low as water, because of a different molecular composition of the hydrolysates. More recently it was found by Jian et al. [10] that ultrasound can enhance the yield of the digestion by enzymatic hydrolysis of protein waste.

The previous results indicate that efficient enzymatic solubilisation of fleshing meat is indeed possible with enzymes such Alcalase and papain, although a complete information on the chemical composition of the obtained hydrolysates is lacking. Moreover, the reasons why these enzymes are efficient and others (such as trypsin) less is still unclear. The aim of the present work was to explore the enzymatic digestion of untreated fleshing meat in a systematic way, using common commercial enzymes: pepsin, trypsin, dispase, Alcalase, pancreatin, papain. Efficiency of hydrolysis was assessed by measuring the solubilisation of the fleshing meat. Hydrolysates were deeply characterized at the molecular level by determining free and total amino acid content and the most abundant peptides. Our results clearly indicated that the most efficient enzymes in solubilizing fleshing meat are the ones able to efficiently degrade collagen.

2. Material and Methods

2.1 Samples

20 samples of cattle hides, with fleshing meat, were provided by Inalca Industria Alimentare Carni SpA (Castelvetro di Modena, Italy) and stored at -20°C. The pieces came from a single animal, and were taken from different parts of the body, in order to average the intrinsic anatomic variability of the raw material. After thawing, fleshing meat was obtained by mechanical removal with a scalpel. The samples were finely minced into one batch.

2.2 Reagents and solvents

Deionized water was obtained from a Millipore Alpha Q-Waters purification system (Billerica, MA, USA). Enzymes were used as commercially available and the units indicated are the ones provided by the companies, together with the batch number. Pepsin from porcine gastric mucosa (250 U/mg, 117K0811), trypsin from porcine pancreas (10 U/g, 029K7012), dispase from *Bacillus polymyxa* (10 U/g, BCBM8270V), Alcalase from *Bacillus licheniformis* (2.4 U/g, SLBL2953V), pancreatin from porcine pancreas (4 x USP, SLBM4075V), papain from papaya latex (1.5-10 U/g, SLBJ6115V), sodium dihydrogen phosphate, hydrochloric acid, calcium chloride, acetonitrile, ethylenediamminetetraacetic acid, DL-cystine, sodium acetate anhydrous, dichloromethane, diethylether, calcium acetate hydrate, tyrosine, phenylalanine, N-acetyl-L-cysteine, sodium tetraborate decahydrate, DL-isoleucine and D-asparagine, L-glutamine, D-arginine, DL-norleucine,

hydrogen peroxide solution 30%, sulfuric acid, formic acid and trifluoroacetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Copper oxide and hydrobromic acid were obtained from Carlo Erba (Milan, Italy).

Ammonium bicarbonate, o-phthalaldehyde, boric acid, D-cystein were bought from Fluka (Buchs, Switzerland). Sodium dodecyl sulphate was purchased from Biorad (Hercules, CA, U.S.A). Kjeldahl tablets defoamers and catalyst 3.5 g/tablet were purchased from Merck (Darmstadt, Germany). AccQ-Fluor reagent kit was obtained from Waters (Milford, MA, U.S.A).

All analyses were done in triplicate.

2.3 Moisture and fat content determination

Moisture content of fleshing meat was determined using an official method [11]. Four samples of 4.5 g each were dried in previously weighed glass dishes in a vacuum oven (Thermair, MSL srl, Lissone, Italy) at 105 °C for 24 h. After cooling in a desiccator the dried samples were weighed again. The moisture content was calculated as the difference in the weight before and after desiccation.

Crude fat content was also determined using the standard A.O.A.C [11] procedures. 4.5 grams of samples in pre-weighed flasks were extracted with diethylether (60 mL) using an automatized Soxhlet fat extractor (SER 148/3 VELP SCIENTIFICA, Usmate Velate, Italy).

2.4 Protein content

Protein content was determined using standard procedures [11] with a rapid Kjeldahl system (VELP SCIENTIFICA DKL heating digester and VELP SCIENTIFICA UDK 139 semiautomatic distillation unit; VELP SCIENTIFICA,) from total nitrogen content (%N x 5.81). This coefficient was obtained by total amino acid composition, assuming an equimolar amount of Asn/Asp and Gln/Glu.

2.5 Enzymatic hydrolysis

Fleshing meat (5 g) was mixed with each enzyme (1% w/w) in 45 mL of the specific buffer solution, and left standing for 12 hours. The hydrolysis was performed by applying for each enzyme the optimal conditions indicated by Sigma Aldrich [12], as described in Table 1.

Table 1. Reaction conditions used in the enzymatic hydrolysis, as indicated by the enzyme suppliers.

enzyme	T = °C	pH	solution buffer
Pepsin	37	3.0	HCl 10 mM
Pancreatin	37	7.8	NH_4HCO_3 25 mM, $CaCl_2$ 2.5 mM
Trypsin	37	7.8	NH_4HCO_3 25 mM, $CaCl_2$ 2.5 mM
Alcalase	60	6.5	Na_2HPO_4 10 mM
Papain	60	7.5	Na_2HPO_4 10 mM, EDTA 2 mM, DL-Cystine 4 mM
Dispase	37	7.3	CH_3COONa 10 mM, $(CH_3COO)_2Ca$ 5 mM

Fleshing meat was homogenized with Ultra-turrax T 50 basic (IKA-WERKE, Staufen, Germany) for 2 minutes and the enzyme was added up to a final ratio of 1% (weight enzyme/weight raw material). The hydrolysis reaction was left overnight under stirring. The day after the hydrolysed mixtures were centrifuged at 4000 x g for 30 min and pellet was separated from the supernatant. These two fractions were stored at -20°C until required for analysis. This procedure was repeated for blanks in the same conditions without enzymes.

2.6 Degree of hydrolysis using o-phthaldialdehyde (OPA) method

The determination was done according to a previous study, with slight modification [13]. Briefly the OPA/NAC (N-acetyl-cysteine) reagent (100 mL) was prepared by combining 10 mL of 50 mM OPA (in methanol) and 10 mL of NAC 50 mM, 5 mL of 20 % (w/v) SDS, and 75 mL of borate buffer (0.1 M, pH 9.5). The reagent was covered with aluminium foil to protect from light and allowed to stir for at least 1 h before use. The OPA assay was carried out by the addiction of 20 µL of sample (or standard) to 2.4 mL of OPA/NAC reagent. The absorbance of this solution was measured at 340 nm with JASCO B-530 Uv-Vis-Spectrophotometer (JASCO, Oklaoma City, OK, U.S.A). A standard curve was prepared using L-isoleucine (0-2 mg mL^{-1}). The degree of hydrolysis (DH) was calculated using formulas (1) and (2) assuming that every amino acid was involved in a peptide bond at the beginning of the reaction and calculating in the DH only the proteinaceous materials present in solution after hydrolysis:

1. % DH = moles of free nitrogen atoms from alpha amino groups in amino acids after hydrolysis (in solution, OPA determination) / moles of nitrogen atoms in amide bonds before hydrolysis
2. moles of nitrogen atoms in amide bonds before hydrolysis = g total proteins (determined by as reported in section 2.4) / average residual amino acid molecular mass

In order to calculate with a high degree of reliability, what was the contribution of the side chain to OPA reactivity for several amino acids, model experiments were performed using amino acids

standard solutions of cysteine, arginine, glutamine and asparagine. Cysteine side chain was found to be nonreactive with the OPA-reagent (Cys:OPA ratio 1:1, thus only the alpha amino group is reacting), in accordance with the literature data [14]. As expected, also the amidic groups of Asn and Gln did not react with OPA. On the other side, in the case of Arg, two N-sites of the guadinium group reacted in addition to the alpha amino group (Arg:OPA ratio 1:3). The data was properly used for correction in order to exactly calculate the free alpha nitrogen groups.

2.7 Free and total amino acids by high-performance liquid chromatography with fluorescence detection (HPLC/FLD) after AccQ•Tag derivatization

The determination of free and total amino acids was performed according to Nollet [15], with slight modifications. For free amino acids, 50 µL (in case of high phosphotungstic acid (PTA)-soluble nitrogen values) or 100 µL (in case of low PTA-soluble values) of sample were mixed with 34 µL of Norleucine (5 mM in deionized water) as internal standard, and then the volume was brought to 1 mL with deionized water. Calibration was performed with standard solutions prepared mixing 40 µL of Norleucine (2.5 mM in HCl 0.1 N), 40 µL of amino acids hydrolysate standard mixture, 40 µl of cysteic acid (2.5 mM in HCl 0.1 N) and 880 µL of deionized water. 10 µL were transferred into 1.5 mL tubes, and 70 µL of borate buffer were added in order to keep the optimal pH range for the derivatization (pH = 8.5), then the solution was vortexed. 20 µL of reconstituted AccQ•Fluor reagent were finally added and the mixture was vortexed for few seconds. The tube was left closed and at room temperature for 1 minute and it was then inserted in a heated bath at 55°C for 10 min. The derivatised samples were diluted with 400 µL of deionised water before injecting in the HPLC system. For total amino acids, 0.5 g of pellet or 3 mL of supernatant were weighed into a 18 mL Pyrex glass tube fitted with Teflon-line screw caps. 6 mL of HCl 6 N were added and mixed. The tube was flushed with nitrogen for 1 min in order to remove air. Hydrolysis was carried out at 110°C for 23h. After letting the tubes cool at room temperature, the internal standard (7.5 mL of Norleucine 5 mM in water) was added. The mixture was filtered through filter paper and collected into a 250 mL volumetric flask. Acid hydrolysis was used for the determination of all amino acids except tryptophan (Trp), cysteine (Cys) and methionine (Met). In particular during acid hydrolysis tryptophan is degraded because of its high sensibility to acids, and for this reason it was not detected. For Cys and Met performic acid oxidation followed by acid hydrolysis was used. In this case, an amount of 0.5 g of pellet or 3 mL of supernatant was weighed in a 18 mL Pyrex glass tube fitted with Teflon-line screw caps. After adding 2 mL of performic acid freshly prepared (by mixing 9 volumes of formic acid with 1 volume of hydrogen peroxide),

samples were kept in an ice bath for 16 h at 0°C. Then 0.3 mL of hydrobromidric acid was added in order to remove excess performic acid. The bromine formed during the reaction was removed with nitrogen flow. Then acid hydrolysis was performed as described above.

All samples were analysed with an Alliance 2695 separation system with a Waters AccQ•Tag amino acid analysis column (3.9mm×150mm). The column was heated at 37°C and the flow rate was set at 1.0 mL/min. The injection volume of samples was 10 µL, while standard calibration solution was injected at several volumes: 2.5, 5, 10, 15, 20, 25 and 30 µL, corresponding to 5, 10, 20, 30, 40, 50 and 60 pmoles of amino acids hydrolysate standard mixture injected. Mobile phase A consisted of AccQ•Tag eluent A (100 mL AccQ•Tag A concentrate + 1L deionized water). Mobile phase B was a 60:40 (V/V) solution of acetonitrile and deionized water respectively. Gradient elution was performed according to the following steps: 0 min 100%A, 1 min 97%A, 13 min 93%A, 18 min 90%A, 38 min 67%A, 51 min 67%A, plus washing step and reconditioning. Detection was carried out by Waters 470 fluorescence detector ($\lambda_{excitation}$ = 250 nm and $\lambda_{emission}$ = 395 nm). Quantitative analysis was carried out by using the internal standard method.

2.8 Peptide identification

High resolution mass spectrometry was performed on the samples for peptide identification using a µHPLC DIONEX Ultimate3000 interfaced with a LTQ-Orbitrap XL Thermo Fisher Scientific was used (Thermo Fisher Scientific, Waltham, MA, U.S.A). Column: Jupiter C18 4 µm, Proteo 90 Å 150×0.30 mm, Phenomenex; eluent A: H_2O + 0.1 % FA; eluent B: ACN + 0.1 % FA; flow: 5 µL/min, gradient: 0–4 min from 100 % A to 95 % A, 4–60 min from 95 % A to 50 % A, 60–62 min from 50 % A to 10 % A, 62–72 min 10 % A, 72–74 min from 10% A to 95 % A, 74–90 min 95 % A; analysis time (min): 90; column temperature (°C): 30; injection volume (µL): 5; acquisition time (min): 0-75; ionization mode: ESI+; scan range (m/z): 200–1,800; source voltage (kV): 3.5; capillary voltage (kV): 35; source temperature (°C): 275. Scan event details: (Fourier transform) FTMS+p res=30,000 or (250.0–2000.0); (ion trap) ITMS+c Dep MS/MS Most intense ion form; activation type: CID; isolation width: 2.00; normalized coll. energy: 35.0; default charge state: 2; activation Q: 0.250; activation time: 30.000; dynamic exclusion enabled; repeat count: 2; repeat duration (s): 10.00; exclusion duration (s):30.00. Charge state rejection: enabled; unassigned charge states: rejected; charge state 1: rejected; charge state 2: not rejected; charge state 3: not rejected; charge states 4+: not rejected; ion signal threshold: 10,000.

3. Results and discussion

3.1 Enzymatic hydrolysis of the samples and solubilized nitrogen

The average moisture and fat percentage was determined to be 78.9% (± 2.5) and 1.6% (± 0.4) respectively, the protein content was found to be 19.7% (± 2.8) on the wet weight, differently from what reported in the literature, with more proteins and less fat and ashes present. The samples were hydrolysed for 16h with common commercial enzymes derived from vegetal (papain), bacterial (Alcalase, dispase) or animal sources (pepsin, trypsin, pancreatin). All the reaction conditions used, according to the provider specifications, are reported in table 1.

In order to calculate the efficiency of the process, the amount of nitrogen released after 16h of reaction was determined by the Kjeldahl method. As a blank control, the same determination was done on the samples in the same conditions without enzymes. The nitrogen in the remaining pellet was also determined in all cases, in order to check the mass balance. The solubilisation ratio (nitrogen in solution/total nitrogen) with and without enzyme is reported in table 2, together with the DH determined in the supernatants by the OPA method. The DH was calculated on the proteinaceous materials released in solution, so only counting the solubilized proteins.

Table 2 Solubilisation ratio and DH of enzymatically hydrolysed fleshing meat

	Solubilisation ratio (N in solution/total N) without enzyme[a]	**Solubilisation ratio (N in solution/total N) with enzyme[a]**	**Remaining material (N%left in the pellet)**	**DH% with enzyme[b]**
Pepsin	0.03	0.18	-[c]	1.2
Pancreatin	0.05	0.25	75%	6.4
Trypsin	0.05	0.38	64%	5.0
Alcalase	0.03	0.97	4%	30.9
Papain	0.04	0.97	4%	6.5
Dispase	0.03	0.18	86%	7.1

a. Same conditions reported in table 1 applied with and without enzymes for 16h. Results from three replicates. Maximum RSD = 0.17
b. The DH was calculated taking into account only the proteins present in solution after enzymatic hydrolysis. Results from three replicates. Maximum RSD = 0.09. %DH on samples without enzyme was <0.8% in all cases. Maximum RSD = 0.05
c. Impossible to determine due to gel formation

Alcalase and papain were the most efficient, releasing in solution 97% of the proteins originally present. Differently trypsin and pancreatin gave slightly better results than pepsin and dispase

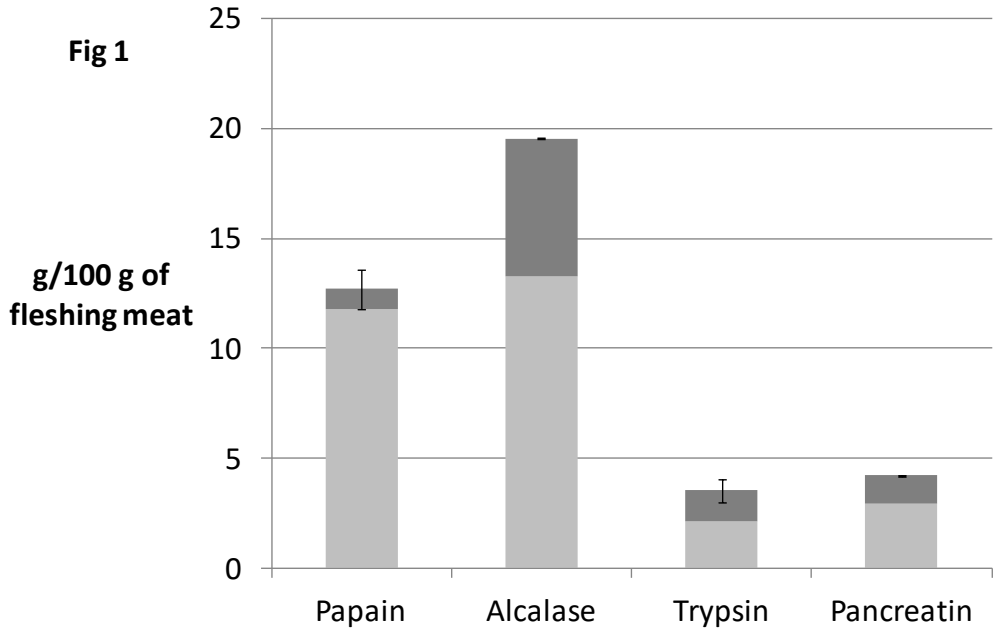
(38%, 25%, and 18%, 18%). Bajza and Vrcek [6] and Dramrongsakkul et al. [9] also studied the efficiency of the Alcalase and papain in solubilizing fleshing meat material and reported the total solubilisation of fleshing meat with these enzymes. Also consistently with our data, Fernandez-Hervas et al. [8] observed a quite inefficient solubilisation of fleshing meat with trypsin.

While Alcalase and papain treatments both resulted in a high solubility, there was a significant difference between the DH of the two resulting hydrolysates (Alcalase: 30.9% and papain: 6.5%). The final DH after Alcalase hydrolysis indicated that the nitrogen released was mainly in the form of free amino acids or very small peptides (average peptide length released in solution 3.2 residues), whereas in the case of papain hydrolysis, albeit giving the same solubilisation ratio, the lower DH indicates that the nitrogen released is mainly in form of medium and long peptides (average peptide length released in solution 15.4 residues).

3.2 Amino acid composition of the solubilized nitrogen fractions

Total and free amino acids were determined on the solutions obtained with the four most active enzymes, i.e. Alcalase, papain, trypsin and pancreatin (Figure 1).

Figure 1 Distribution of total and free amino acids in the supernatant after enzymatic hydrolysis: sum of the total amino acids (full bar) and free amino acids (darker part of the bars). The lower part of the bar, in a lighter grey, indicates amino acids which are not part of the free pool, thus contained in soluble peptides.



From the amount of total amino acids it was confirmed that basically all the proteins contained in the fleshing meat materials were indeed solubilized in case of papain and Alcalase treatments (the sum of total amino acids matched with the total protein content measured by Kjeldahl), whereas less than half of them were solubilized in case of trypsin and pancreatin. The solution obtained by Alcalase treatment had the highest relative proportion of free amino acids, and papain the lowest, with the other two lying in between.

The total amount of every single amino acid measured in the four different solutions is reported in Table 3.

Table 3 Total amino acid content of the solutions obtained by enzymatic hydrolysis of fleshing meat (content expressed as mg/mL solution. RSD percentage <11%)

Total AA	Alcalase hydrolysis (mg/mL)	Papain hydrolysis (mg/mL)	Trypsin hydrolysis (mg/mL)	Pancreatin hydrolysis (mg/mL)
Cys	23.7	26.5	7.6	7.1
Hpro	144.7	92.9	12.2	20.9
Asp	61.8	41.7	14.5	11.6
Ser	34.3	24.2	7.9	6.7
Glu	103.0	62.9	28.8	30.6
Gly	137.8	88.9	6.4	6.5
His	20.1	13.1	9.5	8.9
Arg	65.1	44.9	9.1	10.2
Thr	29.1	18.0	5.5	8.7
Ala	58.1	41.3	13.1	25.4
Pro	78.8	53.3	6.3	8.3
Tyr	20.8	15.7	4.9	4.8
Val	32.7	19.6	10.3	11.8
Met	17.2	10.2	4.6	4.5
Lys	56.7	30.0	14.3	18.4
Ile	24.4	13.7	6.9	6.9
Leu	42.7	25.1	9.8	11.0
Phe	25.7	12.3	5.1	8.5

In the case of the solutions deriving from the action of Alcalase and papain, the two most abundant amino acids in the total pool were glycine and hydroxyproline, a strong indication that the solubilized proteins were mostly constituted by collagen peptides and amino acids, since these latter two amino acids are the most abundant in the collagen proteins. In case of the solutions deriving from trypsin and pancreatin action, less hydroxyproline and glycine were present in relative terms, indicating a less efficient solubilisation of collagen.

The above presented data indicate that Alcalase and papain, the most efficient in solubilizing fleshing meat, generated hydrolysates rich in glycine and hydroxyproline. These data suggest that, at the molecular level, the ability to efficiently hydrolyse collagen is the requisite required for also having efficient fleshing meat solubilisation.

3.3 Peptide composition of the solubilized nitrogen fractions

The solutions were also analysed by high resolution mass spectrometry in order to identify the most abundant peptides present in them and the proteins of origin (Table 4).

In the solutions obtained by the hydrolysis with Alcalase and papain most of the peptides found in solutions were collagen-derived peptides. On the other side, in trypsin- and pancreatin-derived solutions, the most abundant peptides identified derived from muscular protein, such as myosin. This is in agreement with the previous data suggesting that the ability to hydrolyse collagen seems to be the characteristic of the enzymes most able to efficiently solubilize fleshing meat.
Moreover, the collagen-derived peptides identified in case of papain hydrolysis were, on average, longer than the ones identified in case of Alcalase hydrolysis, which is consistent with the higher DH observed in the latter case.

Table 4 Identification by LTQ-OrbiTrap of the main peptides present in the solutions derived from enzymatic cleavage on fleshing meat, and of the proteins of origin. Proteins are listed in order according to the highest number of peptides generated.

Enzyme	Identified Peptides	Protein	Average peptide length[a] (N°AA)
Trypsin	106	Myosin-2	13
	92	Myosin-1	12
	92	Myosin-2	12
	75	Myosin-7	12
	63	Myosin-2 (Fragment)	13
	59	Myosin-7	13
Pancreatin	211	Titin	12
	98	Elastin	18
	78	Isoform 7 of elastin	18
	50	Myosin-2	9
	48	Myosin-1	9
	35	Myosin-1 fragment	9
Papain	115	Collagen alpha-1(I) chain	16
	92	Collagen alpha-2(I) chain	14
	48	Actin, alpha skeletal muscle	11
	46	Collagen alpha-1(II) chain	19
	31	Myosin-2	11
	29	Myosin-1	11
Alcalase	64	Collagen alpha-1(I) chain	11
	62	Elastin	18
	58	Isoform 7 of Elastin	18
	57	Collagen alpha-1(III) chain	12
	56	Collagen alpha-1(III) chain	13
	8	Myosin light chain 1/3, skeletal muscle isoform	19

a. the average peptide length is calculated on peptides automatically annotated. Automatic annotation excludes very short peptides, since they cannot be univocally assigned to a specific proteins, thus the average peptide length is not corresponding to the one calculated by DH

4. Conclusion

Alcalase and papain were found to be the enzymes with the best hydrolytic activity on fleshing meat, with 97% solubilisation, yielding solubilized nitrogen mixtures rich in hydroxyproline and glycine (the most abundant amino acids in collagen) and collagen-derived peptides. Quite interestingly, albeit being equally efficient in solubilisation, these two enzymes act on collagen producing hydrolysates with different distribution of the nitrogen substances: Alcalase produces a larger amount of free amino acids and small peptides, whereas papain generates less free amino acids and longer peptides. The different end point composition suggests that the two enzymes can be used to tailor the hydrolysate composition for different purposes, such as gelatine production, thickening agents, texturizers and others. For future industrial implementation, we can evaluate that Alcalase is considerably less expensive than papain.

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Chapter II

Optimization and scale up reaction of bovine fleshing's enzymatic hydrolysis

Optimization and scale up reaction of bovine fleshing's enzymatic hydrolysis

Abstract

A high amount of waste and pollutants are produced every day by leather industries. Due to their impact on the environment, various protocols and processes have been studied for the recovery and the reuse of such products. In the present study we focused on fleshing, the co-product formed by the meat scraped from bovine hides. Enzymatic hydrolysis was applied in order to recover in soluble form the protein fraction from it, for possible further reuse. Surface response methodology was used in order to determine the optimum reaction conditions for achieving the complete hydrolysis of two types of fleshing deriving from calf and steer. Based on these results, a reaction scale-up and a consecutive characterization of the protein fraction of the hydrolysates were performed. The optimized conditions allowed obtaining an almost complete digestion of fleshing with an 8-hour process, also taking into account the variability of the bulk material, resulting in broths rich in digested high quality proteins. The protocol here presented could have a potential application for the recovery of fleshing in the form of protein hydrolysates available for feed industry.

Keywords: fleshing, Alcalase, protein hydrolysate, optimization, scale-up.

1. Introduction

Leather industry produces a high amount of waste and pollutants, rich in substances harmful for the environment and for the human beings. The tannery process is made of several steps, which produce a great quantity of waste. The solid ones are: raw hide/skin trimmings, limed fleshing, hide splits and chrome shavings [1]. Various procedures have been studied in order to make this process more eco-friendly. Some protocols replaced toxic reagents with greener ones [2-4], while other groups focused the attention on the recovery of these waste through their recycle and their valorisation in different fields [5-8]. One of the most interesting parts discarded is fleshing, a term referring, in bovines and other animals, to the meat part located just under the skin. Converting fleshing into enzymatic hydrolysates allows nitrogen solubilisation, because of the production of free amino acids and peptides in solution, at the same time providing protein hydrolysates of sufficient quality to be used in the food and feed industry for multiple applications, such as production of flavours, improvement of nutritional quality, formulation of high-protein feed ingredients, and palatability-enhancing agents for use in animal foods [9].

In literature, various works are found which examined several approaches to recover and reuse fleshing, derived from different animal sources, through the production of protein hydrolysates [10-14]. In particular Raju et al [11] used pancreatic enzymes from chicken intestines and Kumuranguru et al [13] studied the activity of the commercial pancreatic enzymes for the hydrolysis of fleshing. In both cases the complete digestion is reached. Bajza and Vrček [14] and Simenova and Dalev [10] used treated fleshing to obtain protein hydrolysates after enzymatic hydrolysis with protease from *Bacillus licheniformis*, obtaining 80-90% and 95% of solubilisation. Moreover, Jian et al [12] studied the effect of the ultrasound in the hydrolysis of the pig skin fleshing, fixing their attention more on the novel technology aspect, than a deeper analysis of the hydrolysates. All the above papers focused on the proximate analysis of the hydrolysate, without a deeper characterization, also in order to clarify why some enzymes are more efficient than others. This was covered in our recent work [15] where the proteolytic activity of six different enzymes was tested. The complete characterization at the molecular level of the hydrolysates allowed understanding that the high efficiency of Alcalase from *Bacillus licheniformis* and papain from *Papaya latex* derived from their ability to degrade collagen, the main component of fleshing. In that work a complete solubilisation of fleshing in high value protein hydrolysates was obtained. Anyway, from an industrial point of view, before scaling up a process there is the need to find the

optimum conditions in order to obtain the highest yield possible in the most convenient, economically and environmentally friendly conditions.

A useful tool to determine the optimum condition of a process is with Response Surface Method (RSM). This type of analysis was widely applied in the food waste and food co-products recovery with the employment of chemical and enzymatic hydrolysis [16-18]. In the case of fleshing, the optimization of the hydrolysis conditions were tested by Bhaskar et al [19] and Rai et al [20] on fleshing material coming from delimed sheep and goat skins. In particular, Rai et al [20] obtained the optimum conditions with this bulk material by the application of acid hydrolysis using a combination of formic acid and propionic acid 1:1. Although the hydrolysates showed antioxidant activity, the acid hydrolysis is time consuming. Bashkar et al [19] investigated, on the same waste material, the optimisation of the reaction conditions' for both enzymatic hydrolysis and microbial fermentation. In the case of the proteins hydrolysates the yield was 49% and the amino acids profile, the SDS-page profile and the colour were evaluated.

The present study aims to optimize the enzymatic hydrolysis conditions, using the untanned fleshing of calf and steer, as raw materials for the production of hydrolysates of sufficient quality to be used as food or feed additives, to perform a scale up of the reaction by finding the optimized conditions, and to analyse and characterize the N-fraction of the final broths.

2. Materials and methods

Samples of fleshing from calf and steer were provided by Inalca Industria Alimentare Carni SpA (Castelvetro di Modena, Italy) and stored at -20°C. Frozen fleshing was initially coarsely grinded by Breaker and meat cutter RISKO TR160 PSA1222/n 1 (Velati, Tribiano, Italy) and then homogenised by Moulinex DJ2005 Illico for 2 minutes (Moulinex SA, Courbevoie, France). After these treatments it was stored at -20 °C.

2.1 Reagents and solvents

Deionized water was obtained from a Millipore Alpha Q-Waters purification system (Billerica, MA, USA). Alcalase from *Bacillus licheniformis* (2.4 U/g), sodium dihydrogen phosphate, hydrochloric acid, acetonitrile, DL-cystine, dichloromethane, N-acetyl-L-cysteine, sodium tetraborate decahydrate, DL-isoleucine and DL-norleucine, hydrogen peroxide solution 30%, sulfuric acid,

formic acid and trifluoroacetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Copper oxide was obtained from Carlo Erba (Milan, Italy).

O-phthalaldehyde and boric acid were bought from Fluka (Buchs, Switzerland). Sodium dodecyl sulfate was purchased from Biorad (Hercules, CA, U.S.A). Kieldahl tablets defoamers and catalyst 3.5 g/tablet was purchased from Merck (Darmstadt, Germany). AccQ-Fluor reagent kit was obtained from Waters (Milford, Ma, U.S.A).

2.2 Raw material chemical composition

Moisture content of fleshing was determined using the method of A. O. A. C. [21]. 4.5 g of sample were dried in previously weighed glass dishes in a vacuum oven (Thermair, MSL srl, Lissone, Italy) at 105 °C for 24 h. After cooling in a desiccator the dried samples were weighed again. The moisture content was estimated as the difference of weight before and after desiccation. Protein content was determined using standard A. O. A. C. [21] procedures with a rapid Kjeldahl system (VELP SCIENTIFICA DKL heating digester and VELP SCIENTIFICA UDK 139 semiautomatic distillation unit, VELP SCIENTIFICA, Usmate Velate, Italy) from total nitrogen content (%N x coefficient). The coefficients were obtained by total amino acid composition of calf (5.83) and steer (5.88) fleshing. Crude fat content was also determined using the standard A.O.A.C [21] procedures. 5 grams of samples in pre-weighed flasks were extracted with diethylether (60 mL) using an automatized Soxhlet fat extractor (SER 148/3 VELP SCIENTIFICA).

2.3 Quantification of free and total amino acids by high-performance liquid chromatography with fluorescence detection (HPLC/FLD) after AccQ•Tag derivatization

For free amino acid determination, according to Nollet [22], 100 µL of samples were employed. Samples were mixed with 34 µL of Norleucine (5 mM in deionized water) which was added as internal standard, and then the volume was brought to 1 ml with deionized water. In order to perform a calibration curve, a standard solution was prepared: 40 µL of Norleucine (2.5 mM in HCl 0.1 N), 40 µL of amino acids hydrolysate standard mixture (Sigma Aldrich, Saint Luis, Missouri, U.S.A), 40 µl of cysteic acid (2.5 mM in HCl 0.1 N), 40 µl of hydroxyproline (2.5 mM in HCl 0.1 N) and 840 µL of deionized water were mixed. Then 10 µL either of samples or standard solution were transferred into 1.5 mL tubes, and 70 µL of borate buffer. Then the solution was vortexed. 20 µL of reconstituted AccQ•Tag reagent (Waters, Milford, Massachusetts, U.S.A) were finally added

and the mixture was vortexed for few seconds. The tube was left closed and at room temperature for 1 minute and then it was warmed up in a heated bath at 55°C for 10 min. The derivatised samples were diluted with 400 µL of deionised water before injecting in the HPLC system.

Samples were analysed with an Alliance 2695 separation system with AccQ•Tag amino acid analysis column (3.9mmX150mm), (Waters, Milford, Massachusetts, U.S.A). The column was thermostated at 37°C and the flow rate was set at 1.0 mL/min. The injection volume of samples was 10 µL, while standard calibration solution was injected at several volumes: 2.5, 5, 10, 15, 20, 25 and 30 µL, corresponding to 5, 10, 20, 30, 40, 50 and 60 pmoles of amino acids hydrolysate standard mixture (Sigma Aldrich, Saint Luis, Missouri, U.S.A)injected. Mobile phase A consisted of AccQ•Tag eluent A (100 mL AccQ.Tag A concentrate+1L deionized water). Mobile phase B was a 60:40 (V/V) solution of acetonitrile and deionized water respectively. Gradient elution was performed according to the following steps: 0 min 100%A, 1min 97%A, 13 min 93%A, 18 min 90%A, 38 min 67%A, 51 min 67%A, plus washing step and reconditioning. Detection was carried out by Waters 470 fluorescence detector ($\lambda_{excitation}$ = 250 nm and $\lambda_{emission}$ = 395 nm).

Quantitative analysis was carried out by using the internal standard method. To calculate the total amino acid content, according to Nollet [22], 0.5 g of skin or 3 mL of broth were weighed into a 18 mL Pyrex glass tube fitted with Teflon-line screw caps. 6 mL of HCl 6N were added and mixed. The tube was fluxed with nitrogen for 1 min in order to remove air. Hydrolysis was carried out at 110°C for 23 h. After the tubes cooled down at room temperature, the internal standard (7.5 mL of Norleucine 5 mM in water) was added. The mixture was filtered throughout filter paper and collected into a 250 mL volumetric flask. HPLC/FLD analysis after AccQ•Tag derivatization was performed as described above for free amino acid determination. Then the derivatization and the analysis were performed as described above.

2.4 Enzymatic hydrolysis optimization

An amount of 5 g of fleshing was mixed with the appropriate percentage from 1% to 0.1% of Alcalase from *Bacillus licheniformis* for 16 h with different amount of distilled water (45, 25 and 15 mL) at 60°C. The hydrolysis reaction was left overnight under stirring. The day after the hydrolysed mixtures were centrifuged at 4000 x g for 30 min at 4°C and fat was separated from the supernatant by filtration. The Kjeldahl analysis was immediately performed.

2.5 Statistical analysis

Analysis of the data obtained through the response surface method (RSM), to achieve the optimized conditions was accomplished using the same STATISTICA™ software (Statsoft, Tulsa, OK, U.S.A). RSM data was analysed to acquire response surface and desirable levels of independent factors in order to find optimum protein content by least square method.
The fitting curve was executed with Sigmaplot™ software (Sysat, San Jose, USA).

2.6 Enzymatic hydrolysis scale-up reaction

After the determination of the optimum conditions with RSM, a scale-up reaction was performed. The hydrolysis reactions were carried out in a reactor (Steroglass s.r.l, Perugia, Italy) of 5L of capacity with a heating shell and agitator blade. The sampling was executed by collecting of supernatants every 10 minutes in the first hour and later every hour (from 2 to 16, the last sampling was at 25 h). The enzyme was inactivated by boiling the solution at 90°C for 2 minutes. Each solution coming from calf and steer fleshing samples, were analysed with Kjeldahl method.

2.7 Determination of degree of hydrolysis (DH) using OPA (o-phthaldialdehyde) method

The OPA method used was the one reported by Spellman et al [23], with slight modification. Briefly, the OPA/NAC (N-acetyl-cysteine) reagent (100 mL) was prepared by combining 10 mL of 50 mM OPA (in methanol) and 10 mL NAC, 5 mL of 20 % (w/v) SDS, and 75 mL of borate buffer (0.1 M, pH 9.5). The reagent was covered with aluminium foil in order to be protected from light and was allowed to stir for at least 1 h before use. The OPA assay was carried out by the addiction of 20 µL of sample (or standard) to 2.4 mL of OPA/NAC reagent. The absorbance of this solution was measured at 340 nm with JASCO B-530 UV-Vis-Spectrophotometer (JASCO, Oklaoma City, OK, U.S.A). A standard curve was prepared using L-isoleucine (0-2 mg mL^{-1}).

2.8 Peptide identification by LTQ-Orbitrap analyses

For high resolution mass spectrometry, a µHPLC DIONEX Ultimate3000 interfaced with a LTQ-Orbitrap XL Thermo FisherScientific was used (Thermo Fisher Scientific, Waltham, MA, U.S.A). Column: Jupiter C18 4 µ, Proteo 90 Å 150×0.30 mm, Phenomenex; eluent A: H2O + 0.1 % FA; eluent B: ACN + 0.1 % FA; flow: 5 µL/min, gradient: 0–4 min from 100 % A to 95 % A, 4–60 min from 95 % A to 50 % A, 60–62 min from 50 % A to 10 % A, 62–72 min 10 % A, 72–74 min from 10%

A to 95 % A, 74–90 min 95 % A; analysis time (min): 90; column temperature (°C): 30; injection volume (µL): 5; acquisition time (min): 0-75; ionization mode: ESI+; scan range (m/z): 200–1,800; source voltage (kV): 3.5; capillary voltage (kV): 35; source temperature (°C): 275. Scan event details: (Fourier transform) FTMS+p res=30,000 or (250.0–2000.0); (ion trap) ITMS+c Dep MS/MS Most intense ion form; activation type: CID; isolation width: 2.00; normalized coll. energy: 35.0; default charge state: 2; activation Q: 0.250; activation time: 30.000; dynamic exclusion enabled; repeat count: 2; repeat duration (s): 10.00; exclusion duration (s):30.00. Charge state rejection: enabled; unassigned charge states: rejected; charge state 1: rejected; charge state 2: not rejected; charge state 3: not rejected; charge states 4+: not rejected; ion signal threshold: 10,000.

3. Results and discussion

3.1 Fleshing bulk characterization

The percentage of proteins, fat and moisture of the samples used in the present work are reported in Table 1. Steer fleshing had the higher percentage of proteins than calf fleshing, otherwise calf fleshing presented the higher percentage of fat. These differences derived both from the different ages of the two animals and also from the liming treatment which altered the fat and protein content in the residual fleshing.

Table 1 Chemical characterization of the bulk materials

	Calf		**Steer**	
	Average	Dev. st	Average	Dev. st
% proteins	8	1	12	2
% moisture	34	1	47	1
% fat	54	2	39	3

The percentage distribution of total amino acids in calf and steer fleshing (Table 2) was similar; in particular it was found a great amount of Hydroxyproline, Glycine and Proline. The presence of these amino acids suggests that a great amount of collagen is in fleshing because of its proximity to the skin in agreement with Rai et al [20].

Table 2 Amino acids percentage distribution analysis by HPLC-FLD

	Calf	Steer
Hpro	13	14
Asp	6.8	6.1
Ser	3.5	3.2
Glu	11.3	10.4
Gly	13	11
His	2.0	2.3
Arg	7.5	6.9
Thr	3.1	3.2
Ala	8.4	8.8
Pro	8.0	7.1
Tyr	2.0	2.5
Val	4.3	4.3
Met	1.1	1.6
Lys	4.8	6.5
ile	2.8	3.0
Leu	5.1	5.1
Phe	3.1	3.4

Results in three replicates. Maximum RSD = 0.12.

3.2 Enzymatic hydrolysis

Enzymatic hydrolysis was used to recover and reuse fleshing through the production of protein hydrolysates, according to our previous results [15]. In the present work, optimized conditions were determined by experimental design, and protein hydrolysates were produced by modulating different reaction parameters. The influence of volume of solution added to the fleshing (X1) and % of enzyme (X2) on the hydrolysis reaction performed by Alcalase was determined for calf and steer fleshing using factorial design, as described in material and methods section. The reaction temperature were kept for all the experiments at 60°C, and the amount of starting material was also kept constant at 5g. The observed values of both steer and calf fleshing, for solubilised proteins at different combination of the independent variables are showed in table 3.

Table 3 Independent variables along with the observed values for the response variables, protein content derived from the hydrolysis of calf and steer fleshing

Volume (ml)	% enzyme	Proteins (g/1 g of calf fleshing)	Proteins (g/1 g of steer fleshing)
45	1	0.078	0.121
25	1	0.077	0.119
15	1	0.073	0.118
45	0.8	0.076	0.118
25	0.8	0.075	0.115
15	0.8	0.073	0.112
45	0.5	0.073	0.114
25	0.5	0.072	0.112
15	0.5	0.070	0.108
45	0.3	0.071	0.112
25	0.3	0.071	0.110
15	0.3	0.068	0.107
45	0.1	0.069	0.109
25	0.1	0.067	0.108
15	0.1	0.067	0.107

According to variance analysis, the model used for the two different type of fleshing was significant. The R^2 values for both models were higher than 0.75, indicating a good fit. In particular for fleshing calf R^2 was 0.93 and for fleshing steer 0.92. The analyses of coefficients for each type of fleshing used to fit the data are given in table 4. The results obtained by fleshing calf demonstrated that, among the independent variables, volume had significant effect ($p<0.05$) and volume^2 owned high significant effect ($p<0.01$). The percentage of the enzyme added seems not to influence the analysis. For the interaction variable, model coefficient for solubilised proteins gained high significance ($p<0.01$). For fleshing steer only the percentage of Alcalase influenced the analysis with significant effect ($p<0.05$), as for the interaction between volume and enzyme percentage. The coefficients of independent variables (volume and enzyme percentage) with significance were positive values both for calf and steer fleshing. This fact explained that when these variables increased, the solubilised protein values have the same trend.

In Figure 1A and 1B the response for the interaction between enzyme percentage and volume during the hydrolysis of calf and steer fleshing for the protein content is presented. The optimum levels of different independent variables were determined by employing the desirability profile

(figure 1C and 1D) by assigning the highest desirability of 1.0 to the highest solubilised proteins observed while a desirability of 0 was assigned to the lowest solubilised proteins. The desirability profile indicated that an optimum solubilised proteins could be achieved from both calf and steer with a volume of 28.3 mL (fleshing:solution ratio 1:6) and 0.54% of Alcalase.

The model equations for solubilised proteins in calf and steer fleshing, the response variable of calf fleshing as a function of the independent variables and their interactions, using the constant, linear and quadratic regression coefficients was derived as:

Y_{calf}= 0,0686474735157+0,000357454986439*"VOLUME (mL)"-8,13981296127e-006*"VOLUME (mL)"^2-0,00327465127613*"% ENZYME"+2,55892689807e-005*"% ENZYME"^2+0,000281556051499*"VOLUME (mL)*% ENZYME"

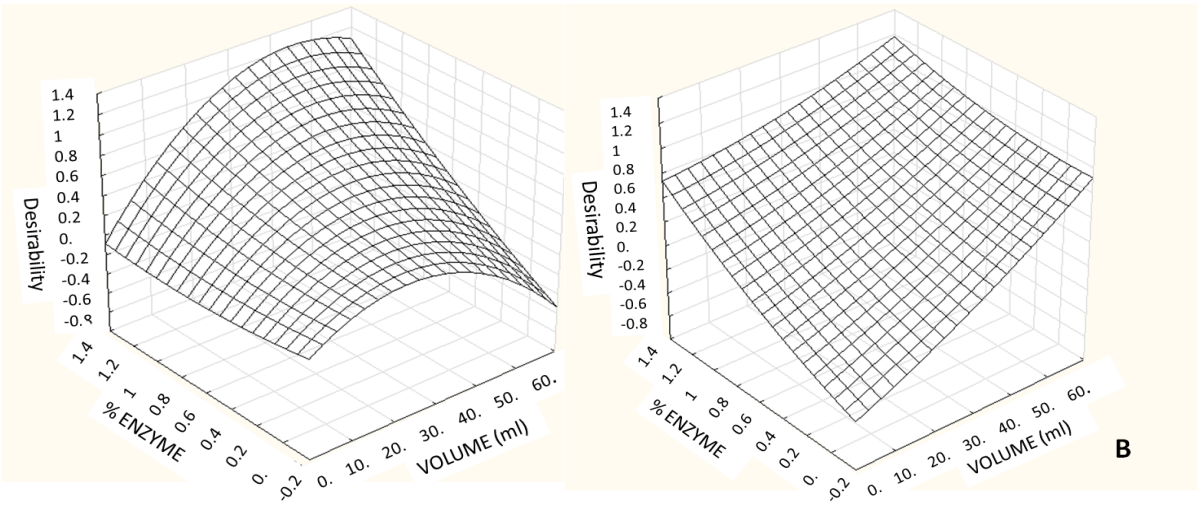
Y_{steer}= 0,0944663972221+0,000460598746233*"VOLUME (mL)"-8,05050971553e-008*"VOLUME (mL)"^2+0,015694680205*"% ENZYME"+0,000584811642178*"% ENZYME"^2-0,000244859175282*"VOLUME (mL)*% ENZYME"

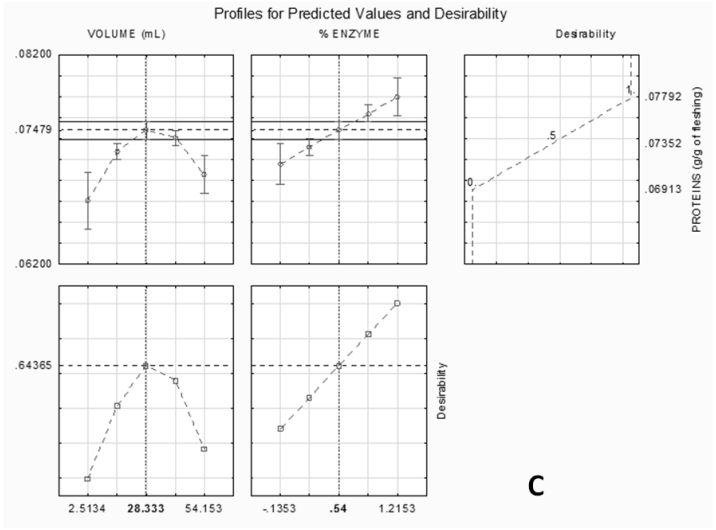
Fig 1a Three dimensional response surface plot of the effects of enzyme (Alcalase) and volume on desirability for calf fleshing hydrolysis

Fig 1b Three dimensional response surface plot of the effects of enzyme (Alcalase) and volume on desirability for steer fleshing hydrolysis

Fig 1c Profile of desirability level for different factors for optimum DH for hydrolysis of calf fleshing

Fig 1d Profile of desirability level for different factors for optimum DH for hydrolysis of steer fleshing


Desirability
% ENZYME
VOLUME (ml)
B


Profiles for Predicted Values and Desirability
VOLUME (mL)
% ENZYME
Desirability
PROTEINS (g/g of fleshing)
Desirability
C

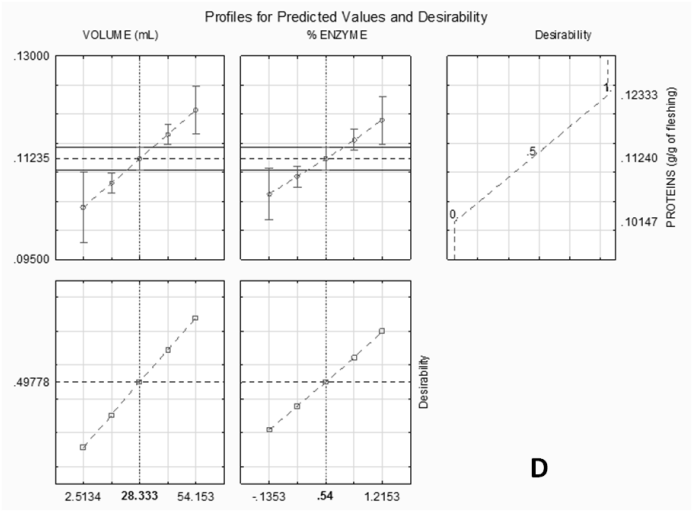

Profiles for Predicted Values and Desirability
VOLUME (mL)
% ENZYME
Desirability
PROTEINS (g/g of fleshing)
Desirability
D

Table 4 Analysis of variance (ANOVA) of response variables as affected by independent variables and their interactions based on experimental runs

Calf fleshing	**SS**	**df**	**MS**	**F**	**P**
Independent variables					
Volume	0.000004	1	0.000004	10.413	0.010375
% enzyme	0.000001	1	0.000001	1.662	0.229477
Interactions					
Volume^2	0.000009	1	0.000009	21.448	0.001227
% enzyme^2	0.000000	1	0.000000	0.0000	0.990066
Volume *% enzyme	0.000020	1	0.000020	49.643	0.000060c
error	0.000004	9	0.000000		
Steer fleshing	**SS**	**df**	**MS**	**F**	**P**
Independent variables					
Volume	0.000007	1	0.000007	3.4117	0.097805
% enzyme	0.000015	1	0.000015	7.5338	0.022670
Interactions					
Volume^2	0.000000	1	0.000000	0.0004	0.984196
% enzyme^2	0.000000	1	0.000000	0.0169	0.899459
Volume *% enzyme	0.000015	1	0.000015	7.4092	0.023532
Error	0.000018	9	0.000002		

3.3 Scale-up reaction

The results of the optimum conditions for the hydrolysis of fleshing, obtained by the RSM method with Statistica™ software, were applied in a scale up reaction. The experiments were performed in the same conditions [0.54 % (w/v) of Alcalase, fleshing: solution ratio 1:6, 60°C] starting from calf and steer fleshing in a reactor of 5 l of capacity. The kinetics reactions were studied following the solubilisation of the proteins. In particular in figure 2 the kinetic profiles of the solubilisation of fleshing calf and steer as function of time of hydrolysis are showed. After 2 hours the calf fleshing, which has the less protein amount, was completely solubilised, otherwise the steer fleshing, which was more abundant in proteins, needed 8 hours to be entirely solubilised. This method is less time consuming and with higher nitrogen recovery than the acid protocol proposed by Rai et al [20], where after 9 day only the 42,5 % of nitrogen was obtained

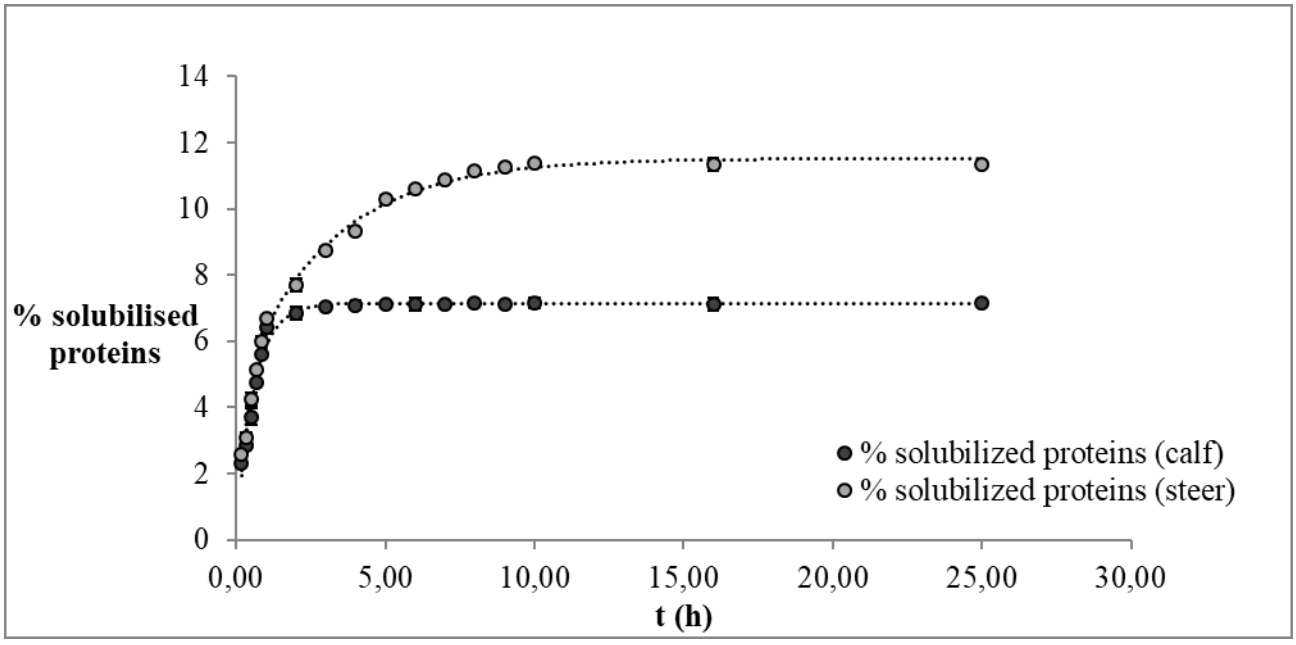


Figure 2 The hydrolysis reaction of calf and steer fleshing as function of time

Figure 3 shows the increase of the degree of hydrolysis (DH) as function of time, calculated on the protein material released in solution, determined by monitoring the reaction of the free amino groups with OPA. In both cased DH reached a value of 25%, with similar trends in time. This indicates that in spite of the variability of the starting raw materials, the two type of fleshing underwent the attack of Alcalase with the same mechanism of hydrolysis.

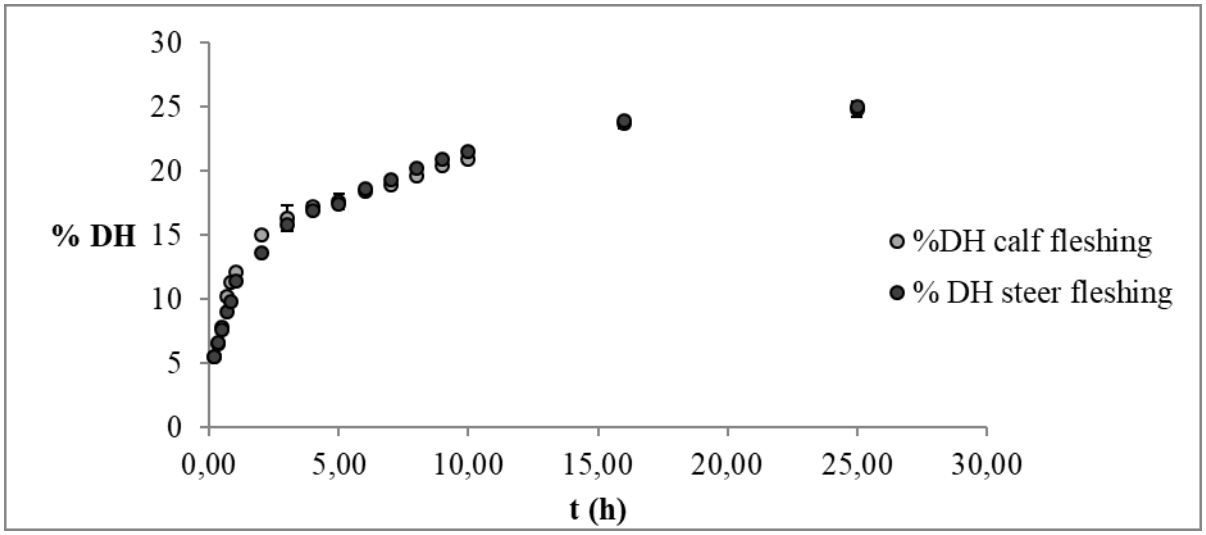


Figure 3 The increase of the DH by time passing for the hydrolysis of calf and steer fleshing.

In Figure 4 total (full bar) and free amino acid (dark grey part of the bar) content in solution after 16 and 25 hours of the hydrolysis reaction are shown. The results are perfectly consistent with the bulk nitrogen content presented in Figure 2, and indicated that a considerable part of the released nitrogen fraction (more or less half) was in form of free amino acids, consistently with the observed high DH. The rest of the solubilized nitrogen (light grey part of the bar) is represented by soluble peptides.

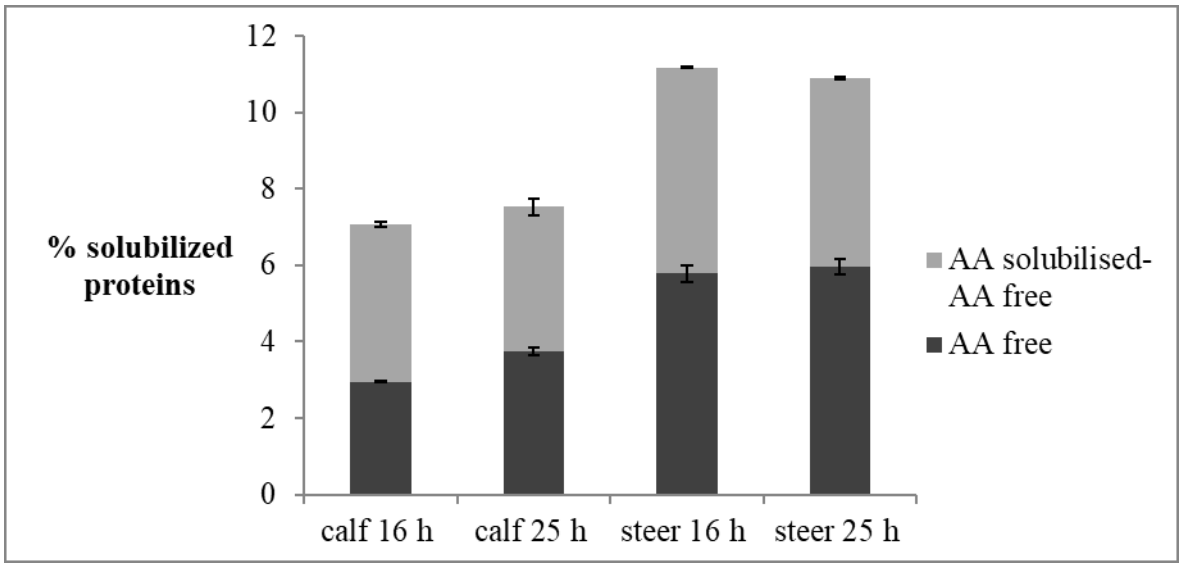


Figure 4 Distribution of total and free amino acids in the supernatant after enzymatic hydrolysis: sum of the total amino acids (full bar) and free amino acids (darker part of the bars)

A deeper analysis on the identity of these peptides was performed on both peptide fractions at 16h and 25h by LTQ-orbitrap mass spectrometry. The results reported in Table 5 show that most abundant peptides came from collagen, demonstrating again that Alcalase efficiency is strictly linked to its ability to degrade collagen, the major protein constituent of the fleshing, as already demonstrated by us in a previous work [15].

Table 5 Identification by LTQ-OrbiTrap of the main peptides present in the solutions derived from enzymatic cleavage on fleshing, and of the proteins of origin. Proteins are listed in order according to the highest number of peptides generated

Sample	Peptides	Protein
Calf 16 h	71	Collagen alpha-1(I) chain
	40	Collagen alpha-1(III) chain
	37	Collagen alpha-2(I) chain
Calf 25 h	80	Collagen alpha-1(I) chain
	43	Collagen alpha-1(III) chain
	41	Collagen alpha-2(I) chain
Steer 16h	102	Collagen alpha-1(I) chain
	79	Elastin
	53	Collagen alpha-2(I) chain
Steer 25h	105	Collagen alpha-1(I) chain
	66	Elastin
	58	Collagen alpha-2(I) chain

4. Conclusion

In this work, we showed the optimization of a hydrolysis method which could be usefully applied to recycle and valorise fleshing, a co-product of meat and leather industry. In particular, the proteolytic activity of Alcalase from *Bacillus licheniformis* was evaluated on fleshing samples coming from calf and steer. Taking into account the variability of the raw material, the results acquired by the experimental design allowed obtaining the optimum conditions to have a complete digestion of fleshing. The best selected procedure needs at least 8 hours for a complete degradation, with a fleshing: solution ratio of 1:6 and Alcalase concentration of 0.54%. This preliminary study could be considered in future for the setting up of green methodologies for the recovering of fleshing through protein hydrolysates and for the eventual reuse of them in feed/food industry.

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Chapter III

Towards environmentally friendly skin unhairing process: a comparison between enzymatic and oxidative methods and analysis of the protein fraction of the related wastewaters

Towards environmentally friendly skin unhairing process: a comparison between enzymatic and oxidative methods and analysis of the protein fraction of the related wastewaters

Abstract

Environmental problems due to the great amount of pollutants released during leather processing are generated in particular for the unhairing step.

For this reason, several strategies are studied today to reduce its impact. In this work two different cleaner methods, developed at a pilot scale level in semi-industrial reactors, were compared: an unhairing method based on proteolytic enzymes (Alcalase from *Bacillus licheniformis*) and an oxidative one based on chemical agents (sodium hydroxide and hydrogen peroxide). Apart from the unhairing efficiency, also the impact of both treatments on the nitrogen fraction in wastewaters was determined in order to assess the suitability of these co-products for a possible recovery in food/feed industry: the protein amount, the total and free amino acids, peptides and the percentage of amino acid racemisation were determined on broths, washing residues and skins. The oxidative method seemed to be the most efficient and less expensive unhairing methodology. On the other side, the wastewaters of enzymatic treatments, quite rich in good quality proteins, could be recovered for feed/food formulations, whereas the wastewaters coming from the oxidative treatments, due to the oxidative damage, only for lesser value applications. The results indicated that both methods have the potential to be more sustainable alternatives to the current ones.

Keywords:

Skin processing; enzymatic unhairing; oxidative unhairing; protein fraction; wastewaters

Abbreviations

Abbreviations	Extended Name
Ala	Alanine
Arg	Arginine
Asp	Aspartate
BOD	Biological Oxygen Demand
CID	Collision Induced Dissociation
Cys	Cysteine
COD	Chemical Oxygen Demand
FTMS	Fourier Transform Mass Spectrometry
GC-MS	Gas Chromatography-Mass Spectrometry
Gly	Glycine
Glu	Glutamate
His	Histidine
HPLC/FLD	High Performance Liquid Chromatography with a Fluorescence Detector
Ile	Isoleucine
ITMS	Ion Trap Mass Spectrometry
Leu	Leucine
Lys	Lysine
Met	Methionine
MS	Mass Spectrometry
NAC	N-acetyl-L-cysteine
OPA	O-phthalaldehyde
Phe	Phenylalanine
Pro	Proline
SEM	Scanning Electron Microscopy
Ser	Serine
SIMS	Secondary Ion Mass Spectrometry
Thr	Threonine
TSS	Total Suspended Solids
Tyr	Tyrosine
Val	Valine

1. Introduction

The most pollutant step in leather processing is the unhairing process. Strong chemicals are used, such as sodium sulphide and lime, which represent approximately 80-90 % of total pollution of leather manufacturing (Dettmer et al., 2012a). Despite these problems, unhairing is a fundamental step in leather industry, because it has two main aims: the removal of the hair and the opening-up of the collagen fibrous structure for the following processes on the skin (Alexander et al., 1986). Thus, sulphide and lime used in traditional unhairing should be better replaced by biotechnological processes with a lower impact on the environment.

A strategy developed to decrease the environmental impact of tanneries implies replacing sodium sulphide with other chemicals, in order to avoid the release of H_2S, because of neurological and respiratory problem (Dettmer et al., 2012a). Shi et all, (2003) investigated the role of hydrogen peroxide in the destruction of the hair and Marmer and Dudley (2004 and 2006) examined the oxidative chemicals degradation of keratins (wool and bovine hair); on the other hand, Kanagaraj et al. (2016) examined the remediation of sulphide on goat skins. Among the new technologies, the use of proteolytic enzymes has also been widely experimentally tested (Kanagaraj et al., 2015). The enzymatic unhairing was investigated starting from different raw materials with the addition of several enzymes. Different techniques were used to understand the quality of the process, analysing the mechanical and physical properties of the leather and the morphology of the unhaired skin by optical microscopy and SEM. The research group of Jian et al.l, (2009 and 2010) employed an enzymatic unhairing method on pigskin also with the ultrasound support.

Dettmer et al, (2012a, 2012b and 2011) screened the proteolytic activities of bacteria, using isolated strains derived from the sludge of local tannery, respectively over azocasein, keratin and azocoll and then on bovine hides. A similar study was performed by Senthivelan et al. (2012) starting from goat skins using 2% of protease for 16 h at 45 °C. Saurabh et al. (2013) tested the unhairing and the defatting efficiency of protease and lipase reaching the complete unhairing and degreasing of the skin. These experiments were also done for the bovine hide unhairing with commercial enzymes (Borras et al, 2008). Moreover, the combination of enzymes and chemicals was also studied by several research groups. In particular, Crispim and Mota (2003) used commercial enzymes (Riberzyme MPX, Erhavit MC), together with environmental unfriendly chemicals (calcium hydroxide and sodium sulphide). Also, Andrioli and Gutteres (2014) tested the

contemporaneous action of the enzyme and the hydrogen peroxide obtaining good unhairing results.

George et al. (2014) isolated an extracellular alkaline protease (*Vibrio metschnikovii* NG 155) from soil samples of leather industry (George et al., 2013). After the purification and the characterization, this protease was used in the unhairing of buffalo hides and goat skin. The morphological and physicochemical properties of the dyed crust were characterised (George et al., 2014). Anyway, even this work, like the previous ones, focused on the quality of the unhairing process, and did not give any indication on the quality of the wastewaters released, and on the possibility to recover and reuse the organic materials there contained. In particular, in some of the previous research works, the sludge was only partially characterized. The determination of biological oxygen demand (BOD), chemical oxygen demand (COD), total dissolved solids (TDS) and total suspended solids (TSS) were evaluated, but there was a lack of deeper chemical and quality analyses of the wastewaters for a possible recycle as food/feed ingredients or other applications.

The aim of this work is to compare two alternative unhairing methods, executed at a semi-industrial level, which have less environmental impact than the traditional one, using less aggressive chemicals. This not only would prevent the use of toxic compounds and their discard in the environment, leading to a cleaner production, but would allow a further advantage: indeed, the obtained wastewaters, instead of being discarded, could be recycled (mostly considering their nitrogen content) in various applications, actually leading, in the respect of the principles of circular economy, to a further valorisation, improving the overall of the process. Anyway, all previously reported works were done at a laboratory scale, whereas the application at industrial level was not deeply investigated yet.

In particular, the unhairing of bovine skin was performed in a pilot plant in semi industrial reactors, using both an oxidative method, based on the action of sodium hydroxide and hydrogen peroxide, and an enzymatic method, based on the action of Alcalase from *Bacillus licheniformis*. Polypropylene drums were used for the oxidative process and stainless steel drums for the enzymatic unhairing. These two reactions have been studied by first observing the unhairing efficiency. Then, the nitrogen fraction in broths was deeply characterized at the molecular level. This last step was performed in order to assess the impact of their release in the environment and to investigate the possible recycling of hydrolysed broths as potential additives to feed/food products in order to increase their nutritional value.

2. Materials and Methods

Samples made by pieces of bovine skin were provided by Inalca Industria Alimentare Carni SpA (Castelvetro, Modena, Italy). 15 kg of skin were used for oxidative unhairing method and 11 Kg for the enzymatic one. The experiments were performed in Po.Te.Co s.c.r.l Technogical centre (Santa Croce sull´Arno, Pisa, Italy).

2.1 Reagents and solvents

Alcalase from *Bacillus licheniformis* (2.4 U/g), sodium dihydrogen phosphate, hydrochloric acid, acetonitrile, dichloromethane, N-acetyl-L-cysteine, sodium tetraborate decahydrate, sodium thiosulfate, DL-isoleucine, DL-norleucine, 2-propanol, trifluoroacetic anhydride, sulfuric acid, formic acid, acetic acid and trifluoroacetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Copper oxide, starch paste 1% and potassium iodide were obtained from Carlo Erba (Milan, Italy).

O-phthalaldehyde and boric acid were bought from Fluka (Buchs, Switzerland). Sodium dodecyl sulphate was purchased from Biorad (Hercules, CA, U.S.A). Kjeldahl tablets defoamers and catalyst 3.5 g/tablet was purchased from Merck (Darmstadt, Germany). AccQ-Fluor reagent kit was obtained from Waters (Milford, Ma, U.S.A). Soy lecithin was purchased in a local market.

2.2 Enzymatic unhairing protocol

The processes were done in cylindrical stainless steel laboratory drums (80 cm diameter, 55 cm length, capacity 70 L) rotating in a temperature-controlled bath (Puccini et al., 2014). According to the method, with slight modifications, of the procedure proposed by Song et al (2007), the pieces of skin (11 kg) were washed and degreased twice. In the first step of washing, 33L of water containing 2% Na_2CO_3 and 1% of soy lecithin were added, and discharged after the washing, and in the second one the same amount of water containing 1% Na_2CO_3 and 1.5% of soy lecithin were used, and again discharged after the washing. Both these washing steps required 1 hour of agitation, with rotation speed of 10 rpm. After these steps, the skins were swollen in 33L of 10 mmol L^{-1} Na_2HPO_4 for 1 hour to remove the traces of Na_2CO_3, and the wastewaters were discharged again.

Enzymatic hydrolysis reaction started by suspending the prepared skins in the buffer solution (33 L of Na_2HPO_4 10 mmol L^{-1} at pH=7). 110 ml of enzyme solution (Alcalase from *Bacillus licheniformis*,

2.4 U/g solution, 1% v/w as related to the skin mass) were added. The unhairing procedure was performed at 40°C for 19 hours, with the following rotation cycle: an initial rotation speed of 10 rpm for 5 hours, then, for the next 10 hours, 30 rotation cycles by alternating 5 minutes with rotation speed at 10 rpm and 15 minutes of pause. At the end of this period the reaction was left rotating at 10 rpm for an hour.

At the end of each step, 250 mL of the solutions were collected for the analyses. The samples were instantly boiled for 10 minutes in order to inactivate enzyme.

2.3 Oxidative hair removal

In these tests, because of the stress generated by oxidative unhairing, the reactor used was completely covered by polypropylene, including the inner bolts, the door and the hatch, to make sure that the liquid never got in contact with any metallic part (Italprogetti srl, Sospiro, Cremona, Italy). The drum was equipped by a cooling/heating plant and it had a system for the measurement of temperature in continuous (Italprogetti srl, Sospiro, Cremona, Italy).

According to the method, with slight modifications, of Song et al (2007), the pieces of skin were washed in 15L of water under rotation speed of 10 rpm for 1 hour. Then the water was discharged and in the second step a surfactant was added, in order to degrease the skins, in 15L of water under rotation speed of 10 rpm for 1 hour, followed by discharging of solutions. In the real oxidative unhairing step the protocol used by Puccini et al (2014) was followed. Other 15L of water were added, and then several additions of H_2O_2 solution were performed, with the simultaneous addition of a NaOH solution in order to maintain the pH stable around the value of 13. After 13 hours the unhairing reaction was considered complete, and at this point the pH was brought in the range 6-7 by several consequential additions of citric acid solution.

At the end of each step, 250 mL were collected for the analyses.

2.4 Determination of protein content in broths and skins samples

Protein content was estimated using standard A. O. A. C. (2002) procedures with a rapid Kjeldahl system (VELP SCIENTIFICA DKL heating digester and VELP SCIENTIFICA UDK 139 semiautomatic distillation unit) from total nitrogen content. Proteins were determined as %N x 6.25.

2.5 Determination of free amine groups on the protein fraction using OPA (o-phthaldialdehyde) method

The degree of peptide bond hydrolysis was determined, with slight modifications, by the OPA method reported by Spellman et al (2003). Briefly the OPA/NAC (N-acetyl-cysteine) reagent (100 mL) was prepared by combining 10 mL of 50 mmol L^{-1} OPA (in methanol) and 10 mL NAC, 5 mL of 20 % (w/v) SDS, and 75 mL of borate buffer (0.1 mol L^{-1}, pH 9.5). The reagent was covered with aluminium foil to protect from light and allowed stirring for at least 1 h before use. The OPA assay was carried out by the addiction of 20 µL of sample (or standard) to 2.4 mL of OPA/NAC reagent. The absorbance of this solution was measured at 340 nm with JASCO B-530 UV-Vis-Spectrophotometer. Since OPA reacts with free nitrogen groups, to calculate the concentration, a standard curve was prepared using L-isoleucine (0-2 mg mL^{-1}). The results were given by considering the numbers of moles of free amine groups, equal to the moles of OPA, by the skin weight.

2.6 Quantification of free and total amino acids by HPLC/FLD after ACQ derivatization

For free amino acid determination, according to Nollet (2004), 100 µL of samples were employed. Samples were mixed with 34 µL of Norleucine (5 mmol L^{-1} in deionized water) which was added as internal standard, and then the volume was brought to 1 ml with deionized water. In order to perform a calibration curve a standard solution was prepared: 40 µL of Norleucine (2.5 mmol L^{-1} in HCl 0.1 mol L^{-1}), 40 µL of amino acids hydrolysate standard mixture (Sigma Aldrich, Saint Luis, Missouri, U.S.A), 40 µl of cysteic acid (2.5 mmol L^{-1} in HCl 0.1 mol L^{-1}), 40 µl of hydroxyproline (2.5 mmol L^{-1} in HCl 0.1 mol L^{-1}) and 840 µL of deionized water were mixed. Then 10 µL either of samples, blanks or standard solution were transferred into 1.5 mL tubes and 70 µL of borate buffer were added, then the solution was vortexed. 20 µL of reconstituted AccQ•Tag reagent (Waters, Milford, Massachusetts, U.S.A) were finally added and the mixture was vortexed for few seconds. The tube was left closed and at room temperature for 1 minute and it was inserted in a heated bath at 55°C for 10 min. The derivatised samples were diluted with 400 µL of deionised water before injecting in the HPLC system.

Samples were analysed with an Alliance 2695 separation system with AccQ•Tag amino acid analysis column (3.9mm × 150mm), (Waters, Milford, Massachusetts, U.S.A). The column was set at 37°C and the flow rate was set at 1.0 mL/min. The injection volume of samples was 10 µL, while standard calibration solution was injected at several volumes: 2.5, 5, 10, 15, 20, 25 and 30 µL,

corresponding to 5, 10, 20, 30, 40, 50 and 60 pmoles of amino acids hydrolysate standard mixture (Sigma Aldrich, Saint Luis, Missouri, U.S.A) injected. Mobile phase A consisted of AccQ•Tag eluent A (100 mL AccQ.Tag A concentrate+1L deionized water). Mobile phase B was a 60:40 (V/V) solution of acetonitrile and deionized water respectively. Gradient elution was performed according to the following steps: 0 min 100%A, 1min 97%A, 13 min 93%A, 18 min 90%A, 38 min 67%A, 51 min 67%A, plus washing step and reconditioning. Detection was carried out by Waters 470 fluorescence detector ($\lambda_{excitation}$ = 250 nm and $\lambda_{emission}$ = 395 nm).

Quantitative analysis was carried out by using the internal standard method. To calculate the total amino acid content, according to Nollet (2004), 0.5 g of skin or 3 mL of broth were weighed into an 18 mL Pyrex glass tube fitted with Teflon-line screw caps. 6 mL of HCl 6 mol L^{-1} were added and mixed. The tube was fluxed with nitrogen for 1 min in order to remove air. Hydrolysis was carried out at 110°C for 23 h. After letting the tubes stand cool at room temperature, the internal standard (7.5 mL of Norleucine 5 mmol L^{-1} in water) was added. The mixture was filtered throughout filter paper and collected into a 250 mL volumetric flask. HPLC/FLD analysis after AccQ•Tag derivatization was performed as previously described for free amino acid determination.

For Cys and Met analysis, performic acid oxidation followed by acid hydrolysis was used. In this case, an amount of 0.5 g of pellet or 3 mL of supernatant was weighed in an 18 mL Pyrex glass tube fitted with Teflon-line screw caps. After adding 2 mL of performic acid freshly prepared (by mixing 9 volumes of formic acid with 1 volume of hydrogen peroxide), samples were kept in an ice bath for 16 h at 0°C. Then 0.3 mL of hydrobromic acid was added in order to remove excess of performic acid. The bromine formed during the reaction was removed with nitrogen flow. Then the derivatization and the analysis were performed as described above.

2.7 Determination of amino acids racemization.

0.5 g of skin or 3 mL of broth were hydrolysed overnight in 2 ml hydrochloric acid (6 mol L^{-1}) at 100°C. The residue was filtered, evaporated to dryness. The residue was dissolved in 4.0 ml 2 mol L^{-1} HCl in 2-propanol and kept in a screw capped tube at 90°C for 1 h. The sample was evaporated, dissolved in 1.0 ml of dichloromethane, and treated with 0.5 ml of trifluoroacetic anhydride in a screw-capped tube at 50°C for 1 h. The tube was cooled and opened, so the solvent evaporated. The residue was dissolved in 0.5 ml of dry dichloromethane and injected (1 µl) in GC-MS. D,L amino acids have been resolved and quantified using a chiral capillary column (Chirasil-LVal 30 m 3 0.25 mm I.D. 3 0.25 lm film thickness Chrompack). GC-MS analysis was performed on an Agilent

Technologies 6890N gas chromatograph coupled to an Agilent Technologies 5973 mass selective detector (Agilent Technologies, Santa Clara, CA, USA). According to the method of Caligiani et al (2007) with slight modifications, GC conditions were as follows: oven temperature increased from 80 to 190 °C, at 5 °C/min, after an initial hold at 80 °C for 1 min; injector temperature: 250 °C; auxiliary temperature: 210 °C; carrier: helium. MS conditions were as follows: ion source temperature: 230 °C; electron impact: 70 eV; acquisition mode: Scan (m/z 40–400). SIM (m/z: 140, 168, 126, 166, 182, 184, 198, 91, 203, 171, 180).

2.8 Peptide identification by LTQ-Orbitrap analyses

For high resolution mass spectrometry analysis on the nitrogen fractions for peptide identification, a µHPLC DIONEX Ultimate3000 interfaced with a LTQ-Orbitrap XL Thermo Fisher Scientific was used. Column: Jupiter C18 4 µ, Proteo 90 Å 150×0.30 mm, Phenomenex; eluent A: water + 0.1 % formic acid; eluent B: acetonitrile + 0.1 % formic acid; flow: 5 µL/min, gradient: 0–4 min from 100 % A to 95 % A, 4–60 min from 95 % A to 50 % A, 60–62 min from 50 % A to 10 % A, 62–72 min 10 % A, 72–74 min from 10% A to 95 % A, 74–90 min 95 % A; analysis time (min): 90; column temperature (°C): 30; injection volume (µL): 5; acquisition time (min): 0-75; ionization mode: ESI+; scan range (m/z): 200–1,800; source voltage (kV): 3.5; capillary voltage (kV): 35; source temperature (°C): 275. Scan event details: (Fourier transform) FTMS+p res=30,000 or (250.0–2000.0); (ion trap) ITMS+c Dep MS/MS Most intense ion form; activation type: CID; isolation width: 2.00; normalized coll. energy: 35.0; default charge state: 2; activation Q: 0.250; activation time: 30.000; dynamic exclusion enabled; repeat count: 2; repeat duration (s): 10.00; exclusion duration (s):30.00. Charge state rejection: enabled; unassigned charge states: rejected; charge state 1: rejected; charge state 2: not rejected; charge state 3: not rejected; charge states 4+: not rejected; ion signal threshold: 10,000.

PEAKS software (Bioinformatics Solutions Inc) was used for the identification of the peptides and their structural modifications.

2.9 Peroxide value determination

According to A.O.A.C (2002), in a 250 mL flask 5 g of sample were dissolved in 25 mL of solvent mixture (60 % glacial acetic acid, 40 % chloroform) and 0.5 mL of saturated potassium iodine solution is added. The mixture was stirred by giving a rotary motion to the flask. After the addition of the potassium iodine, the solution was leaved in dark for 5 minutes and then 75 mL of water

were added. The liberated iodine was titrated with the addition of indicator (1% starch paste solution) with 0.1 N or 0.02 N sodium thiosulfate, depending on the amount of iodine liberated.

$$(3)\ \text{Peroxide number} = \frac{C_{tit} * V_{tit} * 16 * 1000}{2 * W}$$

Where: C_{tit} is titrant concentration, V_{tit} is titrant volume used, 16 is the molecular weight of oxygen and W is the mass (g) of sample weighed.

$$(4)\ \text{Peroxide number contained in the skin} = \frac{peroxide\ number}{g\ of\ skin}$$

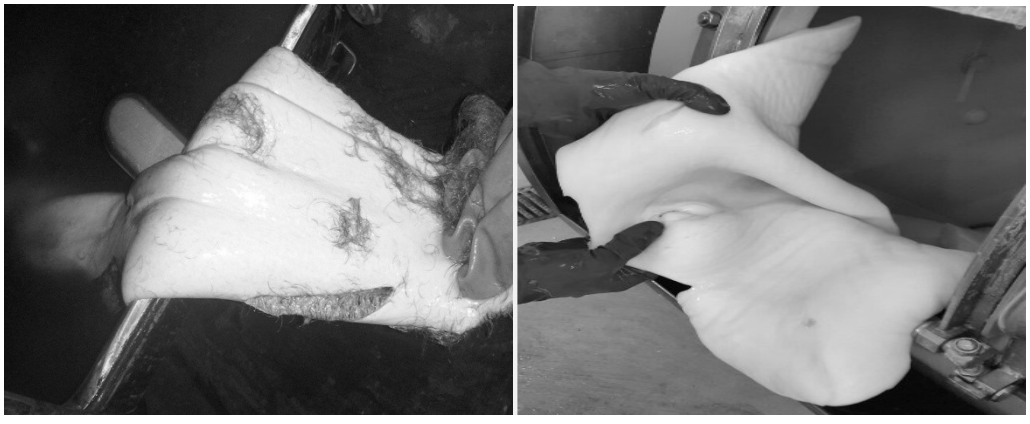
3. Results and discussion

3.1 Implementation of the two unhairing processes

Both unhairing methods tested in this paper were performed in pilot systems: for the oxidative methodology, polypropylene-coated machinery were used, for the enzymatic one stainless steel equipment was employed.

The enzymatic unhairing process needed 22 hours, which included two steps of washing and one of swelling. The two washing steps (Na_2CO_3, soy lecithin) were necessary to remove the lipid layer of the hair and to make the enzyme attack possible. A swelling step, where the skin was conditioned with the buffer used for the subsequent hydrolysis, was introduced, because of the need to reach the optimum pH for Alcalase. The use of not sanitized skin generated a sharp odour during the hydrolysis reaction, probably derived from a high bacterial activity (mesophilic bacteria) which was not hindered by low temperature (40°C). Anyway, at the end of the enzymatic hydrolysis, the skin was completely shaved and reduced in the thickness, even if some hairs, which were cleaved by Alcalase, remained attached to the sticky subcutaneous layer (Figure 1).

Figure 1 The skin obtained after the enzymatic (left) and oxidative (right) unhairing process



Cantera et al (2004) suggested that the enzymatic unhairing process causes the digestion of the cells in the hair bulb, also degrading the medulla, but not the cortex. In particular proteoglycans are cementing substances, composed of polysaccharide and core proteins, contents of the skin fibrous structure and basement membrane, (Jian et al., 2010); in addition they contribute to the cohesion and to the stability of the structure and to the junction of hair and skin (Jian et al., 2009). It is likely that the enzyme damages the protein backbone of the proteoglycans, leading to their hydrolysis. The hair adhesive attachment to the subcutaneous layer could be considered as a consistent limitation of this process.

A possible solution to solve this problem could be the addition of further machinery able to eliminate mechanically the thin subcutaneous layer. However, this implementation would not only lead to an increase in the costs of the enzymatic process, but also it could damage the skin, lowering its quality. A further solution could be to project different pilot plant equipment, not with a rotating drum, but with a reactor with a rotating blade. This equipment would leave the hair floating at the top of the solution and the separation of the hair would be more straightforward by performing simple filtration.

The experimental protocol of the unhairing oxidative method was similar to the one applied by Puccini et al (2014). The hairs were completely disintegrated by aggressive chemicals (NaOH, H_2O_2), and the skin was swollen and white, as it is shown in Figure 1. The chemical method promotes the polypeptide backbone hydrolysis, but it also helps the swelling of the hair shaft. The latter phenomenon allows the chemicals to penetrate the core area of the hair shaft (Marmer and Dudley, 2006). On the other hand, the role of hydrogen peroxide is to cleave the disulphide linkages in cysteine residues (Marmer and Dudley, 2006).

Marmer and Dudley (2006) observed three different types of outcomes in the oxidative unhairing processes: there was the traditional type, in which the hair was still attached to the skin and it could be easily removed by scraping or pulling; an intermediate case, in which the hair was not attached to the skin, but it could be found intact, floating in the unhairing solution; an extreme case, in which the hair was burnt and it was completely pulped. The oxidative unhairing clearly belonged to the last case, since no hairs were observed in the solution anymore.

3.2 Molecular composition analysis of the nitrogen fraction in the reaction broths

For each step of the unhairing reactions, the broths were collected and the protein content was determined by Kjeldahl method (Figure 2A). The two steps of washing, essential for the enzymatic unhairing, requested the addition of soy lecithin, thus its contribution was taken into account in the analysis of nitrogen content. The protein content in the final broth was found to be 21.5±0.7 mg of protein/g of skin in enzymatic broth and 18±1 mg of protein/g of skin in oxidative broth. In particular the enzymatic broth showed a higher protein content than the oxidative broth, in agreement with the findings of de Souza and Gutterres (2012).

The total amino acids analysis performed by HPLC-FLD (Figure 2B) indicated nevertheless a completely different amino acidic composition of the two final broths, clearly indicative of the different mechanisms of the unhairing process. In particular, in the broth from the enzymatic reaction hydroxyproline and glycine were present in high amounts (14.4% and 10.2% respectively of the total amino acidic content); these amino acids are particularly abundant in collagen, indicating that Alcalase attacked and degraded this protein, something which did not happen in the chemical reaction. These findings are in agreement with the total amino acids analyses performed by Mu et al, (2003) showing a great amount of Gly and Pro, as an indicator of high collagen presence in the sludge. On the other side, the amino acid fraction of the chemical method was rich in cysteine, an amino acid particularly abundant in the keratin constituting the hairs, which was found in its oxidized form (cysteic acid), due to the action of the oxidative reagents. Afterwards, the free amino acids analyses were performed in same conditions and the results are showed in Figure 2C.

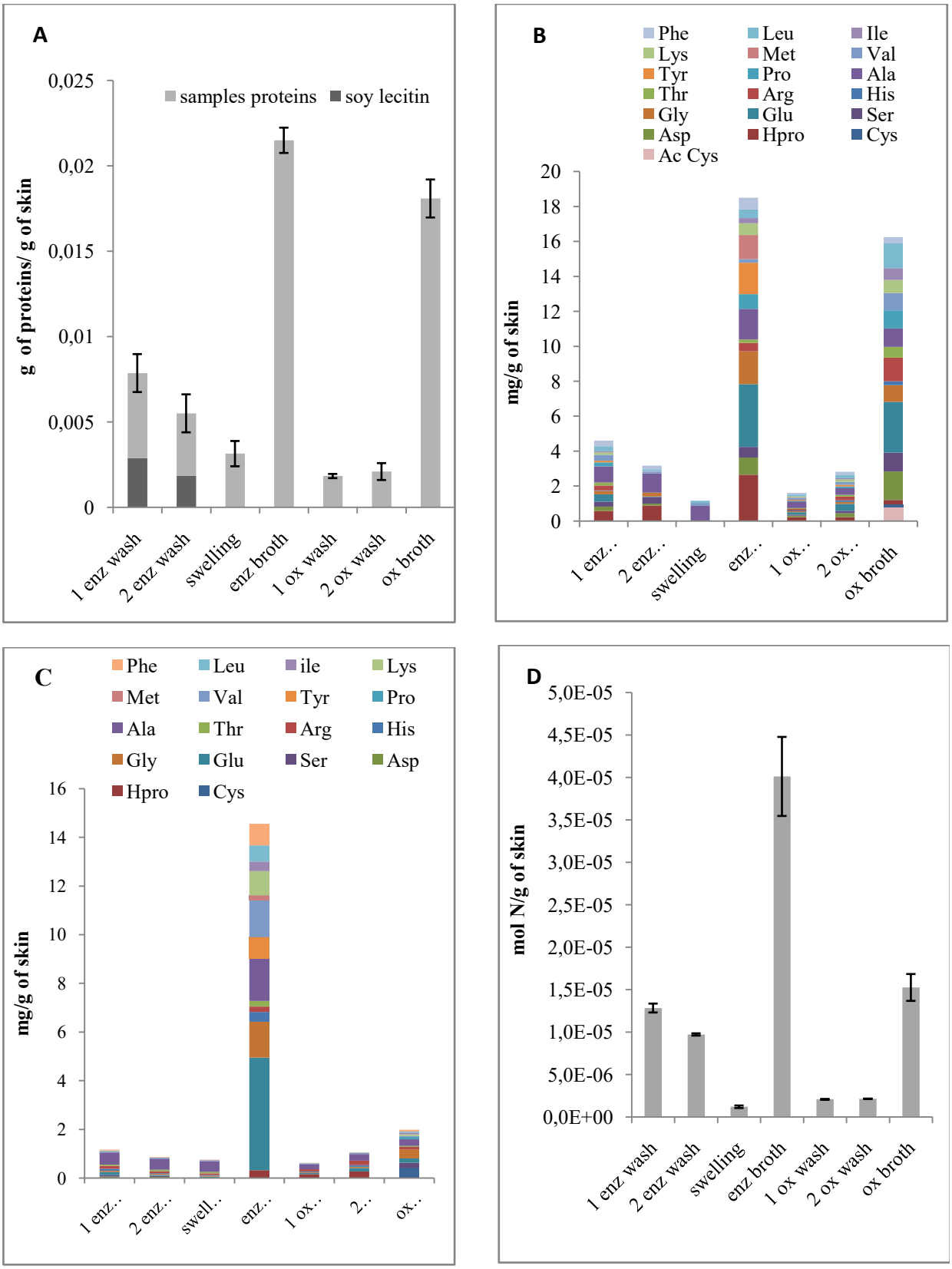


Figure 2 Protein content determined by Kjeldahl method (A), total amino acids analysis performed by HPLC-FLD, results from three replicates: maximum RSD = 0.10 (B), free amino acid analysis performed by HPLC-FLD, results from three replicates: maximum RSD = 0.8 (C), free amine groups determination by OPA method (D).

The enzymatic broth was the richest in free amino acids, even if the amount was somehow limited (1.5% as compared to the amount of skin), while the other liquid fractions appeared to have a negligible amount of free amino acids compared to the total ones (less than 0.2%). These results could be associated with the protease activity of Alcalase, whose main enzyme component is subtilisin Carlsberg, an industrial and food-grade enzyme preparation produced by a selected strain of *Bacillus licheniformis.* Adamson and Reynolds (1996) observed that Alcalase cleaved peptide bonds on the carboxyl side of Glu, Met, Leu, Tyr, Lys and Gln. Because of the broaden specificity and the preference for hydrophobic residues, Alcalase was able to release a higher amount of free amino acids.

In addition OPA method was used to determine the amount of free nitrogen-containing species in each broth.

Figure 2D indicates that the amount of the OPA reactive free nitrogen-containing species is higher in the broth obtained by the enzymatic hydrolysis, in agreement with the free amino acid analysis.

All the above data indicate that the mechanism of unhairing was clearly different in the two cases: a degradation of the collagen part of the skin by using the enzymes (and then a subsequent detachment of the hairs), and a degradation of the hair themselves by using the chemical method.

Amino acids may racemase as a consequence of technological process, such as severe heating, irradiation, or treatment at extreme pH (Caligiani, et al., 2007). Another cause of racemization could be the microbial contamination or the action of microbial enzymes. In order to assess the integrity of the amino acidic fraction, chiral amino acids analysis was performed on each broth by GC-MS. Table 1 showed the comparison of the results between oxidative and enzymatic broth; the analyses of the other liquid fraction did not show any racemization. Only in the case of the oxidative broth many amino acids were found to be quite racemised. The percentage of D-amino acids was likely due to the treatment with extreme basic pH (14) for long time. The presence of D-lys only in the enzymatic broth could derive from the activity of a bacterial lysine racemase enzyme (Chien et al., 2009).

Table 1 D,L ratio of amino acids in the broth treated with enzymatic and oxidative unhairing method by GC-MS analysis. In bold the amino acids which underwent extensive racemization.

	enzymatic broth	**oxidative broth**
D-Ala	0.9 ± 0.3	**6.6 ± 0.5**
L-Ala	99.1 ± 0.4	**93.4 ± 0.5**
D-Asp	2.4 ± 0.2	**24 ± 1**
L-Asp	97.6 ± 0.2	**76 ± 1**
D-Glu	1.3 ± 0.2	**10.6 ± 0.2**
L-Glu	98.7 ± 0.2	**89.4 ± 0.2**
D-Lys	**9.8 ± 0.2**	4.7 ± 0.3
L-Lys	**90.2 ± 0.2**	95.4 ± 0.3
D-Pro	2 ± 1	1 ± 1
L-Pro	98 ± 1	99 ± 1
D-Phe	0.8 ± 0.4	**11 ± 1**
L-Phe	99.2 ± 0.4	**89 ± 1**

The molecular characterization of the peptide fractions for both broths obtained with the two unhairing methods was performed by high resolution mass spectrometry. The data processing, executed by Peaks software, allowed identifying the proteins of origin; table 2 showed the results obtained.

Table 2 Identification by LTQ-OrbiTrap of the main peptides present in the solutions derived from enzymatic and oxidative unhairing methods. Proteins are listed in order according to the highest number of peptides generated.

Sample	**Peptides identified**	**Protein of origin**
Enzymatic broth	77	Collagen alpha-1(III) chain
	77	Collagen, type III, alpha 1
	77	Collagen alpha-1(III) chain
	74	Collagen alpha-1(III) chain
	7	Myosin light chain 1/3, skeletal muscle isoform
Oxidative broth	35	KRT33A protein
	35	KRT33B protein
	33	Keratin 31
	13	Keratin, type II cuticular Hb1
	9	Keratin 85

Peptides produced in the oxidative broth derived mainly from keratins, indicating that this method mostly degraded the hairs. On the other side, peptides coming from enzymatic broth were originated from the degradation by Alcalase of collagen, the main component of the skin. These results confirmed that the enzymatic hydrolysis degrade the hair bulb, leading to hair releasing together with a small percentage of collagen hydrolysis. Oxidative unhairing, instead, destroys directly the hair, leading to the release of keratin peptides in solution.

The effect of the addition of hydrogen peroxide in the oxidative unhairing method was finally also evaluated by the determination of the number of peroxides, which turned out to be 319 meq/Kg of skin. As a comparison the peroxide content in the enzymatic broth is completely absent.

The peroxide content, together with the above presented data, indicate that wastewaters of the oxidative unhairing process contains an extensively damaged, peroxide-rich, protein fraction, thus quite unsuitable for further possible feed/food applications. The lower quality of the oxidative broth is certainly a limitation of this process, preventing its use in high quality applications such as food, feed or cosmetics, but anyway this proteinaceous material could be reused for some low added value applications, such as soil amendment or biogas production.

The real innovation of this study, besides the testing of the new method at the semi-industrial scale level, is also a deeper analysis and characterization, at the molecular level, of all the wastewaters generated during the unhairing process. Such a level of molecular details for the wastewater composition from unhairing is unprecedented in the literature, and allows understanding the mechanism of action of the unhairing process in both methods. This is of help for eventually deciding their possible subsequent re-use, tailored according to the high (food/feed) or low (soil amendment, biogas production) quality.

Anyway, in general, the results showed a great amount of nitrogen compounds in the wastewaters, in particular proteins, peptides and free amino acids. The possibility to recover these nitrogen mixtures could decrease the amount of nitrogen content in the overall wastewaters released in the environment during all the process. As a consequence the load of COD and BOD, and also the environmental impact could be reduced (Nazer et al., 2006).

3.2. Molecular composition of the skin obtained with oxidative and enzymatic unhairing

The protein content of the skins treated with oxidative and enzymatic unhairing methods was determined by Kjeldahl method. The difference of the total nitrogen content of the skin after both

the unhairing treatments is indicative of a higher degradation of the skin in the former case, which is consistent with the hypothesis done on the mechanism of unhairing.

The distribution of total amino acids in both skins was also investigated; the table 3 showed that the most abundant amino acids were Hpro, Gly and Pro (respectively 21.1/21.4% of Hpro, 21.5/21.1% of Gly, 11.5, 11.2% of Pro), consistently with a high collagen presence, and the distribution was not different in the two cases, indicating that the two processes did not differentially affect skin composition.

Table 3 Amino acids percentage distribution in the skin after the two processes, given as percentage of the total amino acid pool**.**

	Skin after enzymatic unhairing	**Skin after oxidative unhairing**
Hpro	21.1	21.4
Asp	5.3	5.3
Ser	3.2	3.2
Glu	9.7	9.4
Gly	21.5	21.1
His	0.0	0.0
Arg	7.3	7.7
Thr	1.7	1.8
Ala	6.7	6.0
Pro	11.5	11.2
Tyr	0.0	0.7
Val	2.3	2.2
Met	0.4	0.9
Lys	3.2	3.1
ile	1.4	1.3
Leu	2.8	2.6
Phe	1.7	2.0

Analysis by HPLC-FLD. Results in three replicates. Maximum RSD = 0.13

As discussed in section 3.2 the analysis of amino acids racemization was performed also for skin samples and in the table 4 it could be observed that in this case there was no racemization, indicating that the oxidative stress did not affect the collagen proteins remaining on the skin, but only those released in solution.

Table 4 D,L ratio of amino acids in the skins treated with enzymatic and oxidative unhairing method by GC-MS analysis.

	Skin after enzymatic unhairing	Skin after oxidative unhairing
D-Ala	1.87 ± 0.06	1.2 ± 0.5
L-Ala	98.13 ± 0.06	98.8 ± 0.5
D-Asp	5 ± 2	6 ± 1
L-Asp	95 ± 2	94 ± 1
D-Glu	2 ± 1	2 ± 1
L-Glu	98 ± 1	98 ± 1
D-Lys	1.90 ± 0.07	1.49 ± 0.07
L-Lys	98.10 ± 0.07	98.51 ± 0.07
D-Pro	4.1 ± 0.3	0.720 ± 0.004
L-Pro	95.9 ± 0.3	99.280 ± 0.004
D-Phe	1.86 ± 0.06	2.59 ± 0.04
L-Phe	98.14 ± 0.06	97.41 ± 0.04

4. Conclusion

Both unhairing methods described here, studied at a semi-industrial level, may be considered as a sustainable cleaner alternative of the traditional protocols, strongly improving the sustainability of the skin industry. The most efficient method turned out to be the oxidative one, allowing to have the hairs completely disgregated and the skin completely unhaired. In the enzymatic method the major limitation was found to be the adhesive attachment of the hairs left in the solution to the subcutaneous layer. The removal of the subcutaneous layer could actually solve the problem, but with an increased cost of the process. Further to the sustainability of these methods, the chemical analyses of the wastewaters indicated a high nitrogen content in both cases, due to peptides and amino acids, allowing their exploitation in food/feed preparations or ingredients. In particular, the mild reaction conditions of the enzymatic method allowed obtaining wastewaters with food- and feed-grade protein derivatives. The oxidative broth, despite the high amount of racemised and oxidized amino acids, and a high peroxide content (preventing any re-use in food or feed), could be used for lesser agricultural applications, such as soil amendments.

Although cost data of the two methods have not been fully assessed, some considerations can be done. The enzymatic method is more time demanding than the chemical one, which of course translates in additional industrial costs. Moreover, in the chemical unhairing method the reagents are very cheap (NaOH, H_2O_2), whereas the enzymes are more costly. On the other side, the wastewaters obtained by the enzymatic method have possibility of reusing in high added value ingredients, which could help to gain some more value from the process, whereas the wastewater of the chemical method are to be used in lesser applications of lower added value. The output, as indicated, is constituted, beside the unhaired skin, by wastewater rich in nitrogen which has a high (for enzymatic) or fair (for oxidative) possibility to be recovered in further applications. This is to be compared with the output of strongly contaminated wastewaters usually obtained with the normal treatment.

In conclusion, the enzymatic unhairing method seems to have much more potential for the recycle and valorisation of the broth mixtures, but it was found to be less efficient and more expensive than the chemical one. The high efficiency of the oxidative method could lead to an effective use in industrial plants as a cleaner alternative to the traditional processes and the wastewater could be also recovered as source of nitrogen.

The three ways that leather tanneries have followed in the last years to make their process greener are actually: reducing water use (or recycling water), substituting dangerous chemicals with less aggressive ones, and saving energy by finding alternative energy sources. The results here reported might contribute in all these fields, since they provide a process of unhairing which uses less aggressive chemicals, also requiring less energy, and generates reusable wastewaters.

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Chapter IV

Enzymatic hydrolysis as a way to recovery bovine hides: optimization, characterization of the hydrolysates and scale-up to semi-industrial scale.

Enzymatic hydrolysis as a way to recovery bovine hides: optimization, characterization of the hydrolysates and scale-up to semi-industrial scale.

Abstract

Bovine hides are one of the considerable co-products in meat industry which could be recovered for other applications. The methodology here investigated in unprecedented detail, first on lab scale, then on medium scale, then on a pilot plant, is based on an enzymatic hydrolysis of full bovine hides with Alcalase, in order to produce protein hydrolysates. Alcalase turned out to be very efficient due to its ability to degrade collagen, the main protein of hides. In the optimized protocol here proposed, hides were hydrolysed at lab and medium scale, obtaining about 90% of protein solubilisation after 6 hours, as demonstrated by Kjeldahl analysis, with a consistent release of free amino acids (determined by liquid chromatography methods) and a degree of hydrolysis of 17-19% (as determined by the O-phtaldialdehyde method). The method was scaled up to a semi-industrial level, where solubilisation efficiency decreased due to less efficient mixing, but still indicating that this procedure could be applied at industrial scale.

Keywords: bovine hides, Alcalase, protein hydrolysate, optimization, scale-up.

1. Introduction

Beef meat is a primary source of energy and protein. However, only 44% of live animal weight ends up in the food chain (Russ and Pittroff, 2004). The other is 56% usually discharged, and is composed of edible (e.g. offal) and non-edible (e.g. specified risked materials SRM) parts. Nowadays, there is an increasing interest in studying these non-meat products in order to extract or recover additional value from the meat processing chain.

Bovine hide is one of such product: it accounts for 7% of the total animal weight, and it is a protein-rich material which might provide a feedstock for the generation of protein ingredients to be used in different ways (Mullen et al., 2015). Hides chemical composition is characterized by the presence of a large amount of collagen, a structural fibrous protein. Due to collagen content, bovine hides are often converted into gelatines by thermal denaturation and/or chemical degradation (Bajza and Vrcek, 2001). Gelatine preparation is conventionally based on chemical degradation and solubilisation of collagen (acidic or alkaline). The downside of this traditional method is mainly the long period required to extract collagen and the use of strong polluting chemicals, together with chemical degradations of the proteins involved. In the alkaline process usually applied to bovine hides or bones, type B gelatine is obtained, but this method requires 30-100 days. As a comparison, pig skin is the raw material for the production of gelatine type A through acid process, which needs 10-45 hours to be completed (Damrongsakkul et al, 2008; Morimura et al, 2002). Moreover, amino acids are modified in both processes: during acid hydrolysis, asparagine and glutamine are completely hydrolysed to aspartic acid and glutamic acid, respectively, while tryptophan is completely destroyed (Fountoulakis and Hans-Werner, 1998). With basic hydrolysis serine, threonine, arginine and cysteine are degraded (Ravindran and Bryden, 2005). In both cases new, even obnoxious compounds can be formed (e.g. lysinoalanine).

Alternatively to the chemical method, enzymatic hydrolysis can be used (Damrongsakkul et al, 2008). The enzymatic methods provide shorter timing, milder conditions, and few or no undesirable side reactions (Guerard et al, 2001). Another advantage is that amino acids are preserved and not degraded, as it happens in traditional chemical processes (Alvarez et al., 2012). Several factors, like pH, time, enzyme to substrate ratio, temperature and water to substrate ratio might affect enzymatic activity and thus the efficiency of the process: due to this reason the conditions of hydrolysis must be carefully controlled (Alvarez et al, 2012).

The aim of the present research was to pave way to an economically viable, environmentally friendly, simple method, which can be applied at the industrial scale, for the solubilisation and

recovery of bovine hides proteins with enzymatic hydrolysis. Because of the high request of readily available amino acids in soil fertilizers, in food/feed preparation and in pharmaceutical field, we focused our attention on the production of protein hydrolysates with a high degree of hydrolysis in order to obtain a high amount of free amino acids and small peptides. For this aim, we selected Alcalase as the hydrolysing enzyme, a serine alkaline protease produced by a selected strain of *Bacillus licheniformis*. Alcalase has previously used to produce soluble hydrolysates starting from different raw materials (Doucet et al., 2003, Liu et al, 2010, Haslaniza et al, 2013, Anzani et al., 2017a). The extensive used of this particular enzyme derives from its low cost and its food grade behaviour (Doucet et al., 2003; Kristinssons and Rasco, 2000; Muzaifa et al., 2012, Saidi et al., 2016). The commercial preparation also contains several proteinases with different specificities, and due to this reason this enzyme has broad specificity, hydrolysing most of peptide bonds, preferentially those containing aromatic amino acids residues (Doucet et al., 2003). Therefore Alcalase usually generates protein hydrolysates with high DH, as compared with other enzymes such as pepsin and trypsin. The kinetic of the process was evaluated at lab scale and at a medium scale, and the obtained hydrolysates were fully characterized in molecular details. Finally, the optimized method was applied in a pilot plant, in order to verify its applicability at industrial scale for the production of protein hydrolysates to be used in high quality applications.

2. Materials and Methods

Samples, constituted by pieces of bovine hides, were provided by Inalca Industria Alimentare Carni SpA (Castelvetro di Modena, Italy).

2.1 Reagents and solvents

Alcalase from *Bacillus licheniformis* (2.4 U/g), sodium dihydrogen phosphate, hydrochloric acid, acetonitrile, dichloromethane, N-acetyl-L-cysteine, sodium tetraborate decahydrate, sodium thiosulfate, DL-isoleucine, DL-norleucine, trifluoroacetic anhydride, sulfuric acid, formic acid, acetic acid and trifluoroacetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Copper oxide was obtained from Carlo Erba (Milan, Italy).

O-phthalaldehyde and boric acid were bought from Fluka (Buchs, Switzerland). MA, USA).

Kjeldahl tablets defoamers and catalyst 3.5 g/tablet was purchased from Merck (Darmstadt, Germany). AccQ-Fluor reagent kit was obtained from Waters (Milford, Ma, U.S.A).

Soy lecithin was purchased from the local market.

2.2 Enzymatic hydrolysis protocol at lab and medium scale

The processes were performed in a reactor of 5 L of capacity with a heating shell and agitator blade. The experiments were performed in lab and medium scale; the details are reported in table 1.

Table 1 Amount of bovine hides, solution used in the enzymatic protocols and hydrolysates volume recovered

Experiment	Bovine hides (g)	Volume of added buffer (mL)	Volume of final hydrolysate after reaction (mL)
Lab scale hydromodule 1:2	500	1000	1460
Medium scale hydromodule 1:2	1500	3000	4400
Lab scale hydromodule 1:3	422	1265	1670
Medium scale hydromodule 1:3	1000	3000	3950

The pieces of hides were washed and degreased twice. In the first step of washing 2% Na_2CO_3 and 1% of soy lecithin were added and in the second one 1% Na_2CO_3 and 1.5% of soy lecithin were used. Both these washing steps required 30 min of agitation at 60ºC, with rotation speed of 100 rpm; after both washing the wastewaters were discarded. After degreasing, the hides were swollen in 10 mmol L^{-1} Na_2HPO_4 for 1 hour at 60ºC to remove the traces of Na_2CO_3; the wastewater was discarded.

Enzymatic hydrolysis reaction started by suspending the prepared hides in the buffer solution (Na_2HPO_4 10 mmol L^{-1}). 1% (w/w) of enzyme solution was added after monitoring that the pH of the solution (7-7.5). The enzyme used was Alcalase from *Bacillus licheniformis* and the procedure was performed at 60 °C under stirring. The digestion was sampled every 30 min for 6 hours and, at the end, boiled for 10 minutes (90º) to inactivate the enzyme.

2.3 Determination of protein content in broths and hides samples

Protein content was estimated using standard A. O. A. C. (2002) procedures with a rapid Kjeldahl system (VELP SCIENTIFICA DKL heating digester and VELP SCIENTIFICA UDK 139 semiautomatic distillation unit (SER 148/3 VELP SCIENTIFICA, Usmate Velate, Italy)) from total nitrogen content (%N x 6.25). In particular for the evaluation of the protein content of the raw material, 12 pieces were analysed in order to take into account the biological variability of the hides. In the case of the

broths the protein content was estimated after centrifugation at 4000 rpm for 30 min on the supernatant. The percentage of solubilised proteins was determined as grams of the proteins present in the supernatant over the total protein content (grams) in the starting material (calculated from the average amount of the protein of bovine hides).

2.4 Determination of free amine groups on the protein fraction using OPA (o-phthaldialdehyde) method

The degree of peptide bond hydrolysis was determined, with slight modifications, by the OPA method reported by Spellman et al (2003). Briefly the OPA/NAC (N-acetyl-cysteine) reagent (100 mL) was prepared by combining 10 mL of 50 mmol L^{-1} OPA (in methanol) and 10 mL NAC, 5 mL of 20 % (w/v) SDS, and 75 mL of borate buffer (0.1 mol L^{-1}, pH 9.5). The reagent was covered with aluminium foil to protect from light and allowed stirring for at least 1 h before use. The OPA assay was carried out by the addiction of 20 µL of sample (or standard) to 2.4 mL of OPA/NAC reagent. The absorbance of this solution was measured at 340 nm with JASCO B-530 UV-Vis-Spectrophotometer (JASCO, Oklaoma City, OK, U.S.A). Since OPA reacts with free nitrogen groups, to calculate the concentration, a standard curve was prepared using L-isoleucine (0-2 mg mL^{-1}). The results were given by considering the numbers of moles of free amine groups, equal to the moles of OPA, by hides' weight.

2.5 Quantification of free amino acids by HPLC/FLD after ACQ derivatization

For free amino acid determination, according to Anzani et al (2017a), 50 µL of samples were employed. Samples were mixed with 34 µL of Norleucine (5 mmol L^{-1} in deionized water) which was added as internal standard, and then the volume was brought to 1 ml with deionized water. In order to perform a calibration curve a standard solution was prepared: 40 µL of Norleucine (2.5 mmol L^{-1} in HCl 0.1 mol L^{-1}), 40 µL of amino acids hydrolysate standard mixture (Sigma Aldrich, Saint Luis, Missouri, U.S.A), 40 µl of hydroxyproline (2.5 mmol L^{-1} in HCl 0.1 mol L^{-1}) and 880 µL of deionized water were mixed. Then 10 µL either of samples, blanks or standard solution were transferred into 1.5 mL tubes and 70 µL of borate buffer were added, and then the solution was vortexed. 20 µL of reconstituted AccQ•Tag reagent (Waters, Milford, Massachusetts, U.S.A) were finally added and the mixture was vortexed for few seconds. The tube was left closed and at room temperature for 1 minute and it was inserted in a heated bath at 55°C for 10 min. The derivatised samples were diluted with 400 µL of deionised water before injecting in the HPLC system.

Samples were analysed with an Alliance 2695 separation system with AccQ•Tag amino acid analysis column (3.9mm × 150mm), (Waters, Milford, Massachusetts, U.S.A). The column was set at 37°C and the flow rate was set at 1.0 mL/min. The injection volume of samples was 10 µL, while standard calibration solution was injected at several volumes: 2.5, 5, 10, 15, 20, 25 and 30 µL, corresponding to 5, 10, 20, 30, 40, 50 and 60 pmoles of amino acids hydrolysate standard mixture (Sigma Aldrich, Saint Luis, Missouri, U.S.A) injected. Mobile phase A consisted of AccQ•Tag eluent A (100 mL AccQ•Tag A concentrate+1L deionized water). Mobile phase B was a 60:40 (V/V) solution of acetonitrile and deionized water respectively. Gradient elution was performed according to the following steps: 0 min 100%A, 1min 97%A, 13 min 93%A, 18 min 90%A, 38 min 67%A, 51 min 67%A, plus washing step and reconditioning. Detection was carried out by Waters 470 fluorescence detector ($\lambda_{excitation}$ = 250 nm and $\lambda_{emission}$ = 395 nm).
Quantitative analysis was carried out by using the internal standard method.

2.6 Peptide identification by LTQ-Orbitrap analyses

For high resolution mass spectrometry analysis on the nitrogen fractions for peptide identification, a µHPLC DIONEX Ultimate3000 interfaced with a LTQ-Orbitrap XL Thermo Fisher Scientific was used (Thermo Fisher Scientific, Waltham, MA, U.S.A). Column: Jupiter C18 4 µ, Proteo 90 Å 150×0.30 mm, Phenomenex; eluent A: water + 0.1 % formic acid; eluent B: acetonitrile + 0.1 % formic acid; flow: 5 µL/min, gradient: 0–4 min from 100 % A to 95 % A, 4–60 min from 95 % A to 50 % A, 60–62 min from 50 % A to 10 % A, 62–72 min 10 % A, 72–74 min from 10% A to 95 % A, 74–90 min 95 % A; analysis time (min): 90; column temperature (°C): 30; injection volume (µL): 5; acquisition time (min): 0-75; ionization mode: ESI+; scan range (m/z): 200–1,800; source voltage (kV): 3.5; capillary voltage (kV): 35; source temperature (°C): 275. Scan event details: (Fourier transform) FTMS+p res=30,000 or (250.0–2000.0); (ion trap) ITMS+c Dep MS/MS Most intense ion form; activation type: CID; isolation width: 2.00; normalized coll. energy: 35.0; default charge state: 2; activation Q: 0.250; activation time: 30.000; dynamic exclusion enabled; repeat count: 2; repeat duration (s): 10.00; exclusion duration (s):30.00. Charge state rejection: enabled; unassigned charge states: rejected; charge state 1: rejected; charge state 2: not rejected; charge state 3: not rejected; charge states 4+: not rejected; ion signal threshold: 10,000.
Proteom Discoverer™ software (Thermo Fisher Scientific) was used for the identification of the peptides.

2.7 Protocol scale-up.

The scale up reaction was performed in Po.Te.Co s.c.r.l Technogical center (Via San Tommaso, 119/121/123 56029 - Santa Croce sull´Arno (Pisa), Italy). The drum (80 cm diameter, 55 cm length, capacity 70 L) was equipped by a cooling/heating plant and it had a system for the measurement of temperature in continuous (Italprogetti srl, Sospiro, Cremona, Italy). 10 Kg of pieces of hides were employed and the volume of solution used was 30 L. The protocol described in previous sections was followed. In the case of the pilot plant scale-up the rotation speed was 10 rpm and the samples were collected every 30 min for 5 hours and the enzyme was inactivated at 90º for 10 minutes.

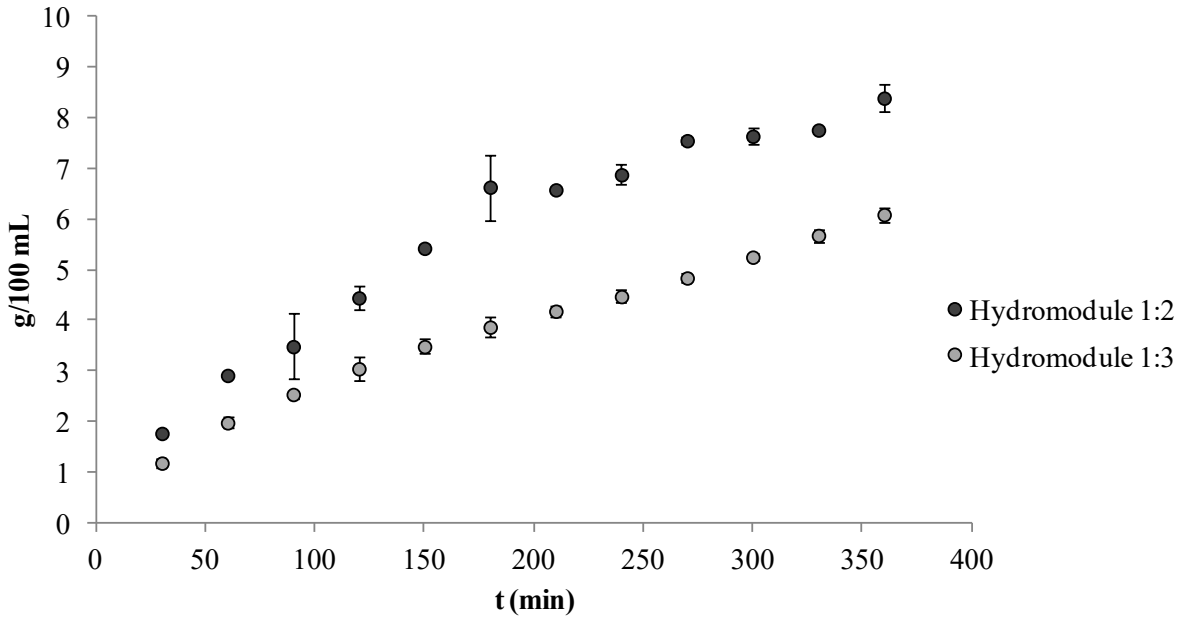
3. Results and discussion

The protein content in the bovine hides used as starting material was determined by complete digestion and Kjeldahl analysis. Also taking into account the biological variability of the starting material, the determined amount was 29.4 ± 3.6 g/100g hides (on wet basis). This value was considered as a reference for the calculations of the % percentage of the protein solubilised during the enzymatic hydrolysis.

For the complete enzymatic hydrolysis of bovine hides, 1% of Alcalase (w/w of hides) was used and two different hides : buffer ratio (w/v) solutions were tested, both in lab and medium scale, in order to assess the best hydrolysis conditions for the further scale up in a semi industrial plant. In the first experiment a hides : buffer ratio 1:2, both at lab and medium scale (see table 1 in material and method section 2.2) was used. The soluble protein content released in solution during reaction was monitored with Kjeldhal method. The solubilized proteins at the end of the reaction were respectively 8.7g/100ml (small scale) and 8.4g/100 ml (medium scale) of the final hydrolysate. Considering the volume increase at the end of the reaction (see Table 1), this value corresponded to 127 ± 2 g and 370 ± 10 g of solubilised proteins respectively, corresponding to about 85% of protein solubilisation in both cases. In the second experiment a ratio hides : buffer solution 1:3 was used. In this latter case, the solubilized proteins at the end of the reaction were respectively 6.0g/100ml (small scale) and 6.1g/100 ml (medium scale) of the final hydrolysate. Considering the volume increase at the end of the reaction (see Table 1), this value corresponded to 100 ± 2 g and 240 ± 4 g of solubilised proteins respectively, corresponding to about 80% of protein solubilisation in both cases. The kinetics profiles of the enzymatic hydrolysis were studied by characterising the soluble protein content released in solution during reaction. Figure 1 shows

the kinetic profile, obtained by measuring protein concentration in solution (determined by Kjeldahl analysis of the solubilized nitrogen). The kinetic profile of both hides/water ratios showed similar trends, with a sharp increase of the solubilised nitrogen starting to flatten after 5-6 hours.

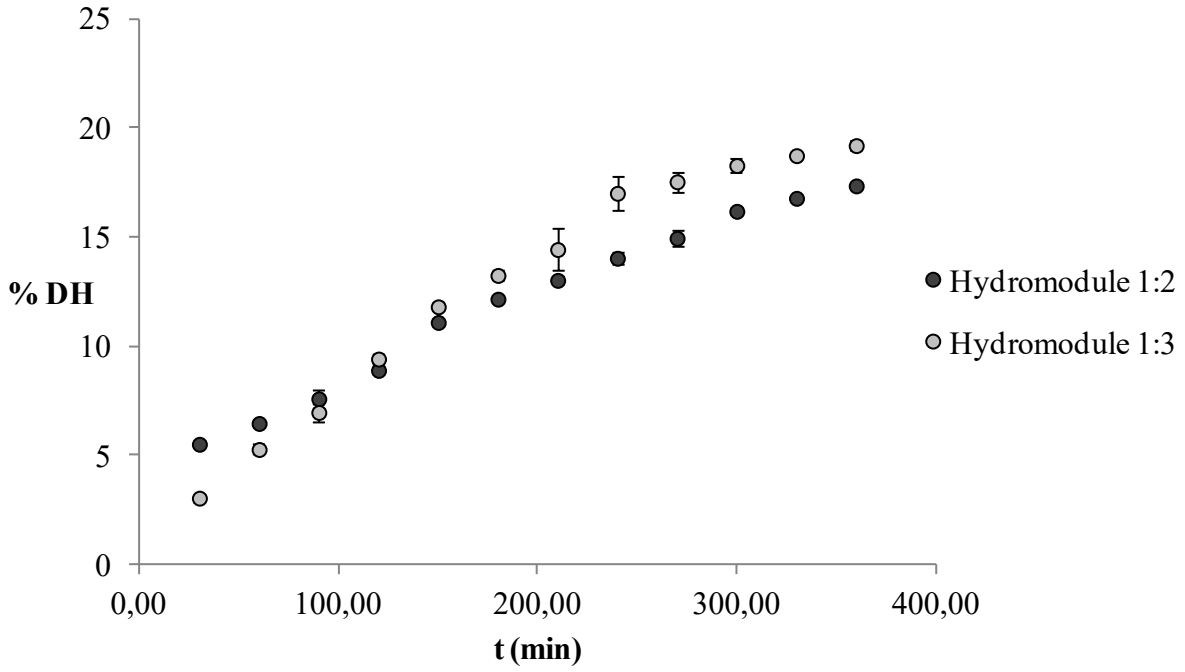
Figure 1 Kinetic profile of the concentration (g/100mL) of the solubilised proteins at different substrate:buffer solution ratios, over the total amount of proteins, as function of time.



The OPA analysis of the solutions indicated an extensive protein hydrolysis, reaching a degree of hydrolysis of about 20% (calculated on the solubilized proteins) in both water/substrate ratios (figure 2), but with some difference between the two conditions. In particular in the hydromodule 1:2, the higher substrate concentration lead to a higher release of N-species during the first hour, compared to the other ratio, but with a lower value at the end of the reaction, (%DH=17.3 ± 0.1). Instead, the more diluted solution with hydromodule 1:3, generated more N-free species at the end of the hydrolysis reaction (%DH=19.2 ± 0.2). This was in agreement with the findings of Butrè et al., 2014, who found that the water availability plays a crucial role in the hydrolysis of proteins at constant enzyme/substrate ratio: by increasing the protein concentration, the overall rate of enzymatic protein hydrolysis decrease.

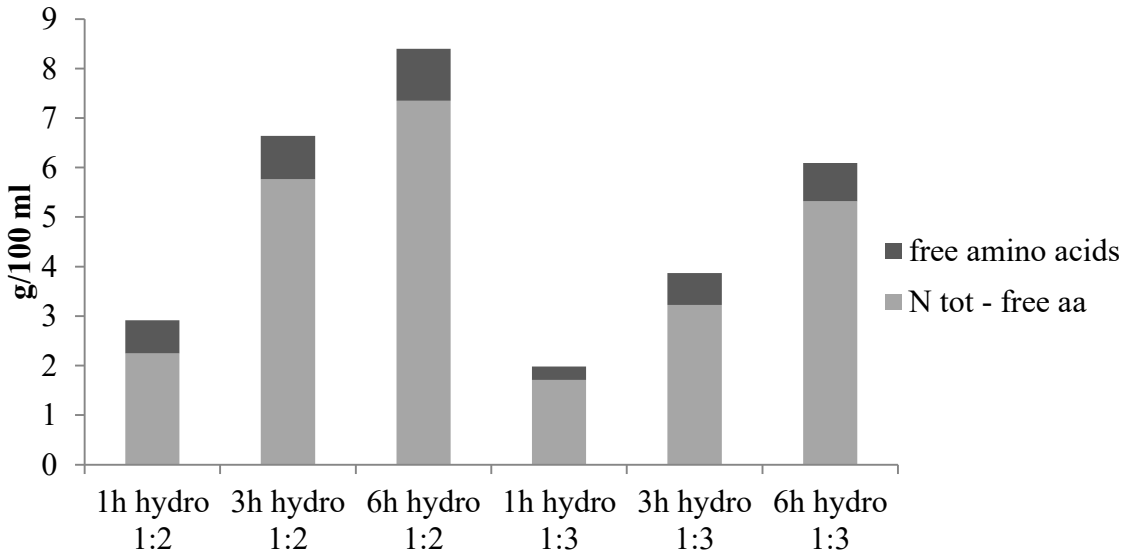
Since Alcalase is known to generate many free amino acids, as also indicated by the high DH observed, also free amino acid content was evaluated by chromatographic methods, as showed in figure 3.

Figure 2 Kinetic profile of the percentage of the degree of hydrolysis at different substrate:buffer solution ratios, as function of time.



The analysis of free amino acids indicated a continuous increase of this species over time, together with the solubilized nitrogen, with a high final amount at the end of the reaction (1.03 g/100 ml for 1:2 hydromodule and 0.77 g/100 ml for the 1:3 hydromodule). The high amount of free amino acids released by Alcalase is in agreement with our previous study and it is due to the broad specificity of this enzyme (Anzani et al., 2017 b) and also accounts for the high degree of hydrolysis measured for these hydrolysates. It is interesting to notice that the experiments, yielding the highest degree of hydrolysis, lead to the lower amount of free amino acids, and vice versa. This indicates that, on average, the 1:2 ratio leads to more free amino acids, but also to longer peptides, whereas the experiment performed with the hydromodule 1:3 leads to less free amino acids, but shorter peptides. This indicates that in concentrated solutions the enzyme attacks preferentially smaller peptides, whereas in more diluted solutions, also longer peptides are cleaved.

Figure 3 Distribution of free amino acids in the broths after enzymatic hydrolysis: sum of the total nitrogen, determined by Kjeldahl method (full bar), and free amino acids (darker part of the bars). The lower part of the bar, in a lighter grey, indicates the nitrogen fraction which is not part of the free pool, thus contained in soluble peptides and proteins.



For further understanding the mechanism of the proteolytic process, proteomic analyses were performed on the solubilized nitrogen fraction. The LTQ-Orbitrap analyses of the peptides present in the hydrolysates are exhibited in table 2. The data indicated that the peptides derived mainly from collagen in both experiments. This fact is indicative that the high efficiency of Alcalase in solubilizing the hides is mostly due to its ability to degrade collagen, as already previously found (Anzani et al, 2017a).

Table 2 Identification by LTQ-Orbitrap of the main peptides present in the solutions derived from enzymatic cleavage on bovine hides, and of the proteins of origin. Proteins are listed in order according to the highest number of peptides generated.

Sample	Protein	Score	Nº peptides
Hydromodule 1:3	Collagen alpha-1(I) chain	914	231
	Collagen, type III, alpha 1	477	130
	Collagen alpha-2(I) chain	398	119
	Elastin	101	53
Hydromodule 1:2	Collagen alpha-1(I) chain	967	250
	Collagen, type III, alpha 1	513	129
	Collagen alpha-2(I) chain	460	150
	COL5A1 collagen type V alpha 1 (Fragment)	50	41

[a]The average peptide length is calculated on peptides automatically annotated. Automatic annotation excludes very short peptides, since they cannot be univocally assigned to a specific proteins, thus the average peptide length is not corresponding to the one calculated by DH.

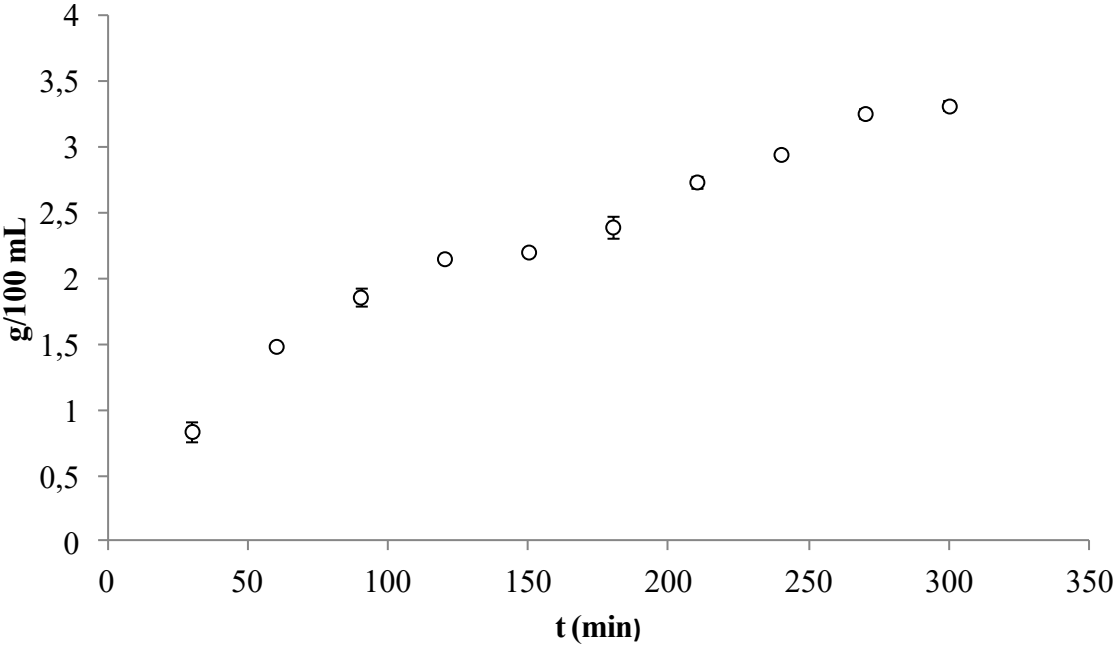
3.1 Enzymatic hydrolysis at semi- industrial scale

An experimental scale-up was performed in order to understand if this process could be applicable in an industrial plant. The same protocol used in the lab scale was followed. The hydromodule 1:3 was selected because slightly more efficient than the other one (as measured by the degree of hydrolysis). An additional reason was the difficulty experienced by the stirring blade in the experiments performed with the hydromodule 1:2, where the rotation movement of the blade was slowed down by the high amount of hides. The semi industrial plant employed was constituted by an agitation drum with an integrated heating/cooling system. The rotation speed was set at 10 rpm, because of the equipment limitations, implying that the mixing efficiency was much less than the one obtained at lab and medium scale. The experiment protocol was designed to be executed within 9 hours, but some delays were experienced due to the bigger scale used,

since the warm up of 30 L required significantly more time, and so the pH adjustment. Therefore the hydrolysis reaction time was reduced to 5 hours in order to accommodate the experiment inside a full working day (9 hours).

The hides solubilisation was lower (45%, considering the recovery of 5.5 kg of unhydrolysed hides) than the trials performed using the lab scale, yielding 3.3 g proteins/100 ml solution. Considering the final volume at the end of the reaction (34500 mL), this value corresponded to 1140 ± 10 g of total solubilised proteins respectively, corresponding to about 40% of protein solubilisation. The kinetic profile of the proteins solubilisation was calculated on the average of the protein content in the bovine hides' - Figure 5.

Figure 4 Kinetic profile of the concentration (g/100mL) of the solubilised proteins over the total amount of proteins, as function of time in the pilot implant.



This slight decrease in the efficiency of the process could depend on two main reasons. The first one is related to the different agitation system between the lab and the semi industrial equipment. In particular in the lab-scale experiments was used an agitator blade and the hides were be completely cover by the buffer solution, therefore the enzyme was directly and always in contact with hides. By contrast, in the semi industrial plant an intermittent agitation drum was employed, and the hides were not always immersed in the solution. The second reason is likely due to the lower rotation speed in the semi-industrial plant. Particularly, in the lab scale experiments the speed was 100 rpm, in pilot plant was set at 10 rpm, because of the equipment

limitations. In order to obtain a solubilisation around 80-90% with the pilot plant equipment, the hydrolysis time would probably needing to be increased of at least another 5 hours. On the other hand, this process is unfeasible because of its high cost and time demanding. Beside this fact, this industrial experiment demonstrated the feasibility of the enzymatic hydrolysis of hides at industrial scale. Clearly, some equipment modifications would be needed to make the process as efficient as in the lab scale, particularly in the efficiency and speed of mixing, but already the preliminary results allowed to achieve a recovery of most of the proteins contained in hides in a soluble, high-quality form. Thus, this process has the potential to be applied at industrial scale for the recovery of protein-rich material for the productions of hydrolysates to be used in the feed or as a food ingredient, or in other high quality applications.

4. Conclusions

The recovery and the valorisation of bovine hides, a major co-product of meat industry, it is possible through the enzymatic hydrolysis, which provides high quality hydrolysates at low cost and low impact. These hydrolysates could be used as ingredients, additives or protein source for feed formulations, in food or in other high quality applications. Alcalase turned out to be very efficient in the digestion of these co-products with a high degree of hydrolysis and a great amount of free amino acids released in solution. Its effectiveness is strictly linked to its ability to degrade collagen, the main protein of hides. Our results showed that Alcalase works efficiently at different hydromodules, with proper mixing in order to perform well in a heterogeneous phase. Despite of the low solubilisation of hides in experiment performed in a semi-industrial implant, this methodology could be applied in industrial scale with an appropriate equipment similar to the one used in the lab scale, in order to decrease the time of the hydrolysis at the same time preserving efficiency.

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Chapter V

Effect of Maillard reaction on the functional properties of collagen based peptides obtained from commercial gelatines and a collagen-rich co-product

Effect of Maillard reaction on the functional properties of collagen based peptides obtained from commercial gelatines and a collagen-rich co-product

Abstract

The recovery of food waste, in particular in the meat sector, has become a hot topic. In this study the enzymatic hydrolysis of bovine hides was proposed as a greener alternative recovery methodology, compared to the traditional ones, for the production of protein hydrolysates, to be used as food ingredients or additives. The resulting hydrolysate was characterised based on their functional properties, and it was found that its performance was very poor. In order to enhance these properties Maillard reaction, using dextran, was proposed as a compatible process with food production. The glycation reaction occurred successfully as proved by the free amino groups' analysis and the size exclusion chromatography before and after the treatment. However, the glycated hydrolysates did not showed an improvement in the techno-functional properties assayed: solubility, gelling ability, water and oil holding capacity, foam and emulsifying capacity.

Keywords: enzymatic hydrolysates, dry-Maillard reaction, enhancing techno-functional properties, protein modification

1. Introduction

Collagen, due to its good gelling properties, is widely used in food industry for the preparation of desserts, candies, bakery, jellied meat, ice cream and dairy products (Toldrà et al., 2012). Other collagen applications in food are as clarifying agent, stabilizer or protective coating material. Additionally, collagen is a biodegradable, biocompatible, and non-cytotoxic protein; it has good ability to support cellular growth and it can be processed into a variety of forms such as: cross-linked films, steps, sheets, beads, meshes, fibres and sponges which have uses in the biomedical field (Zeugolis, Paul, & Attenburrow, 2008). Finally, collagen can be used as protein supplement once converted into peptides (Dybka & Walczak, 2009).

Hides, in general, are a good source of collagen; and because of this its fibrous structures it is used as a raw material for leather production. A further application of hides is their conversion to gelatines by thermal denaturation, mild enzymatic processes and/or chemical degradation. The percentage of bovine hides, produced during slaughtering, is around the 7% of the animal weight (Mullen et al., 2015). However, some pieces of hide are not suitable for leather industry (too small, damaged, etc) and they are disposed, supposing an extra cost for the producer. Because of the increasing interesting of meat industry to reduce and recover of meat co-products, for this research bovine hide trims, with neutral or negative added-value, have been used for protein recovery in order to valorise them as new food or feed ingredient.

A possible way to add value to proteinaceous bovine hides is to convert them into enzymatic hydrolysates for the production of food ingredients. The addition of exogenous enzymes makes the hydrolytic process more controllable, thereby making it reproducible. Several factors, like pH, time, enzyme to substrate ratio and temperature, influence the enzymatic activity co-operatively, thus these parameters have to be strictly controlled (Viera et al., 1995; Liaset et al., 2000). There is currently a wide spectrum of enzymes that can be used for food purposes and their optimum pH, temperature or cleavage points are different, allowing handling many different substrates and generating final product with specific properties. However, all the enzymes, intended to produce protein hydrolysates, have at least one common characteristic: they should be food grade and, if they are of microbial origin, the producing organism has to be non-pathogenic. In this study Alcalase was selected because its ability to degrade the collagen present in meat co-products (Anzani et al., 2017a, Anzani et al., 2017b). In general the choice of substrate, the protease that is employed and the degree to which the protein gets hydrolysed generally affect the physiochemical properties of the resulting hydrolysates (Bhaskar et al., 2008). Moreover, the

hydrolysis reaction in general leads to a gradual cleavage of protein molecule into smaller peptide units, increasing the solubility of the hydrolysed protein, thus facilitating its further processing (Rai et al., 2009). Alcalase was also selected looking at industrial applications due to its low cost (Doucet et al., 2003). Alcalase is an endo-peptidase with a broaden range of specificity; because of the non-specificity of the cleavage site Alcalase activity is very effective releasing in solution high amounts of free amino acids and small peptides, from a variety of sources. Due to this reason the obtained hydrolysates had a very high hydrolysis degree, and were composed by low molecular weight peptides, which usually don't exhibit good emulsifying, gelling or foaming properties; however high solubility is frequently found. This was also observed by Liu et al 2010 on porcine plasma hydrolysates Alcalse-derived. These modifications of the protein properties can affect the functional properties of the final product, the quality and the selection of the process steps (De Oliveira et al., 2016).

A possible way to enhance the techno-functional properties is by means controlled glycation following Maillard reaction; which is a procedure that involves food grade reagents and can be safely used for food industries with minimal changes to colour and flavour (De Oliveira et al., 2016). The glycation of proteins is chemically conducted to produce more amphipathic compounds by adding hydrophilic groups in forms of carbohydrate, generating new glycoproteins. These chemically modified proteins are accomplished by the covalent attachment of monosaccharides or oligosaccharides between the condensation of the carbonyl group of a reducing sugar and an amine group from protein (or amino acid) through the Maillard reaction (Alvarez et al., 2012). The prevention of the formation of harmful components could be achieved by taking under control incubation time, pH and temperature of glycation reaction avoiding its progression to more advanced stages (Sanmartin et al., 2009). Many studies have been reported to modify proteins using a controlled and limited glycosylation to improve the emulsifying and foaming properties, water-holding capacity, and thermal stability of many food proteins. The commonly strategies to obtain conjugate synthesis is heat reaction in dry or aqueous solution. In the first method the reaction can be taken under control and is not time demanding; basically the protein samples is mixed with a saccharide in a proper buffer and heated at a specific temperature (Zhuo et al., 2012; Li et al., 2013). The disadvantages are the water and buffer removal and its unfeasibility for a large scale production. The second methods involve heating of a dry dispersion of protein and saccharide at a specific temperature and relative humidity. The main limitation is the uneven contact between the reagents in case of folded or rigid proteins (Zhuo et al., 2012: Li et al., 2013).

Commercial gelatines, and its Alcalase hydrolysates, were used as a control. Finally the effect of glycation on the techno-functional properties is evaluated on the bovine hides' hydrolysates.

The aims of this project are:

1. The analysis of the techno-functional properties of the bovine hides hydrolysates obtained by hydrolysis reaction with Alcalase.
2. The glycation reaction on the raw gelatines powders, taken as a control, and on the enzymatic hydrolysis of the commercial gelatines.
3. The analysis of the glycation reaction's effectiveness on the techno-functional properties of small-peptides hydrolysates obtained from bovine hides' enzymatic hydrolysis.

2. Material and methods

2.1 Sample preparation

Samples made by pieces of bovine skin were provided by Inalca Industria Alimentare Carni SpA (Castelvetro, Modena, Italy) and stored at −20 °C.

2.2 Enzymatic hydrolysis protocol of the bovine hides and the commercial gelatines powders

The processes were performed in a reactor of 5 L of capacity with a heating shell and agitator blade. The procedure were carried out using two different substrate ratios/ water (hide:water 1:2 and 1:3).

The pieces of hides were washed and degreased twice. In the first step of washing 2% Na_2CO_3 and 1% of soy lecithin were added and in the second one 1% Na_2CO_3 and 1.5% of soy lecithin were used. Both these washing steps required 30 min of agitation at 60ºC, with rotation speed of 100 rpm; after both washing the wastewaters were discarded. After degreasing, the hides were swollen in 10 mmol L-1 Na_2HPO_4 for 1 hour at 60ºC to remove the traces of Na_2CO_3; the wastewater was discarded.

Enzymatic hydrolysis reaction started by suspending the prepared hides in the buffer solution (Na_2HPO_4 10 mmol L-1). 1% (w/w) of enzyme solution was added after monitoring that the pH of the solution (7-7.5). The enzyme used was Alcalase from Bacillus licheniformis and the procedure was performed at 60 °C under stirring. The digestion was stopped after 6 hours and, at the end, boiled for 10 minutes (90º) to inactivate the enzyme. The enzymatic reaction was performed on

the commercial gelatines powders (bovine and porcine) following the previous protocol using the 1:4 hide:buffer solution ratio to prevent gelation process. Finally, the samples were freeze dried (Cuddon FD80, New Zeland) to obtain a powder, and preserved until employed in refrigeration.

2.3 Preparation of protein-dextran/protein-glucose conjugates

Dextran, with an average molecular size of 10 kDa produced by Leuconostoc sp., was supplied by Sigma-Aldrich. Freeze dried proteins were dissolved in distilled water, subsequently adding dextran to the solution in a weight proportion of 1:3 protein/dextran. This proportion was chosen on the basis of bibliographic data (Jung et al., 2006; Alvarez et al., 2012), having been shown to be the most suitable to afford a good yield in the conjugation of BSA/plasma and dextran. The protein/sugar mixture was freeze dried to obtain a homogeneous powder composed of protein and carbohydrates. The samples powder was placed into hermetic vessels and heated at 75 ºC. The process was carried out twice in order to obtain duplicates.

2.4 Determination of free amino groups

The determination was done according to a previous study, with a slight modification. Briefly the OPA/NAC (N-acetyl-cysteine) reagent (100 mL) was prepared by combining 10 mL of 50 mM OPA (in methanol) and 10 mL of NAC 50 mM (in methanol), 5 mL of 20% (w/v) SDS, and 75 mL of borate buffer (0.1 M, pH 9.5). The reagent was covered with aluminium foil to protect from light and allowed to stir for at least 1 h before use. The OPA assay was carried out by the addiction of 20 µL of sample (or standard) to 2.4 mL of OPA/NAC reagent. The absorbance of this solution was measured at 340 nm with JASCO B-530 UV–Vis-spectrophotometer (JASCO, Oklaoma City, OK, U.S.A). A standard curve was prepared using l-isoleucine (0–2 mg mL^{-1}).

2.5 Protein content determination

Protein content was determined based on the measurement of nitrogen by combustion using a LECO FP628 (LECO Corp., MI, USA) Protein analyser based on the Dumas method according to the AOAC method (1996). A factor of 6.25 was used to convert nitrogen to crude protein per cent**.**

2.6 Total amino acids determination

Amino acid profile was analysed following the method reported by Hill (1965). Proteins were hydrolysed in 6M HCl at 110°C for 23 hours and the resulting hydrolysates analysed on the amino

acid as per free amino acids method. Amino acids were quantified using a Jeol JLC-500/V amino acid analyser (Jeol (UK) Ltd., Garden city, Herts, UK) fitted with a Jeol Na+ high performance cation exchange column**.**

2.7 Solubility

The solubility of conjugates was studied in aqueous media. Protein solubility was determined by the method reported by De Vouno et al (1975). This consists in dissolving 0.5 g of the native or conjugated protein sample in 10 mL of distilled water. The solution was centrifuged at 2400 × g for 30 min in a Sorvall Lynx 6000 centrifuge, (Thermo Scientific, Waltham, Massachusetts, U.S.A). pH was varied to test its effect on solubility. Different pH solutions were prepared by adding HCl or NaOH 1 N to the solution to reach the desired pH, the tested pH ranging between 3 and 8. The amount of soluble protein before and after centrifugation was determined by the Nanodrops. All the experiments were carried out in triplicate.

Solubility was calculated as follows:

$\%S=Pd/Pt\times100$

Where %S is the percentage of solubility, Pd is the amount of soluble protein (g) and Pt the amount (g) the total protein used in the assay.

2.8 Water- and oil-holding capacities

The methods of Vioque et al. (2000) were used with modifications. 10 grams of distilled water or rapeseed oil were mixed with 0.5 gram of protein, holed for 30 min, stirring twice gently, and centrifuged at 2,700 × g for 30 min. The volume of the supernatant was weighed again. The water- (WHC) or oil-holding capacities (OHC) were expressed as following:

WHC/OHC= (weight oil or water recovered)/(oil or water initial weight)×100

2.9 Gelling properties

The method of Coffman and Garcia (1977) was followed with slightly modifications. For all the samples the tested proteins range was from 2% to 12% w⁄ v aqueous solutions of freeze dried protein; pH was adjusted to 6 using NaOH and HCl; the solutions were heated in a thermostatic bath at 85ºC for 30 min. After heat-induced gelation, samples were cooled and stored at 4 ºC for 24h. The lowest gelation concentration (LGC) is the lowest concentration of protein in the range tested in which the test tube is inverted and the gel does not slide, i.e. consistent.

2.10 Foam capacity and stability

Foam capacity and stability were assessed according to the method of Lawhon et al (1972) using 1% protein solution in an Ultraturrax (Ultraturrax T25, Janke & Kunkel IKA-Labor technick, Staufen im Breisgau, Germany) at 10000 rpm for 5 min. The percentage increase in foam volume was recorded as foam capacity. The change in the volume of foam after 0,15,30,45 and 60 min of standing at room temperature was recorded as foam stability. Foam capacity (%) was expressed as foam (volume/total volume) × 100.

2.11 Emulsifying capacity

Emulsifying capacity was determined by the Inklaar & Fortuin (1969) method. All the freeze dried samples were dissolved in 10 mL of solvent to reach concentrations ranging between 2 and 10 mg/mL). The solvent used to prepare the protein samples was water with NaCl 0.075% w⁄v; this salt increases the protein-emulsifying capacity by inducing protein folding. Subsequently, 13 mL of rapeseed oil was added to each sample. After stirring the sample for 2 min at 15000 rpm with a homogenizer (omni-prep Homogenizer, OMNI Inc, Kennesaw, Georgia, U.S.A) the samples were centrifuged at 1200 × g for 10 min (Sorvall Lynx 6000 centrifuge, Thermo Scientific, Waltham, Massachusetts, U.S.A). Triplicates of all experimental results were obtained. The percentage of emulsification was calculated as:

$$\%E = Ve/Va \times 100$$

where %E is the percentage of emulsification, Ve the volume of the emulsified oil (mL) and Va the volume of the added oil (mL).

2.12 Ultra-fractionation of sample, SDS-polyacrylamide gel electrophoresis and glycoprotein stain

Protein extracts were fractionated by using Amicon Ultra devices equipped with a 3 kDA MWCO membrane. Ten mL of sample was loaded, the volume of the retentate was kept constant by adding ultrapure water until an 10 mL were obtained for the permeate. SDS-PAGE was performed using a 12% acrylamide separation gel. Conjugates (15 mL, 4 mg/mL) were prepared in a Tris-HCl 0.1 M buffer at 6.8 pH. Electrophoresis was carried out for 60 min at 200 V in a Tris-Glycine buffer. The gels were made in duplicate, and were stained with Coomassie Blue.

2.13 Molecular weight distribution of recovered protein

SEC chromatographic analyses were carried out to determine the molecular size of the hydrolysates. Phosphate buffer (pH 7.0, 0.15 M) was used as mobile phase with a flow of 0.35 mL/min in a Waters HPLC (2795 Separation Module) coupled to two serial-connected columns: AdvanceBio SEC 130A 2.7 µm 4.6x300 mm and AdvancedBio SEC130A 2.7 µm 4.6x50 mm Guard. The result was monitored at 214 nm in a Photodiode Array Detector (Waters 2996) and the retention time of each peak was evaluated using the Empower Pro 2 software (Waters Corporation). A calibration curve was made using blue dextran (2 million Da), albumin (66kDa), carbonic anhydrase (29 kDa), Lysozime (14.3 kDa) and Vit B12 (1.3 kDa).

3. Results and discussion

3.1 Characterization and techno-functional properties of untreated samples

The analysis of the protein content of the hydrolysates after fractionation by using centrifugal filter devices showed that a great content of peptides had a MW less than 3 kDa (figure 1b). Particularly the SDS-PAGE profile of the broths illustrated that in solution the majority of the peptides had a MW~14.4 kDa; however peptides smaller than 3 kDa are not easily detected using this technique. From both the analysis it is clear that there were slightly differences between the both sample/buffer ratios employed. This fact could be attributed to the high efficiency and the broad specificity of Alcalase in the hydrolysis of collagen (Anzani et al., 2017a, Anzani et al., 2017b). The amino acid profile (table 1) showed that there is no significant difference ($F<F_{crit}$) between the two different hides:solution buffer ratio used. The high amount of glycine and proline derived basically from the degradation of the collagen, the main constituent of hides, as demonstrated in a previous study (Anzani et al., 2017a).

Table 1 Amino acids percentage distribution in powders with two different hides:buffer solution ratios, given as percentage of the total amino acid pool.

Amino acids % distribution				
Hydrolysate 1:2		**Hydrolysate 1:3**		**Amino acids**
Avg	St dev	Avg	st dev	
1,48	0,11	0,38	0,01	cysteic acid
1,04	0,76	0,95	0,16	Taurine
1,30	0,04	1,32	0,13	Methionine sulphone
6,74	0,16	7,05	0,18	Asp
2,23	0,18	2,34	0,04	Thre
3,43	0,56	3,72	0,29	Ser
11,88	0,65	11,96	0,08	Glu
24,37	0,67	22,57	0,33	Gly
9,27	0,35	8,78	0,07	Ala
2,91	0,02	3,28	0,11	Val
1,79	0,17	2,13	0,03	Ile
3,81	0,17	4,45	0,00	Leu
1,25	0,19	1,42	0,07	Tyr
2,44	0,02	2,59	0,23	Phe
1,07	0,10	1,98	0,80	His
4,29	0,11	4,73	0,06	Lys
8,18	0,86	8,41	0,12	Arg
13,04	0,39	11,94	0,78	Pro

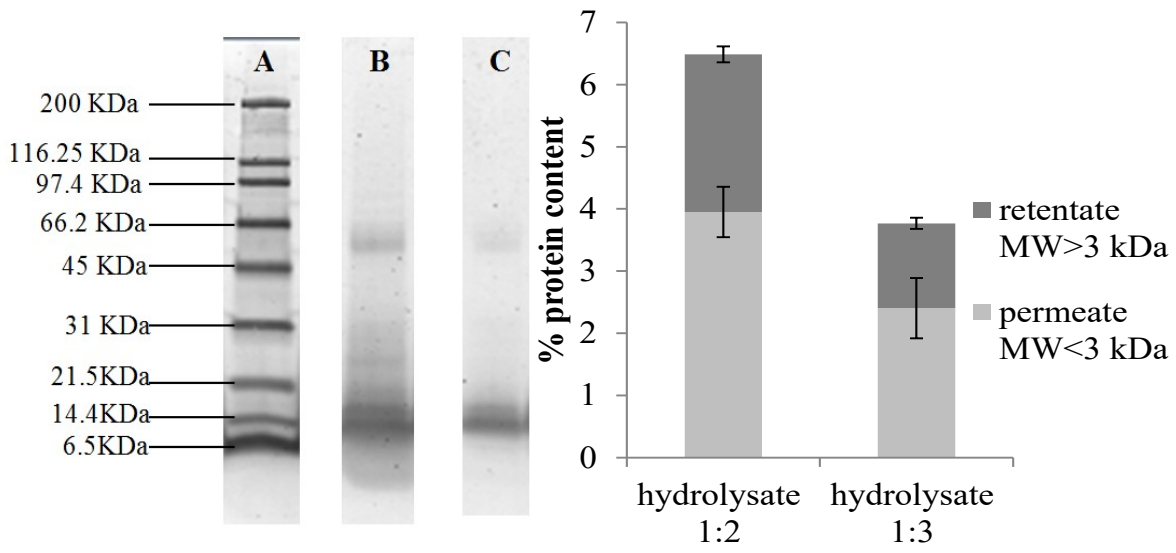


Figure 1 (A) SDS-PAGE profile: A-standard marker, B-hydrolysate 1:2, C-hydrolysate 1:3; (B) protein content distribution after centrifugal devices with a cut off of 3 kDa.

Because of the increasing interest of the research to find application for non-meat co-products originated during the slaughtering as a food component, there is a need to evaluate the techno-

functional properties of these to assess their potential use as food ingredients. The broths were first freeze dried in order to obtain a stable powder, which composition is showed in Table 2. The techno-functional properties of the peptides obtained from hide hydrolysis were analysed and reported in the table 3.

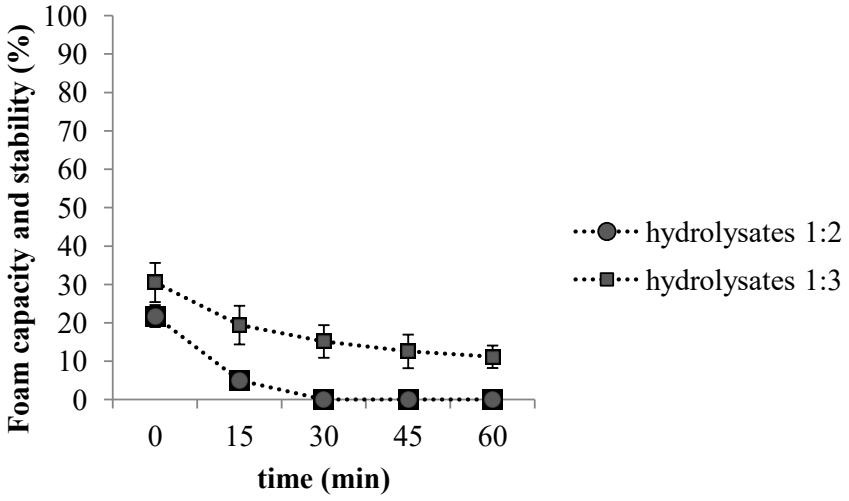
Sample	% protein
bovine commercial gelatines	87.4 ± 0.1
bovine commercial gelatines glycated	29.0 ± 2.3
hydrolysed commercial gelatines bovine glycated	26.0 ± 0.9
porcine commercial gelatines	91.1 ± 0.2
porcine commercial gelatines glycated	26.22 ± 0.04
hydrolysed porcine commercial gelatines glycated	27.8 ± 0.8
hydrolysate 1:3	81.3 ± 0.1
hydrolysate 1:2	86.7 ± 0.2
hydrolysate 1:2 glycated	23.5 ± 0.9

Table 2 Protein percentage in all the sample glycated and non-glycated.

Functional Property	Results
Solubility 3<pH>8	Completely soluble
Water holding capacity	Negative results (high solubility)
Oil holding capacity	4.6 ±0.7 g oil/g protein
Gelling properties (2-12% of protein)	Negative results
Emulsifying capacity	Very poor
Foam capacity	Very poor, slightly better in hydrolysate 1:3

Table 3 The functional properties analysis on both the hide:buffer solution ratios.

Figure 2 Foam capacity and stability in both the hides:buffer solution ratios.



No differences were found between the two hides:buffer solutions ratios. Only in the foam capacity and stability there was a slightly difference, as shown in figure 2. It's clear that the shortening of the peptides chains, due to the advancement of the proteolysis played a negative effect on the majority of the techno-functional properties, in agreement with results reported by Liu et al., 2010., where a loss in emulsifying ability was found after hydrolysing blood plasma proteins. They explained that the less efficiency of the small peptides in stabilizing the emulsion depended on the charge repulsion which not let the peptides to agglomerate producing a fat globule membrane. This happened even though peptides diffuse rapidly towards the interface between the two layers (Liu et al., 2010). Also the foam stability is linked to the molecular properties, because small peptides did not have the strength needed to maintain stable a foam (Shahidi et al., 1995). Despite this poor performing, a good solubility in a broad range of pH was found; it is a positive characteristic that may play an important role in the recovery of these co-products as food additives for liquid formats.

As previously mentioned the increased number of short peptides played a crucial role in the WHC determination; as matter of fact the hydrolysates didn't show any water holding capacity as reported in table 3. Usually long peptides create a hydrophilic matrix that can cause water detention; for this reason compounds with this property may be very useful when incorporated in a food in order to decrease the weight loss or when incorporated in sauces. In our case the high presence of peptides smaller than 3 kDa did not favourite the water retention by not creating an enough strong water-trapping matrix; besides, the high solubility of the peptides impedes the retention of water at the peptide concentration assayed. On the other hand, the OWC test showed very good oil retention (table 3). This positive result may be due again to the very short peptides formed in the hydrolysates: from these results, it is intuitive to think that the fragmentation obtained with the Alcalase enzyme formed a lot more potential lipophilic sites in our material letting the peptides create an internal rearrangement favourable to the oil retention. Hydrophobic groups are usually oriented to the protein core, and after hydrolysis, these groups are exposed thus favouring the interaction with lipids; according to the amino acid profile, around a 55% of total amino acids are hydrophobic. To summarise: the extensive digestion of the hydrolysates gave too short peptides for a positive water retention but at the same time the relative augmentation of the number of binding groups (free amines) gave a very good oil retention.

As discussed, functional properties were completely loss due to the high hydrolysis degree required to recover proteins from hides in a good yield. A possible way to enhance the techno-functional properties is by means of controlled glycation following Mallard reaction, which is a procedure that involves food grade reagents and can be safely used for food industries.

3.2 Changes in free amino groups and molecular size

The glycation of proteins is chemically conducted to produce more amphipathic compounds by adding hydrophilic groups in forms of carbohydrates, generating new glycoproteins (Alvarez et al., 2012). These chemically modified proteins are accomplished by the covalent attachment of monosaccharides or oligosaccharides to the protein free amino groups, especially those in lysine, through the Maillard reaction (De Olivera et al., 2016). Many studies have been reported to modify proteins using a controlled and limited glycosylation to improve the emulsifying and foaming properties, water-holding capacity, and thermal stability of many food proteins (Sanmartın et al., 2009; Corzo-Martinez et al., 2012; Alvarez et al., 2012, Spotti et al.; 2013; Kasran et al., 2013a, 2013b).

In this study the dry glycation reaction protocol was applied instead of the wet Maillard reaction in order to avoid the need to remove the buffer or the water when recovering the products. Initially the protocol was tested on the commercial gelatines powders, either from bovine and porcine sources (Type A and B respectively) and then on the hydrolysed samples. Since no main differences were found in the functional properties, size distribution or amino acid profile of 1:2 and 1:3 sample:buffer rations, the first one was selected; the fact that less water needs to be removed and higher protein recovery yields are achieved makes this process more feasible from an industrial point of view. The reaction time was stopped after 3 hours avoiding the browning and the Amadori compounds' formation. In order to assess the glycation reaction occurred the analysis of the free amino groups and size exclusion chromatography were executed (table 4 and figure). In particular table 4 illustrates the difference of free amino groups of the native protein and after the glycation reaction. As expected, after glycation more available free amino groups were generated. In all of the cases it is reported a decrease in the number of free amino groups, demonstrating that the glycation reaction took place. The conjugated gelatine powders showed a higher decrease of the amount of free amino groups in solution than the same sample hydrolysed, same low glycation degree was observed for hydrolysed hides. Most of free amino groups present in hydrolysates are the amino terminal of the peptide, which is not the preferable site for glycation

(Aoki, Hiidome, Sugimoto, Ibrahim, & Kato, 2001), hence most of them will reamin unaltered. However, in collagen non-hydrolysated, most of free amino groups are susceptible to be conjugated and higher level of free amino groups blocked can be detected.

	Native	Conjugated	Decrease (%)
Bovine	6.24	1.60	74.31
Hydrolysed bovine	7.85	5.00	36.30
Porcine	4.37	0.54	87.58
Hydrolysed porcine	6.05	4.08	32.51
Hydrolysate 1:2	8.86	7.49	20.18

Table 4 Free amino groups (equivalents/mg or g or mol) detected in native and conjugated protein

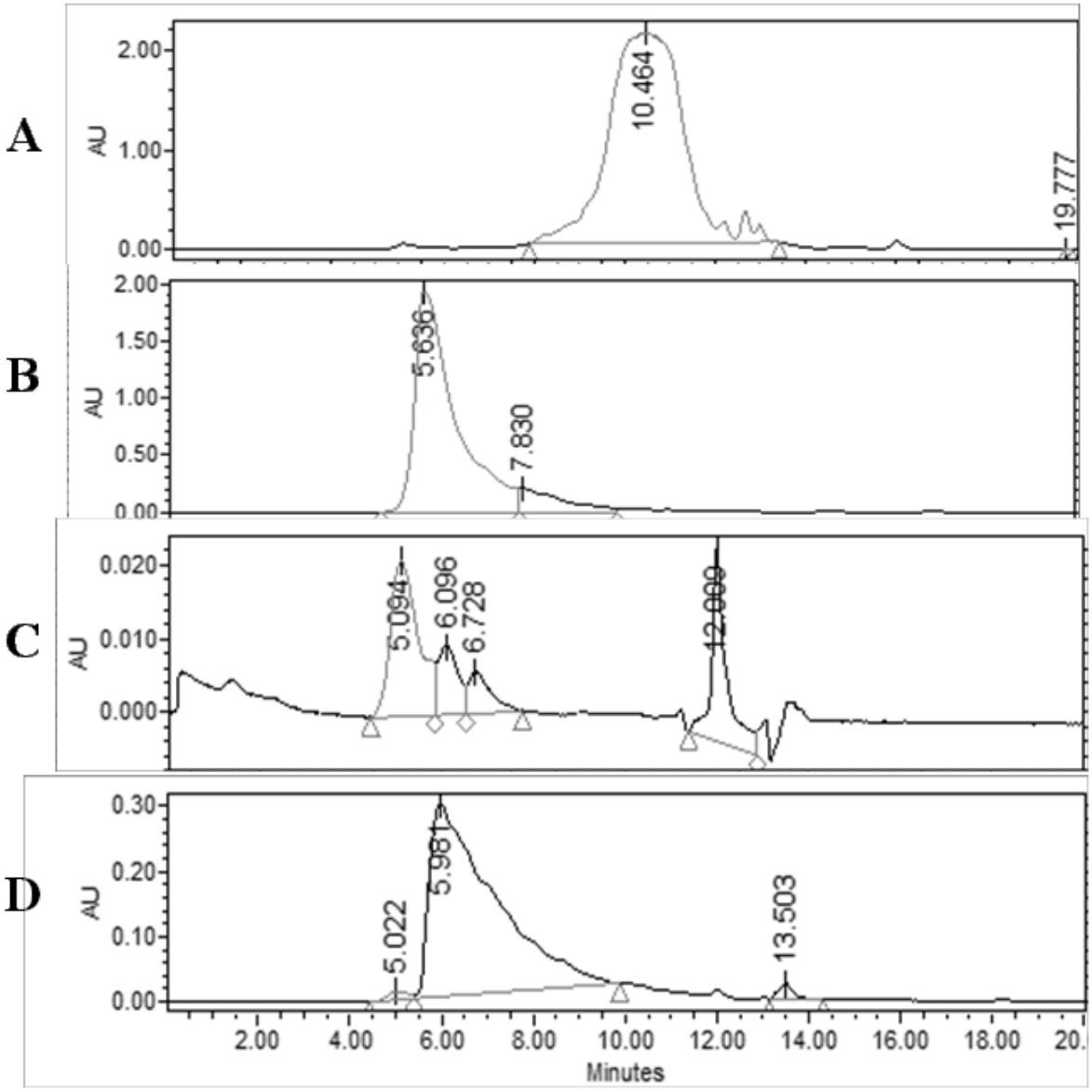


Figure 3 SEC profiles of native and solution-treated samples: A hydrolysate 1:2, B hydrolysate 1:2 glycated, C porcine commercial gelatine, D porcine commercial gelatine glycated

The SEC analysis, carried out on every sample before and after glycation, is further evidence that the glycation reaction occurred. An increasing in the molecular weight was observed. Particularly Figure 3 showed the example of the hydrolysate 1:2 and the porcine commercial gelatines before and after the treatment. The hydrolysate owned an average molecular weight around 6.50 kDa. After the treatment from figure B it was clear that the glycation reaction happened because of the increase of the molecular weight around 150. kDa as a risult of the successful binding with dextran. Additionally the complete disappearance of the peak around 10 minutes demonstrated that all the molecule endure the glycation reaction in a completely or semi-complete way (retention time 7.8 minute). The case of the porcine commercial protein is similar; there was a shift of the molecular weight around 125 kDa after the treatment indicating that the glycation reaction was happened.

3.3 Functional properties of glycated proteins

3.3.1 Solubility

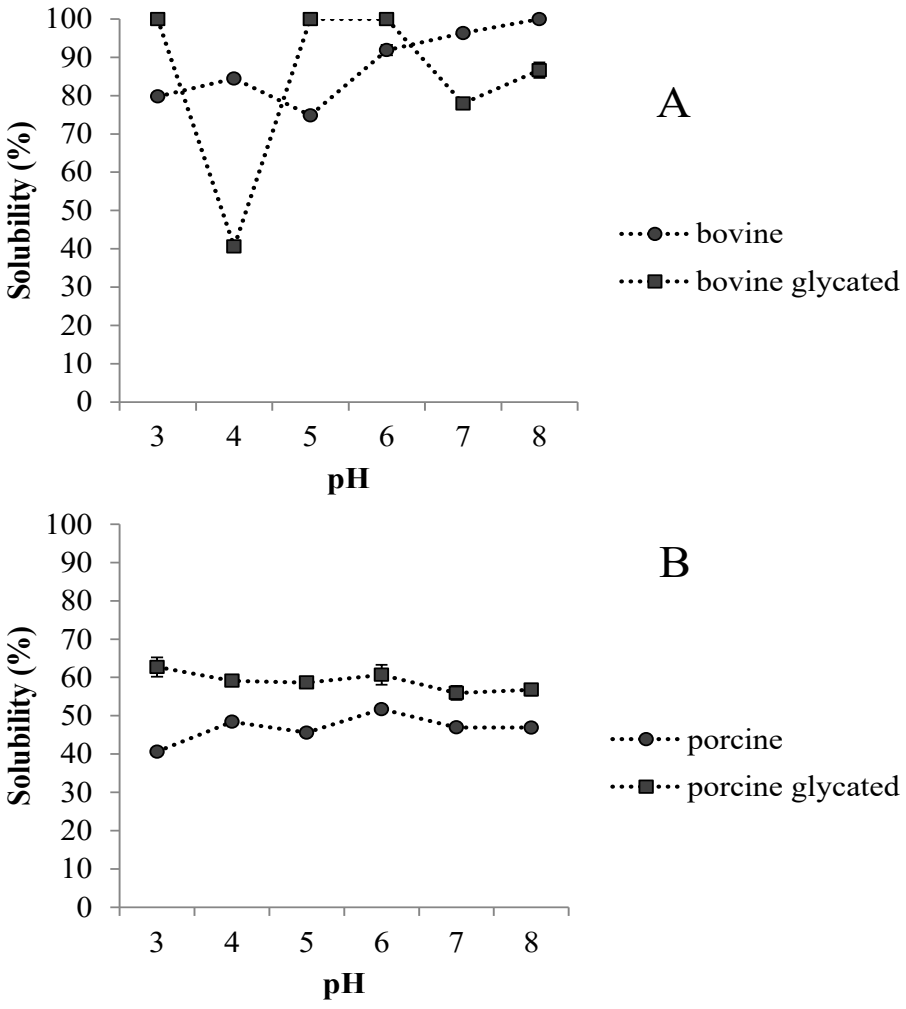


Figure 4 Solubility of native and conjugated samples.

One of the most important properties in proteins to be used in food as additives is its solubility. The behaviour of bovine and porcine commercial gelatines powders is different. The bovine gelatines are more soluble than the porcine one's (figure 4). The glycation had a different effect on both. In the case of the porcine the solubility is increased in all the pH range used (3<pH<8) as observed from other research groups on different started materials. However for the gelatine from bovine source it seemed that the glycation did not affect a lot the solubility a part from a remarkable decrease of solubility at pH 4; a similar trend was observed also by Mulchay et al. (2016) on whey protein isolate treated with maltodextrin. By contrast the glycation reaction did not show effect on the hydrolysates 1:2, which remained completely soluble in the pH tested. After the hydrolysis and the following glycation of the commercial gelatines powders, the solubility increased between pH 3 and 8. Nevertheless this positive contribute in the solubility could not be referred to the conjugation reaction, but is most probably due to the shortening of the peptides length during the enzymatic hydrolysis. It is well known that the hydrolysates, obtained with an enzyme with a broad specificity of cleavage site, owned good solubility properties. This is in agreement with the data in section 3.1.

3.3.2 WHC and OHC

Table 5 showed a significant decrease of the water holding capacity from the commercial gelatines powders and after the glycation and the hydrolysis. This behaviour was found out by Lillard et al., 2009 in the whey protein concentrates glycated with dextran, where each sample exhibited a pellet after low speed centrifugation, attributed to the inability to retain water. In every calculation it was taken into account the effect of the WHC and OHC that dextran owns itself. Therefore it is clear that the improvements in the OHC in all the glycated samples derived from the OHC of dextran and not from the treatment itself. This is the same case in WHC of the hydrolysates 1:2 glycated, where the increase was due to only on the presence of dextran.

Table 5 WHC and OHC determination in the native and the conjugated samples.

	WHC	st dev	OHC	st dev
Bovine	26.81	2.59	5.99	0.46
Bovine glycated	3.08	0.12	7.92	0.37
Hydrolysed bovine glycated	2.24	0.07	8.15	0.11
Porcine	27.68	1.67	7.65	0.37
Porcine glycated	2.52	0.09	7.76	0.20
Hydrolysed porcine glycated	2.46	0.13	8.08	0.23
Hydrolysate 1:2	-	-	4.44	0.69
Hydrolysate 1:2 glycated	2.16	0.07	8.33	0.21
Dextran	2.63	0.61	7.80	0.13

3.3.3 Foam capacity and foam stability

Foaming property is an important attribute of food which can facilitate the mixing, can confer product structure and contribute to product sensory quality. Therefore owing good foam capacity and stability is a good aspect for an industrial point of view, but for most of food products the situation is completely different. In literature there are a lot of examples of improved foam capacity and stability after Maillard reaction (Medrano et al., 2009; Corzo-Martinez et al., 2010), but this is not the case. Figure 5 illustrated the percentage of foam formation during the time passing for both the bovine and the porcine gelatines. The behaviour is completely different between the two different raw materials. In the case of the bovine it seemed that the glycation treatment had a positive effect in the stability of the foam. On the contrary the shortening of the peptide lengths, due to the hydrolysis process with Alcalase, decreased not only the foam capacity but also led to the complete disappearance of the foam after 45 min. In the case of the porcine gelatines powders the glycation after the hydrolysis affected in a negative way the foam capacity and stability without suppressing the foam. From both of the study it is clear that the decreasing of the molecular weight of the peptides played a significant role making the glycation reaction ineffective. The same outcome was found out by Dickinson and Izgi (1996) with the conjugation of casein with dextran.

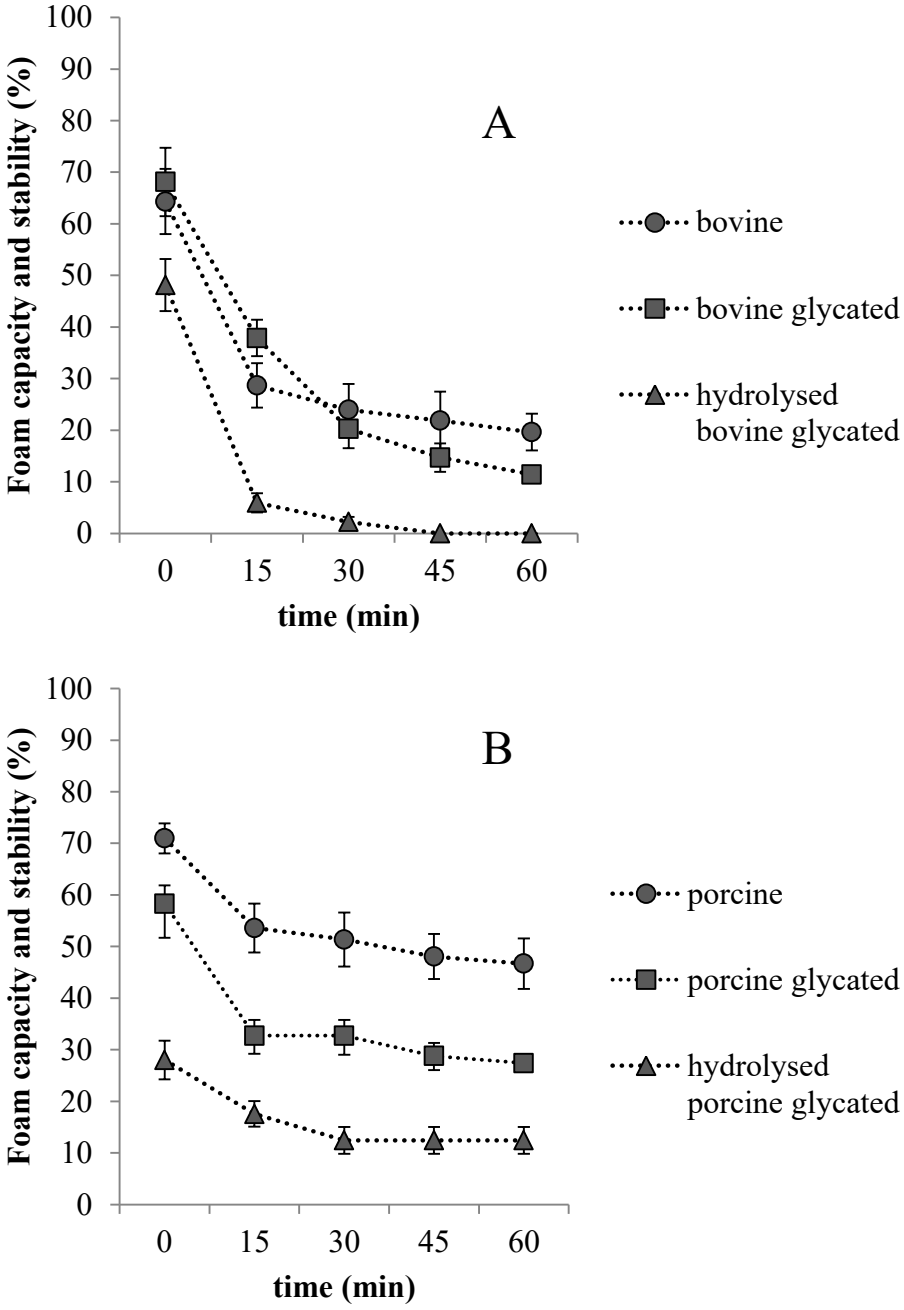


Figure 5 Foam capacity and stability of native, hydrolysed and conjugate samples.

The hydrolysated 1:2 did not show a good foam capacity and stability properties and the result was not changed after the glycation. Also Corzo-Martinez et al. (2012) found out that the glycation of β-lactoglobulin conjugated with galactose did not significantly alter the interfacial characteristics and foaming properties.

3.3.4 Emulsifying capacity

Among the techno-functional properties, the emulsifying properties play an important role in food systems, as they contribute directly to texture and sensory properties of food (De Oliveira et al., 2016). There are several examples in literature that show the increase of the emulsion activity and stability due to the Maillard reaction (Zhu et al., 2010; Li et al., 2013; Kasran et al., 2013a, 2013b). The increment of the emulsifying properties depends on the balance between the hydrophobic and the hydrophilic characters of the protein-polysaccharide conjugates. Moreover, the saccharides attract water molecules around the oil droplet, while the hydrophobic residues of the protein molecules are attached on the oil droplets inhibiting the oil droplets coalescence (Oliver et al., 2006). The gelatines powders from two different sources behaved in a different way, as previously noticed in the foaming properties (figure 6). In the case of bovine glycated gelatines there is a slightly increase in the emulsifying capacity and stability. By contrast after the glycation of the porcine commercial gelatines powders had a great decrease of the emulsifying properties reaching the maximum point at the highest concentration of 10 mg/mL. In the conjugated samples, obtained after hydrolysis, only a very weak emulsion was generated. Moreover, the glycation reaction of the hydrolysates samples 1:2 did not alter the emulsifying properties.

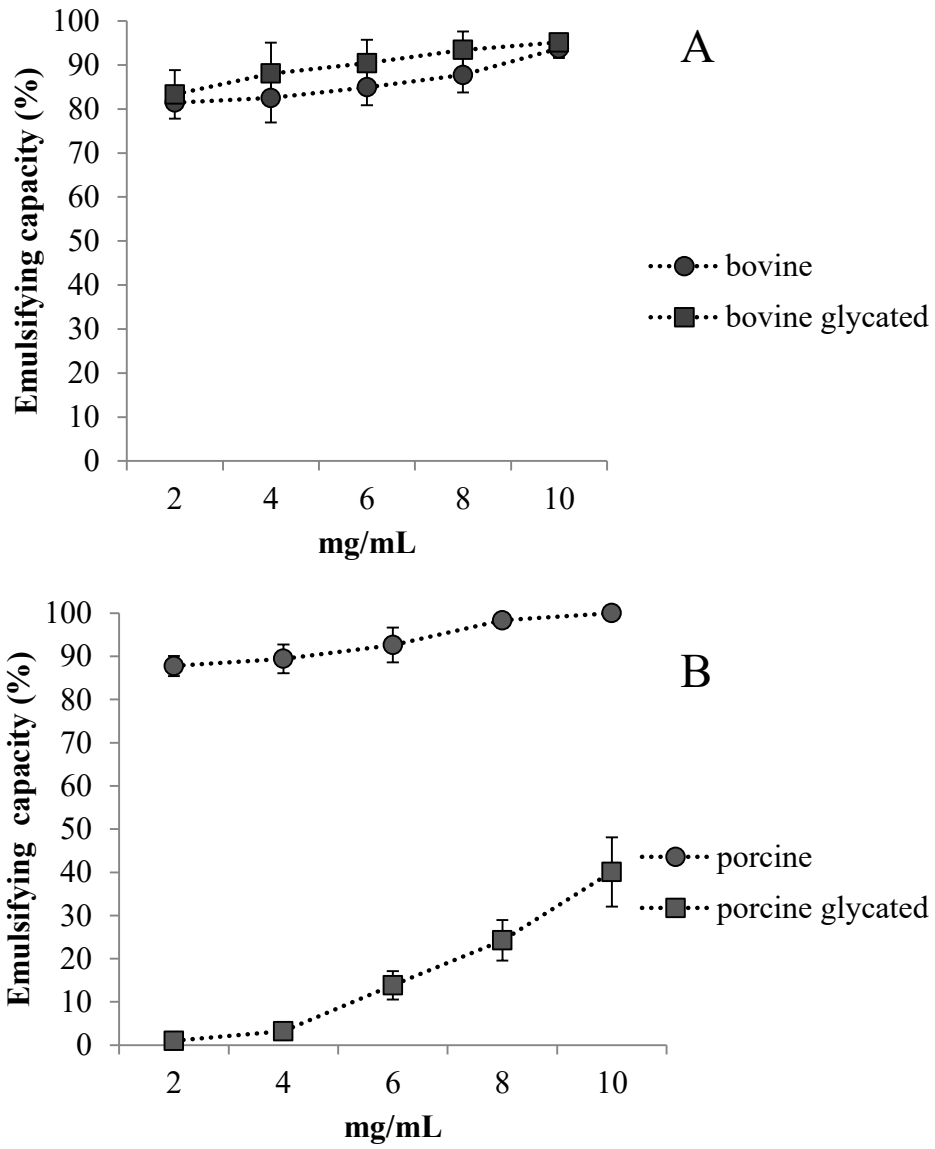


Figure 6 Emulsifying capacity of native and conjugated samples.

3.3.5 Gelling properties

Table 6 Gelling properties in function of the percentage of protein of native, hydrolysed and conjugate samples

Sample	LGC %
Bovine	2
Bovine glycated	4
Hydrolysed bovine glycated	Nd
Porcine	1
Porcine glycated	6
Hydrolysed porcine glycated	Nd
Hydrolysate 1:2	Nd

As shown in table 6 in all of the conjugate hydrolysed samples the gelling properties are not increased, this great diminution depends only on the shortening of the peptides length due to the enzymatic hydrolysis. The case of the commercial gelatines is completely different. The glycation did not increase the gelling properties because the commercial powders owned it themselves, as gelatines, the highest gelification efficiency.

4. Conclusion

Apart from high solubility in a wide pH range (3>pH>8), bovine hides' hydrolysates showed poor techno-functional properties. This behaviour derived from the high efficiency and the broad specificity of Alcalase in the hydrolysis producing low molecular weight peptides. In order to enhance the techno-functional properties of the hydrolysates, the dry glycation reaction with dextran was used to generate glycoproteins following the food grade Maillard reaction. The reaction was carried out firstly on the commercial gelatines powders from porcine and bovine source in order to optimise the condition and then optimised conditions were applied on the hydrolysates. The decreasing of the free amino groups and the presence of high molecular weight molecules in the size exclusion chromatography demonstrated that the glycation happened. From the techno-functional analyses of the hydrolysates it was clear that the glycation did not influence the properties. A future aspect could be the evaluation of other possible strategies to enhance the techno-functional properties of peptides, although current methods are not applicable for a food production point of view.

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Chapter VI

Effect of the replacement of bovine hides hydrolysates and commercial gelatine powders in the technological properties of Irish sausages

Effect of the replacement of bovine hides hydrolysates and commercial gelatine powders in the technological properties of Irish sausages

Abstract

With increasing of meat consumption and the production of meat industry co-products, there is a worldwide interest to recovery and valorise these co-products as food ingredients or in food products formulation. The aim of this study is to evaluate the replacement of enzymatic hydrolysates obtained from bovine hides in Irish sausages and compare them with the commercial gelatines powders used in the Irish sausages production. In particular the protein sources were incorporated in the Irish sausages replacing the meat protein content at 10% and 20% of the protein content. Colour, texture, water holding capacity (WHC), emulsion stability, cook loss were evaluated. The proximate composition (protein, moisture, fat and ash content) were performed on the raw batter and on the cooked sausages. The replacement with the hydrolysates showed a positive effect on the cook loss and on the emulsion stability, but a negative one in the WHC. By contrast the behaviour of the commercial gelatines formulation is the opposite. From a textural point of view, the commercial gelatines sausages exhibit a comparable texture profile like the control. In the case of the hydrolysate replacement, because of the low hardness and chewiness could be employed for elderly consumers.

Keywords: meat industry co-products, bovine hides' hydrolysates, Irish sausages, technological evaluation

Abbreviations

ES	Emulsion stability
BV	Bovine commercial gelatine powder
BV10	Replacement of 10% of bovine commercial gelatine powder
BV20	Replacement of 20% of bovine commercial gelatine powder
HA	Bovine hides hydrolysate hides:buffer solution 1:2
HA10	Replacement of 10% of bovine hides hydrolysate hides:buffer solution 1:2
HA20	Replacement of 20% of bovine hides hydrolysate hides:buffer solution 1:2
HB	Bovine hides hydrolysate hides:buffer solution 1:3
HB10	Replacement of 10% of bovine hides hydrolysate hides:buffer solution 1:3
HB20	Replacement of 20% of bovine hides hydrolysate hides:buffer solution 1:3
PK	Porcine commercial gelatine powder
PK10	Replacement of 10% of porcine commercial gelatine powder
PK20	Replacement of 20% of porcine commercial gelatine powder
SRM	Specified risk materials
TEF	Total expressible fluid
WHC	Water holding capacity

1. Introduction

The meat sector generates a substantial impact on the environment in terms of land occupation, carbon footprint and food wastage. In particular in the meat industry large amounts of waste are generated, especially during slaughtering. Moreover, the amount of waste produced depends on the animal type and the processes applied in the abattoir. For instance, beef meat accounts for approximately 44% of the total live animal weight (Russ and Pittroff, 2004); while remaining 56% is composed of edible (e.g. offal) and non-edible (e.g. specified risk materials or SRM) parts. Hide is one example of a meat industry co-product and concerns the 7% of the total animal weight (Mullen et al., 2015). As matter of fact it is used as a raw material for leather production; however, some pieces of hide are not suitable for leather industry (too small, damaged, etc.) and are disposed. Hide's chemical composition is characterized by the presence of a large amount of collagen, a structural fibrous protein, which is often converted to gelatine by thermal denaturation and/or chemical degradation. Collagen performs excellent gelling abilities and due to this reason it is widely used in food industry for the preparation of desserts, candles, bakery, jellied meat, ice cream and dairy products (Toldrà et al., 2012). Additionally, collagen is biodegradable, biocompatible, non-cytotoxic, it has good ability to support cellular growth and it can be processed into a variety of forms such as: cross-linked films, steps, sheets, beads, meshes, fibres and sponge; which have potential biomedical applications (Zeugolis, Paul et al., 2008). Hide trims, since they are an excellent source of collagen-type proteins and currently are considered to be neutral or negative added-value co-products, have been employed for protein recovery in order to valorise them as new food or feed ingredient. The enzymatic hydrolysis was selected as a recovery method because it is a fast and gentle alternative to other physical or chemical treatments (Vidotti et al., 2003). Enzymatic processes have been recognised as an excellent tool for protein recovery and protein-rich product revalorisation (Galanakis, 2012). The ability of the enzyme to hydrolyse proteins to produce free amino acids and short peptides allows the proteinaceous materials derived from collagen, in this case, to be more soluble and easily recovered and purified, with the aim to be used in the food, feed and pharmaceutical industries (Mullen, Álvarez et al., 2017).

It is well known that because of the gelling and the water binding properties of collagen proteins, when included as ingredient in meat products, the shrinkage is stabilised and cooking yield is increased (Schilling et al., 2003; Tarté et al., 2009). Due to this reason collagen has been employed in meat products or meat preparations to enhance some of their characteristics (Pereira et al., 2011). Particularly Sousa et al (2016) analysed the quality parameters of the frankfurters-type

after replacing fat with tree different levels of commercial collagen hydrolysates. Also the research group of Yeo et al. (2014) replaced fat with duck feet gelatine into low-fat frankfurters, resulting in an increasing of all textural parameters and decreasing in lightness.

Differently the research group of Pereira et al. (2011) evaluated the quality characteristics of the frankfurters-type sausages after the addition of deboned chicken meat and commercial collagen fibres. Moreover, in the experimental design with response surface method the pork trimming were substitute with different percentages of collagen fibres and chicken deboned meat. The aim of this work was to revalorise a meat industry co-product (bovine hides) through the production of protein hydrolysates with the employment of a food grade enzyme: Alcalase from *Bacillus licheniformis*. The enzymatic hydrolysates obtained were employed as protein replacers into a real food matrix (Irish breakfast type sausages). In order to know the effect of replacing meat proteins with hides' hydrolysates, two different replacing levels were tested (10% and 20%). The composition (proximate analysis), the techno-functional properties (emulsion stability and cook loss) and the sensory properties (texture and colour) were evaluated and compared with a control recipe, using a commercial gelatine.

2. Materials and methods

2.1 Enzymatic hydrolysis protocol at lab and medium scale

Samples, constituted by pieces of bovine hides, were provided by Inalca Industria Alimentare Carni SpA (Castelvetro di Modena, Italy). The pieces of hides were washed and degreased twice as follows. In the first step of washing 2% Na_2CO_3 and 1% of soy lecithin were added and in the second one 1% Na_2CO_3 and 1.5% of soy lecithin were used. Both these washing steps required 30 min of agitation at 60°C, with rotation speed of 100 rpm; after both washing the wastewaters were discarded. After degreasing, the hides were swollen in 10 mmol L^{-1} Na_2HPO_4 for 1 hour at 60°C to remove the traces of Na_2CO_3; the wastewater was discarded.

The hydrolysis processes were performed in a outer shell reactor of 5 L of capacity, equipped with an agitator blade. Two different hides:buffer solution ratios have been used:

Hides (1.5 kg):buffer solution (3 L)=1:2

Hides (1.0 kg):buffer solution (3 L)=1:3

Enzymatic hydrolysis reaction was started by suspending the prepared hides' pieces (size of 10x10 cm approx.) in the buffer solution (Na_2HPO_4 10 mmol L^{-1}). Then, 1% (w/hides' w) of enzyme

solution was added after monitoring that the pH of the solution was within 7 to 7.5 values. The enzyme used was Alcalase from *Bacillus licheniformis* and the procedure was performed at 60 °C under stirring. The reaction was stopped after 6 hours and the broth boiled for 10 minutes (90 °C) to inactivate the enzyme. Finally the solutions were freeze dried (Cuddon FD1800, New Zeland) in order to obtain a powder.

2.2 Sausage recipes

In the breakfast sausage formulations, both 10 and 20% of meat was substituted by the equivalent amount of protein coming from hydrolysates powders. Protein replacement was calculated based on the average protein content of meat (18%), which meant that 1.80 and 3.60 g of protein per 100 g of batter was replaced at 10% and 20% replacements levels, respectively. In the same way, moisture content of the replaced meat was maintained (65% on average) and this water was replaced as ice in all formulations. A control was performed as well, resulting in 9 different formulations: PK10, BV10, HA10, HB10, PK20, BV20, HA20, HB20 (PK porcine commercial gelatines powders, BV bovine commercial gelatines powders, HA bovine hides hydrolysate hides:buffer solution 1:2 and HB bovine hides hydrolysate hides:buffer solution 1:3; 10 and 20 mean respectively 10% and 20% of replacement) and control. In the control sausages, the batter composition was 65% bovine meat, 20% bovine fat, 15% water (added as ice) and 1.8% of salt. Table 1 illustrates the composition of all the formulations. One kilogram of each batter was generated. Batters were stuffed into collagen casings, after mincing, and stored at 4 °C until being analysed, within the next 48 hours.

Table 1 Sausage formulations studied in this work

Ingredients	Bovine meat	% protein replaced	Fat	Ice	salt
control	65	0	20	15	2
PK10	55	1,8	20	21,5	2
BV10	55	1,8	20	21,5	2
HA10	55	1,8	20	21,5	2
HB10	55	1,8	20	21,5	2
PK20	45	3,6	20	28	2
BV20	45	3,6	20	28	2
HA20	45	3,6	20	28	2
HB20	45	3,6	20	28	2

2.3 Proximate composition

The proximate analysis of both raw and cooked samples was carried out in order to assess the impact of replacers on sausage properties. Protein content was determined by LECO FP628 (LECO Corp., MI, USA), according to Dumas method (AOAC 1996) based on the measurement of nitrogen by combustion. The crude protein percentage was obtained after the conversion of the nitrogen content for the factor 6.25. As well fat and moisture content were evaluated following AOAC Official Methods (1995) for moisture and fat (1995a). Fat and moisture content were measured using Smart System 5 (Smart Trac 5 Model 907875, CEM Corporation, NC, USA) microwave drying oven and NMR Smart Trac rapid Fat Analyzer (CEM Corporation USA). Ash content was measured using a dry ashing method.

2.4 Technical properties

Five sausages from each treatment were employed for cook loss determination. In particular the samples were gently dried using absorbent paper, then carefully weighed and individually vacuum packed. Following the method of Cofrades et al (2000), each sausage was cooked in a water bath at 80 ^{o}C for 30 minutes, then chilled to room temperature, unpacked, gently dried again and weighed. Cook loss was calculated as follows:

$$\%\ Cook\ loss = ((Raw\ weight - cooked\ weight)/Raw\ weight)\ x\ 100 \qquad \text{Eq1}$$

For the emulsion stability evaluation, according to Hughes et al (1998), approximately 25 g (exact weight recorded) of the raw emulsion was placed in a centrifuge tube (five replicates per formulation). After the centrifugation for 1 min at 2958 g, the samples were heated in a water bath for 30 min at 70°C, and centrifuged again, for 3 min at 2958 g. For each sample, the pellet fraction was removed and weighed. Differently the supernatant portion was poured into pre-weighed crucibles and dried overnight at 100 °C. The volumes of total expressible fluid (%TEF) and the percentage fat were calculated as follows:

$$TEF = Weight\ of\ sample - Weight\ of\ pellet \qquad \text{Eq 2}$$

$$\%TEF = (TEF/sample\ weight)\ x\ 100 \qquad \text{Eq 3}$$

$$\%Fat = (Dried\ supernatant\ /TEF)\ x\ 100 \qquad \text{Eq 4}$$

Water holding capacity (WHC) was measured in the raw batter according to Jin et al (2007). Five grams of sample were mixed with 10 ml water, placed in a 50 ml centrifuge tube, weighted, vortexed for 1 min and centrifuged for 10 min. After pouring the supernatant, samples were reweighed and WHC was calculated as following:

$$\%WHC = ((Final\ weight - Initial\ weight)/Initial\ weight)\ x\ 100 \qquad \text{Eq 5}$$

According to Herrero et al (2007) the textural analysis was performed on five cores (diameter = 2.0 cm; height = 2.0 cm) cut from each sausage. They were axially compressed to 30% of their original height in a two-cycle compression (Instron Universal Testing Machine 4464, Instron Ltd., High Wycombe, UK). The measurements were executed at room temperature using five replicate sausages per treatment. Force-time deformation curves were obtained using a 5 kN load cell applied at a crosshead speed of 50mm min^{-1}. The adhesiveness (N), hardness (N), cohesiveness (dimensionless), springiness (mm), chewiness (Nxmm), and gumminess (N) were evaluated.

The film colour properties were measured in both raw and cooked samples, using a HunterLab UltraScan Pro (Hunter Associates Laboratory, Inc., Reston, VA) with a viewing port of 25.54 mm and illuminant D65, 10°. The specular component was included (RSEX included) and the size of the trap 0.37". Calibration was carried out using a white standard tile (L = 100) and a light trap (L = 0). The white tile was covered in the same material than the sample (transparent cling film) to eliminate any effect on colour readings. Sample was wrapped in the same transparent cling film and five points per sample were measured. L* (lightness), a* (redness) and b* (yellowness) values and averaged. The total colour difference was calculated per the following equation (Salgado et al. 2011):

$$\Delta E = \sqrt{(Ls - Lc)^2 + (as - ac)^2 + (bs - bc)^2} \qquad \text{Eq 6}$$

Were "s" is the value obtained for the sample and "c" is the value measured on the control.

2.5 Statistical analysis

In this study, differences between controls and treatments were analysed by analysis of variance (ANOVA).When analysis of variance revealed a significant effect ($p < 0.05$), means were compared by Tukey test at 5% significance level, using the software SPSS.

3. Results and Discussion

3.1 Characterization of protein hydrolysates and commercial gelatines

The SDS-page profile of the hydrolysates showed that they are mainly composed by short peptides lower than 14 kDa molecular weight, meaning that the hydrolysis process was successful and protein were effectively broken down. The commercial gelatine powder employed in this study were: pork origin, a type A (acid extraction) gelatine with a bloom force of 300 which means that the average weight of collagen fibres is around 50 and 100 kDa; and bovine origin, a type B (alkaline extraction) gelatine with a bloom force between 50-100 which corresponds to 40 and 50 kDa. Type-A gelatine have an isoelectric point at pH∼8–9) and type-B gelatine have an isoelectric point at pH∼ 4–5 (Gómez-Guillén et al., 2011). The powders obtained, along the commercial gelatines, were evaluated based on their techno-functional properties; main results are shown in Table 2.

Table 2 Functional properties analyses of protein replacers: PK porcine commercial gelatines powders, BV bovine commercial gelatines powders, HA bovine hides hydrolysate hides:buffer solution 1:2 and HB bovine hides hydrolysate hides:buffer solution 1:3.

	% protein content	gelling properties	emulsifying capacity	Foam capacity	WHC
PK	105.64±0.45	+	+	+	+
BV	100.30±0.71	+	+	+	+
HA	95.68±0.60	-	-	-	-
HB	90.29±0.81	-	-	-	-

It is well known the high gelling capacity of collagen and also the high WHC which could stabilize the shrinkage and increase the juiciness of meat products (Prabhu et al., 2004). By contrast, because of the shortening of the peptides due to high efficiency of the enzyme, the hydrolysates are expected to have poorer techno-functional properties, mainly those related with gelling abilities.

3.2 Proximate composition

The proximate composition (protein, fat, ash and moisture content) of the raw and the cooked samples are reported in Table 3.

Table 3 Illustrates the proximate analysis of both, raw (a) and cooked (b) samples. The superscript letters (a,b,c,d,e,f) refer to values significantly different from each other (in this case a series of pairwise comparisons with $p \geq 0.05$ have been executed)

a	raw			
	Moisture	Protein	Fat	Ash
control	68.14±0.48^{b,c}	14.73±0.41^{b}	16.33±0.35^{a}	2,55±0,02^{a,b}
PK10	68.80±0.68^{c}	13.22±0.49^{a}	16.26±0.71^{a}	2,54±0,03^{a,b}
BV10	67.99±0.53^{a}	13.08±0.27^{a,b}	19.74±0.75^{c}	2,59±0,01^{a,b}
HA10	66.09±0.55^{b,c}	13.10±0.73^{a}	18.39±0.64^{a}	2,54±0,04^{a,b}
HB10	66.83±0.43^{a,b}	13.68±0.16^{a,b}	16.32±0.71^{b,c}	2,65±0,03^{b}
PK20	68.19±0.44^{b,c}	13.72±0.44^{a,b}	17.35±0.76^{a,b}	2,50±0,02^{a}
BV20	69.35±0.38^{c}	13.46±0.13^{a}	16.11±0.69^{a}	2,56±0,06^{a,b}
HA20	68.26±0.10^{b,c}	13.52±0.62^{a,b}	17.12±0.10^{a,b}	2,596±0,007^{a,b}
HB20	66.95±0.67^{a,b}	12.94±0.25^{a}	19.05±1.06^{b,c}	2,59±0,01^{a,b}
b	Cooked			
	Moisture	Protein	Fat	Ash
control	59.23±0.37^{d}	19.55±0.08^{c}	21.21±0.11^{b,c}	2,465±0,005^{c,d}
PK10	57.45±0.76^{b,c}	19.56±0.35^{c}	24.31±1.09^{d}	2,37±0,12^{b,c}
BV10	58.61±0.19^{c,d}	20.29±0.21^{c}	21.26±020^{b,c}	2,42±0,02^{c,d}
HA10	62.54±0.52^{e,f}	17.76±1.00^{b}	19.90±0.81^{a,b}	2,39±0,02^{c}
HB10	61.12±0.18^{e}	17.38±0.11^{a,b}	22.19±0.20^{c}	2,58±0,05^{d,e}
PK20	54.71±0.19^{a}	19.50±0.11^{c}	27.76±0.13^{e}	2,13±0,01^{a}
BV20	56.64±0.47^{b}	19.89±0.09^{c}	25.74±0.73^{d}	2,203±0,009^{a,b}
HA20	62.75±1.15^{f}	17.01±0.26^{a,b}	21.33±1.24^{b,c}	2,49±0,01^{c,d}
HB20	65.54±0.25^{g}	15.97±0.06^{a}	18.45±0.02^{a}	2,75±0,01^{e}

The moisture, fat, ash and protein content were, although statistically different between treatments ($p < 0.05$), consistent and within a reasonable marge of values. In particular, ash and protein content varied respectively from 2.65%±0.03 (HB10) to 2.50%±0.02 (PK20) and from 13.72%±0.44 (PK20) to 12.94%±0.25 (HB20) (excluding the control in this case). Finally, moisture and fat ranged respectively from 66.09%±0.55 (HA10) to 69.35%±0.38 (BV20) and from 16.11%±0.69 (BV20) to 19.74%±0.75 (BV10). On the other hand, after the cooking treatment, there was a significant decrease of moisture, ranging from 65.54%±0.25 (HB20) to 54.71%±0.19 (PK20). This variation may derive from the different treatment underwent by the powders before the replacement which affected the structure of the proteins. This may be explained by the different length of the collagen fibres observed: the commercial gelatines powders were obtained

after a mild alkaline or acid extraction, so the average molecular weight of the collagen fibres is remarkably larger than the peptides obtained after enzymatic hydrolysis. When collagen rich sources were processed in a similar way, hydrolysates obtained were mainly composed by a high amount of free amino acids and short peptides as previously reported (Anzani et al., 2017a; Anzani et al., 2017b). The highest moisture losses were detected when both commercial gelatine powders were employed as replacers at a 20% level (20% PK and 20% BV). Alternatively, the sausages formulated with hydrolysates showed a low decrease in moisture content, around 6%. This variation was reflected also in the other parameters, as for example protein and fat content in PK and BV formulations, which were more concentrated after cooking because of the significantly lower moisture content. Because of the water loss, there was an increase of the protein and fat content in cooked samples, which were concentrated; besides, a decrease in the ash content was detected, deriving from the loss of the minerals along with the water.

3.3 Textural properties

The effect of the replacement of different protein samples on textural properties of Irish sausages was assessed and the data are shown in Table 4.

Table 4 Texture profile analysis (TPA) of the cooked sausages. The superscript letters (a,b,c,d,e,f,g,h) refer to values significantly different from each other (in this case a series of pairwise comparisons with $p \geq 0.05$ have been executed).

	Hardness (N)	Chewiness (N)	Gumminess (N)	Springiness (N)	Resilience (N)
control	26,19±1,93^{g}	94,63±8,50^{g}	13,38±1,10^{g}	7,07±0,33^{a,b,c,d}	0,51±0,02^{b,c}
PK10	28,17±2,13^{g}	99,86±7,39^{g}	13,48±1,09^{g,h}	7,41±0,20^{c,d}	0,48±0,02^{b,c}
BV10	22,48±1,07^{e}	70,31±2,65^{e}	10,34±0,73^{e}	6,82±0,31^{b}	0,46±0,03^{b,c}
HA10	19,86±1,40^{d}	76,28±9,24^{e}	10,47±1,48	7,31±0,40^{c,d}	0,52±0,05^{c}
HB10	6,84±0,59^{c}	16,05±1,52^{b}	2,33±0,20^{b}	6,88±0,38^{b,c,d}	0,34±0,04^{a}
PK20	28,60±1,83^{g}	113,56±9,56^{h}	15,15±1,16^{h}	7,49±0,17^{d}	0,53±0,03^{c}
BV20	18,93±1,53^{d}	58,85±5,23^{d}	8,44±0,92^{d}	7,00±0,46^{b,c}	0,45±0,04^{b}
HA20	11,45±1,00^{c}	36,64±4,64^{c}	5,21±0,75^{c}	7,05±0,25^{b,c,d}	0,45±0,04^{b,c}
HB20	0,72±0,13^{a}	0,67±0,34^{a}	0,23±0,06^{a}	2,20±0,73^{d}	0,29±0,12^{a}

A significant ($p < 0.05$) effect on all the textural parameters analysed could be observed depending both on the replacement level and the protein used as replacer. It has been previously reported that fat level have an impact on such properties; for instance, Mendoza et al. (2001) found out

that the higher fat replacement leads to the lower values of hardness. However the differences found in the fat content, in the samples here studied, do not correspond with the trend reported by these authors. Therefore, the type of protein employed and the replacement level may play a more important role regarding the textural properties (Pietrasik and Duda, 2000). Particularly, there is a decrease in all the textural parameters when bovine commercial gelatines powders and the hydrolysates generated from hides were employed, when compared to the control. Conversely, when pork gelatine was employed, at both replacements levels, harder products were obtained, this is because even at this replacement level (10%), the amount of gelatin employed is still higher that the LGC; thus a gel can be formed after cooking. When BV is employed, the amount of protein employed is very close to the LGC and the gel formed is very weak. This derived from the different pre-treatment underwent by the raw material. The pre-treatment influenced the molecular weight and the cross-linking of the collagen fibres and it had a direct link with the bloom force which affected the properties of the gelatines (Gómez-Guillén et al., 2011). This can be observed also that there is a high decrease of the hardness as the percentage moves 10% to 20%. Again this trend is similar for both the hydrolysates and the bovine commercial gelatines powders. The reducing in hardness and in the other parameters in the HA and HB samples is related to increasing presence of shorts peptides released by the hydrolytic activity of Alcalase. Differently for PK and BV the values are higher because of the presence of non-hydrolysed collagen which influences the cohesion and the firmness of the final product (Feng at al., 2014). Especially the negative role of the short peptides in the textural properties can be noticed in the sample HB10. It performed the textural properties with the lowest values and in the case of the 20% of replacement the texture was so similar to a pâté that was impossible the evaluation of the textural profile. The case of the porcine commercial gelatine powders showed an opposite trend. In both of the recipes all the textural parameters were higher than the control. Probably the different behaviour of the commercial gelatines powders derived from the different extraction protocol, which modifies the gelling properties as shown in Table 1. It was observed that collagen, by retaining the water through the protein matrix, affects the texture and the cohesion of the batter contributing to the firmness of the sausage (Sousa et al., 2016). Collagen not only has an impact increasing the hardness, but also on the chewiness, requiring higher amount of energy to masticate the sausage (Sousa et al., 2016).

3.4 Emulsion stability and cook loss

The effect of the protein replacement on cooking loss of Irish sausages is shown in Table 5.

Table 5 WHC determination of the raw batters and emulsion stability and cook loss evaluation of the sausages. The superscript letters (a,b,c,d) refer to values significantly different from each other (in this case a series of pairwise comparisons with $p \geq 0.05$ have been executed).

	WHC (%)	Emulsion stability (%)		Cook loss (%)
		%TEF	%FAT	
control	13.21±1.56^{c}	29,06±1,98^{c}	3,56±0,30^{a}	24,67±2,69^{b}
PK10	12.91±0.92^{c}	37,88±0,82^{d}	4,84±0,65^{b}	34,11±1,60^{c}
BV10	18.28±4.48^{d}	40,97±1,12^{e}	5,48±0,81^{b}	37,68±2,86^{c,d}
HA10	8.82±0.60^{a,b}	19,99±0,79^{b}	2,44±0,24^{a}	22,54±2,68^{b}
HB10	9.64±0,33^{b}	20,38±2,18^{b}	3,30±0,57^{a}	20,84±0,83^{a,b}
PK20	13.96±0,76^{c}	45,63±0,86^{f}	5,49±0,47^{b}	40,33±1,25^{d}
BV20	23.53±1.63^{e}	44,83±0,69^{f}	5,25±0,85^{b}	39,94±1,26^{d}
HA20	7.67±0.20^{a,b}	19,39±1,20^{b}	2,62±0,13^{a}	21,73±0,64^{a,b}
HB20	6.50±0,60^{a}	13,66±1,37^{a}	5,63±0,94^{b}	18,31±1,80^{a}

The effect of adding commercial gelatines powders seemed to increase the cook loss values when compared to the control (24.67%±2.69). This behaviour increased with the increment of the percentage of protein replacement. By contrast, the addition of both hydrolysates (HA and HB) decreased the cook loss, (HA10=22.54%±2.69 and HB10=20.84%±0.83). Particularly in the case of the 20% of hydrolysates powders replacement the cook loss decreased slightly (HA20=21.73%±0.64 and HB20=18.31%±1.80). From the cook loss point of view the behaviour of commercial collagen is the opposite than the hydrolysates powders which seemed to have a low cook loss compared to the control. Cook loss and WHC play an important role in the meat industry because both are important parameters to assess the quality of a meat product. In agreement with Sousa et al. (2016), the samples prepared with commercial gelatines powders showed highest WHC value in the formulations with 20% of replacement (PK20= 13.96±0.76% and BV20= 23.53±1.63%) than the control and the 10% samples. That fact demonstrates the high efficiency of collagen in retain water in the raw batter before heating and destabilising the gel structure. The differences in the behaviour of the commercial gelatines could derive from the different animal

source or the different treatment underwent by the raw materials. As described in section 3.1, two different protocols normally used for the commercial production of gelatines: acid and alkali pre-treatment. The trend in the hydrolysates-added samples is the opposite; there was a decrease in the WHC which was even accentuated with the 20% of substitution. This derived from the inability of the short peptides to retain water, previously illustrated in table 1, leading to a negative effect on the WHC of the raw batter. The emulsion stability appeared to be highly linked with cook loss, because both of the properties exhibit the same trend. The samples prepared with the hydrolysates powders were found to have the higher emulsion stability; particularly HB20 showed the lowest value (13.66±1.37). By contrast the addition of commercial gelatines powders indicated that in the Irish sausages they had a negative effect, which turned out to be even worst with the 20% of replacement. Summarising the replacement of the commercial gelatines powders showed a negative effect on the final product about high percentage of cook loss and emulsion stability, however the positive aspect of retaining water. By contrast the samples prepared with hydrolysates powders own better lower cook loss and emulsion stability values, but lower WHC.

3.5 Colour changes

The appearance of the final product is one of the most important parameter to consider from a consumer's point of view; therefore the colour of the raw batter and of the cooked sausages was evaluated. ΔE is the key value to assess the total difference between all the replacements and the control. It was calculated following equation 6.

Table 6 Means and standard deviation of colour parameters of sausages: raw(a) and cooked(b) samples. The superscript letters (a,b,c,d) refer to values significantly different from each other (in this case a series of pairwise comparisons with $p \geq 0.05$ have been executed).

a	Raw			
	L	a	b	ΔE
control	59,77±1,89^{d}	11,68±2,18^{b,c}	17,78±1,27^{c}	0,00
PK10	59,98±1,26^{d}	10,22±1,43^{b,c}	17,19±0,94^{c}	1,58
BV10	53,24±1,34^{a}	9,78±0,79^{b,c}	14,84±0,71^{a}	2,05
HA10	58,54±1,31^{c,d}	11,40±1,69^{b,c}	17,26±0,58^{c}	1,37
HB10	58,44±1,05^{c,d}	10,96±1,14^{a,b,c}	18,29±0,44^{c}	1,60
PK20	56,32±1,57^{b,c}	10,24±1,31^{b,c}	15,44±0,59^{a,b}	4,41
BV20	58,56±1,40^{c,d}	10,27±1,28^{b,c}	16,91±1,27^{b,c}	7,41
HA20	56,82±0,80^{c}	8,55±0,40^{a,b}	15,06±0,88^{a}	5,08
HB20	53,87±0,90^{a,b}	7,60±1,06^{a}	15,10±0,75^{a}	7,66

	Cooked			
	L	a	b	ΔE
control	59,34±1,65[b]	2,95±0,50[a]	12,91±1,45[b,c]	0,00
PK10	58,06±2,60[b]	2,78±0,44[a]	8,73±1,69[a]	4,20
BV10	54,71±64[a]	3,70±0,63[b,c,d]	11,25±1,67[b]	4,06
HA10	59,42±1,43[b,c]	3,24±0,57[b,c]	13,77±1,51[c]	1,41
HB10	60,46±0,97[c]	3,14±0,30[a,b]	12,30±1,09[b,c]	2,22
PK20	54,59±1,65[a]	3,86±0,47[b,c,d]	12,12±1,54[b,c]	3,94
BV20	54,54±2,35[a]	4,08±0,67[d]	13,13±2,11[c]	3,97
HA20	59,83±0,96[b,c]	3,38±0,45[b,c,d]	13,09±2,03[b,c]	1,57
HB20	58,16±1,16[b]	3,67±0,56[c,d]	12,43±1,52[b,c]	0,89

In the classification, according to Mokrzycki and Tatol, 2011, ΔE values lower than one meaning that the differences are not noticeable by necked human eye; values between 1 and indicates that the differences are perceived by experience observers. Inexperienced observer can detect the difference when ΔE values are between 2 and 3.5. A clear difference is observed when ΔE is between 3.5 and 5; the last case when ΔE is major than five two different colours are perceived.

The ΔE values of the raw batter are very variable, particularly is noticeable a great increase of ΔE in all of the samples with 20% of replacement: the values range from 4.41 (PK20) to 7.65 (HB20). The raw batter prepared with the addition of the protein hydrolysates powder showed the higher ΔE value. This may indicate that the powders own an intrinsic colour which affects the appearance of the raw batter. In the case of 10% of substitution the ΔE range from 1.36 (PK10) to 2.05 (BV10), demonstrating that the amount of powder added was not altering strongly the appearance of the raw batter. Highest contributors to the ΔE values in 20% replacement levels are brightness (L) and red-green (a) colours. It was observed that replacing lead to a decrease in the lightness and in the redness of the raw batter; which can be perceived negatively by the consumers. The ΔE results for the cooked sausage were fewer variables than the raw batter. Actually, they can be divided in two different groups: HA and HB had values ranging from 0.89 to 2.22 for 10 and 20% and PK and BV from 3.94 to 4.20. There are minor changes in all of parameters in the raw batter and after the cooking as well. Cooking treatment lead to a general decrease in redness (a) and yellowness (b), which reduced the colour differences because of the browning of the treatment. In particular HA20 and HB20 showed the lowest redness value (respectively 8.55±0.40 and 7.60±1.06), due to the colour of the hydrolysates powders. This effect is missing in the cooked samples.

3.6 Sausage grading based on its properties

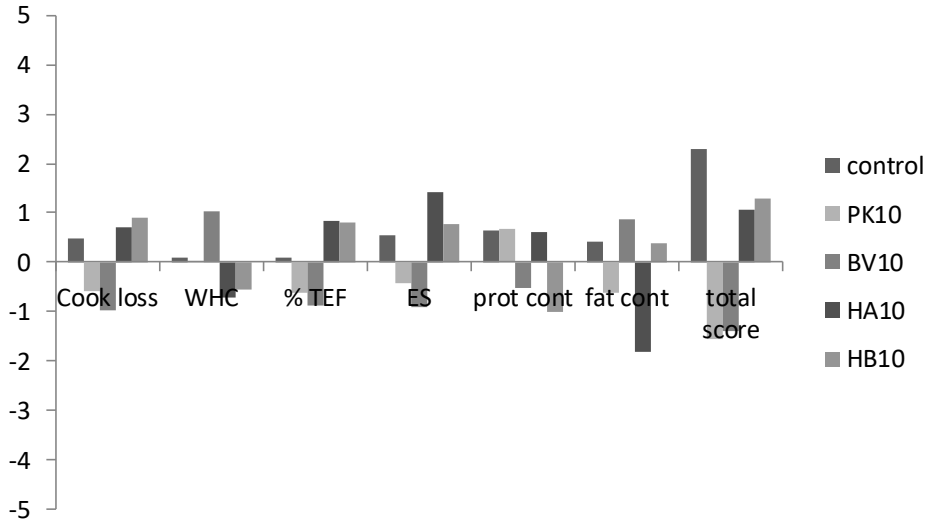
From an industrial point of view is interesting to find which treatment offers the best overall final product. Due to the presence of both negative and positive effects compared to the control, a preliminary study of sausage grading was executed to observe if a particular recipe lead to an improvement in the technological properties. Therefore, a score system was employed taking into account the most important technological parameters measured in all of the recipes. Every treat value was standardized following the below equations:

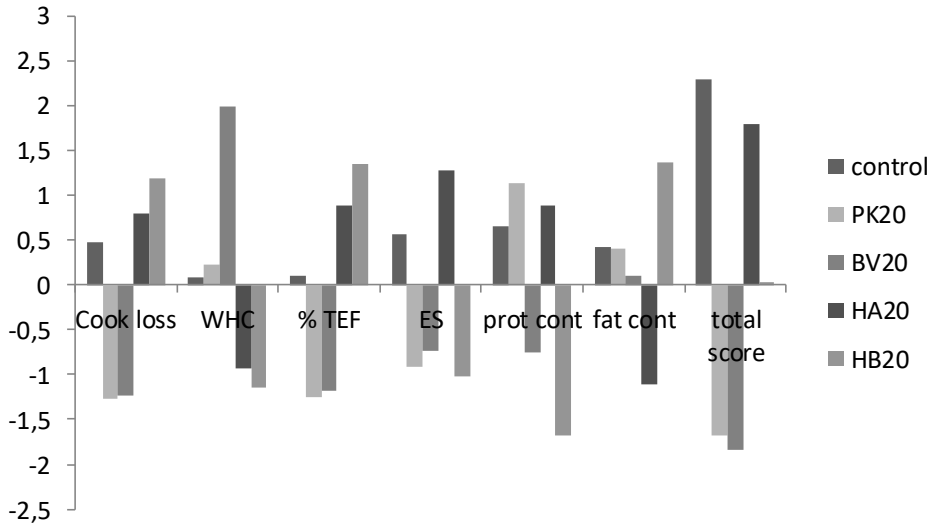
$Z = (X - \mu) / \sigma$ Eq 7

$Z = -(X - \mu) / \sigma$ Eq 8

Where Z is the standardized value, X is the value of the parameter, μ is the average of all samples and σ is the standard deviation. Equation 7 was employed for properties where higher values are considered better (WHC and protein content); while Equation 8 was employed for properties which low values are desirable (cook loss, %TEF, ES and fat content).

Figura 1 Treatments scores for the main technological properties analysed: fig 1a with 10% of protein replacement and fig 1b with 20% of replacement.





In Figure 1a and 1b have been gathered the scores obtained in all the treatments for the main technological properties analysed respectively for 10% and 20% of replacement. These calculations allowed to determine which recipe had the best overall performance according to the technical parameters studied. This score was calculated considering that all the parameters had the same importance from a technological point of view. There are not a lot of difference between 10 and 20% of replacement. In both cases the best recipe is the control with a score of 2.30. In the case of 10% of replacement, apart from the control, the best recipe is HB (1.27) which achieved the lowest score in the 20% of replacement. In the substitution of 20% HA had the best score (1.79) after the control. In the case of the commercial gelatines powders the effect was negative for both the amount of replacement, in spite of the excellent WHC capacities found.

4. Conclusion

The replacement of the enzymatic hydrolysates derived from bovine hides in the Irish breakfast sausages were evaluated. The commercial gelatines powders from two different animal sources were employed as a further comparison object in addition to the control. From the technological analysis, a grading score was calculated in order to compare the different effect of all the treatments. The control showed the best score in both the replacements, followed by the 20% protein hydrolysates obtained with a hides:buffer solution ratio 1:2 and by the 10% with a hides:buffer solution ratio 1:3. This research demonstrates that this particular meat industry co-product, if treated with an enzymatic hydrolysis, could have a good potential to be employed as a replacement in the Irish sausages, previous optimisation and minimisation of the negative effects.

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4. CONCLUSIONS AND FUTURE ASPECTS

In the research work described in this PhD thesis the attention was focused on the recovery and the valorisation of two different meat industry co-products: bovine hides and fleshing. The general aim of this study was to examine the use of enzyme hydrolysis, as a means of recovering protein fraction from these meat co-products. Enzyme hydrolysis offers a fast and gentle alternative to other mechanical or chemical treatments. The ability of the enzyme to hydrolyse proteins to produce free amino acids and short peptides allows the nitrogen to be more soluble and easily recovered and purified; for this reason bovine fleshing meat and hides hydrolysates have the potential to become a new source of valuable proteins. In the first section the activity of six enzymes, deriving from different sources, was evaluated for the hydrolysis of fleshing meat. Alcalase and papain were the most efficient releasing in solution 97% of the proteins originally present, because of their efficiency of degrading collagen, its main component. However they gave rise to hydrolysates with differing distributions of the nitrogen substances: Alcalase produces a larger amount of free amino acids and small peptides, whereas papain generates less free amino acids and longer peptides. An experimental design was established, based on Response Surface Method, to further optimise the hydrolysis conditions using Alcalase. A scale-up reaction was performed following the optimised condition which gave optimal solubilisation/recovery of proteins in form of hydrolysates in few hours of reactions. Therefore, it is possible to recover fleshing in form of hydrolysates in a economical and sustainable way, hydrolysates which could have the potential to be used as supplement in the food industry.

In the second section, the same enzyme was tested using different reaction conditions in the unhairing step of bovine hides and compared with another alternative methodology based on chemical agents (sodium hydroxide and hydrogen peroxide). The goal of this study was to evaluate the efficiency of both unhairing methods and estimate their impact on the nitrogen fraction in the wastewaters. The results indicated that both methods have the potential to be more sustainable alternatives to the current ones. Even if the oxidative method was the most efficient in the unhairing, the wastewaters obtained by the enzymatic unhairing had a higher potential for reuse, being the proteins in wastewaters from the oxidation too much spoiled by oxidative damage. In conclusion, both methodologies are two alternative greener protocols which could be applied in an industrial scale, with different potential applications.

In the last part, the other co-products analysed in this study are pieces of bovine hides not suitable for leather processing (too small, damaged, etc), which was recovered through their complete

enzymatic solubilisation. Since these co-products are protein-rich materials they may provide a feedstock for the generation of peptides with value as a nutritional or food ingredient product. Because of the high efficiency of Alcalase to degrade collagen, the effect of hydrolysis time and water/substrate ratios on the total hydrolysis process was studied using this enzymes. Bovine hides were indeed fully hydrolysed, but the obtained hydrolysates, rich in small peptides, did not showed good techno-functional properties (gelling and foaming). In order to improve their properties, the modification of these hydrolysates via reaction with sugars was studied. The dry Maillard reaction was selected as a food grade methodology and tested to enhance these properties. Unfortunately, even if the reaction occurred successfully, no great improvements were observed in the techno-functional properties. A further step may be the application of other protein modification strategies which own the negative outpoint to not be food grade.

The final study was focused on the replacement of the enzymatic hydrolysates derived from bovine hides in a real food matrix. This study was carried out in order to evaluate their possible incorporation as a food ingredient in Irish sausages. Two levels of protein replacement were executed and the impact on the composition, the techno-functional and sensory of the final product were assessed and compared to a control recipe. The results demonstrated that the sausages with 10% of substitution were closer to the control. In the 20% of replacement the sausages showed a decrease of cohesion and firmness due to the presence of low molecular weight peptides which influenced the texture of the final product. However, this preliminary study indicated that protein hydrolysates from hide have the potential to be used as meat replacers.

Summarising, the results presented in this thesis highlighted that enzymatic hydrolysis is a valuable method to recover meat industry co-products, which are usually discarded, in order to obtain protein hydrolysates rich in peptides and free amino acids. This methodology, optimized in the right conditions with the right enzymes, allowed producing hydrolysates with a good protein content which could be used as food additive or ingredient, obtained through green methodologies applicable at industrial scale.

5. AUTHOR INFORMATION

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WORK EXPERIENCE

Visiting PhD student at Teagasc Ashtown Food Research Centre

02/2017 to present

Teagasc, Ashtown, Dublin 15 D15 KN3K

Initiative capability to find a research group in the same working area and build a future profitable collaboration

Collaboration with Anne Maria Mullen **ReValue Protein** (www.revalueprotein.com)[1] project coordinator
*Evaluation of the techno-functional and the film forming properties of enzymatic hydrolysates, obtained in Parma University (bovine hides hydrolysates) in order to use the protein hydrolysates as food additives
*Assessing the protein modifications (glycation reaction) for improving the functional properties
*Replacement of protein hydrolysates in a real food matrix

1 ReValueProtein, Research Project supported by the Department of Agriculture, Food and the Marine (DAFM) under the National Development Plan20072013 funded by the Irish Government

PhD in Food Science and Technology (currently in 3rd year)

09/2014 to present

Department of Food Science (Parma University, Parco area delle Scienze 59A, 43124, Parma, Italy) associated with **Cluster Agrifood Project 4 "Sustainability of the Italian food chain"**[2]

"Recovery of by-products of meat industry by enzymatic hydrolysis: bovine fleshing and hides"

- Peptide analysis and characterization by Mass-Spectrometry and LTQ-orbitrap
- Protein screening with gel electrophoresis and in-gel digestion
- Analysis and characterization of N-fraction (total N, free and total amino acids)
- Optimisation and industrial scale-up

Some of the experiments and samples were performed in partnership with INALCA S.p.a, the largest meat processing company of Italy.

2 Cluster Agrifood Project 4 "Sustainability of the Italian food chain,Technology network for the recovery and the valorisation of bovine meat by-products" (Project So.FI.A CTN01_00230_450760)

EDUCATION

Master in Chemistry

10/2012 – 07/2014

Department of Chemistry (Pavia University, Viale Taramelli 12, 27100, Pavia, Italy)
"Synthesis and reactivity of neuronal peptides complexed to heme groups and copper ions"

- Solid phase peptide synthesis
- UV-Vis evaluation of binding kinetics activity of cathecols with metal catalyst in presence of α-synuclein
- Spectrophotometric titration of heme and substitute heme in presence of beta-amyloids

Bachelor in Chemistry

10/2009 – 09/2012

At the department of Chemistry (Pavia University, Viale Taramelli 12, 27100, Pavia, Italy)
"Copper's role in Alzheimer disease: its coordination with beta-amyloid"-thesis based on Literature Review

PERSONAL SKILLS

Mother tongue: Italian

Other language	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken interaction	Spoken production	
English	B2	C1	B2	B2	B2

27/09/2014 British Council Examination Services, Via Manzoni 38, 20121, Milan, IT010

Levels: A1/A2: Basic user - B1/B2: Independent user - C1/C2 Proficient user
Common European Framework of Reference for Languages

Job related skills

Good work knowledge (regular use) in:

- Mass Spectrometry, UV-VIS spectroscopy, spectrophotometric titration, NMR, HPLC-FLD, gel electrophoresis, in-gel digestion, peptide identification
- Kjeldahl and Soxhlet methods
- Evaluation of techno-functional properties
- Assess protein modification reaction (glycation reaction)
- Size exclusion chromatography
- Peptide film forming evaluation with the addition of cross linkers
- Replacement of protein hydrolysates in food matrix
- Texture analysis (TPA)
- peptide solid phase synthesis
- Laboratory and class tutor of organic chemistry and workshop for food science students

Computer skills

- Regular use of Microsoft Office tools
- Regular use of software for data acquisition chromatography (MassLynx, Xcalibur)
- Familiar with software for statistical analysis (STATISTICA, SigmaPlot, SPSS)

ADDITIONAL INFORMATION

Publications

*(Submitted) **Anzani, C.**, Prandi, B., Tedeschi, T., Baldinelli, C., Sorlini, G., Dossena, A., Sforza, S. Optimization and scale up reaction of bovine fleshing's enzymatic hydrolysis.

*(Submitted) **Anzani, C.**, Prandi, B., Tedeschi, T., Baldinelli, C., Sorlini, G., Dossena, A., Sforza, S. Enzymatic hydrolysis as a way to recovery bovine hides: optimization, characterization of the hydrolysates and scale-up to semi-industrial scale.

***Anzani, C.**, Prandi, B., Buhler, S., Tedeschi, T., Baldinelli, C., Sorlini, G., Dossena, A., Sforza, S. Towards environmentally friendly skin unhairing process: A comparison between enzymatic and oxidative methods and analysis of the protein fraction of the related wastewaters. Journal of Cleaner Production 2017. 164, 1446-1454.

***Anzani, C.**, Prandi, B., Tedeschi, T., Baldinelli, C., Sorlini, G., Wierenga, P., Dossena, A., Sforza, S. Degradation of Collagen Increases Nitrogen Solubilisation During Enzymatic Hydrolysis of Fleshing Meat. Waste and Biomass Valorization. 2017, 1-7

*Dell'Acqua S, Pirota V, **Anzani C**, Rocco MM, Nicolis S, Valensin D, Monzani E, Casella L. Reactivity of copper-α-synuclein peptide complexes relevant to Parkinson's disease. Metallomics. 2015 7(7), 1091-102.

Conference Presentations

Oral presentations

*__C. Anzani__, B. Prandi, T. Tedeschi, P. Wierenga, A. Dossena and S. Sforza. Molecular Characterization of Protein Hydrolysates from a Meat Industry Byproduct. 18th World Congress of Food Science and Technology, Dublin (Ireland), 21st -25th August 2016

*__C. Anzani__, B. Prandi, T. Tedeschi, A. Faccini, A. Dossena, S. Sforza. LTQ-Orbitrap analyses of protein hydrolysates from meat industry by-products. IV workshop Science at CIM: Young researcher's speech, Parma University, Parma, Italy, 13th June 2016

Poster presentations

*__C. Anzani__, T. Tedeschi, A. Dossena, S. Sforza. Enzymatic hydrolysis of hide 'fleshing' meat: characterisation and optimization. 63rd International congress of meat science & technology (ICoMST), Cork (Ireland), 13th-18th August 2017

*__C. Anzani__, C. Álvarez, T. Tedeschi, A. Dossena, A. M. Mullen, S. Sforza. Meat industry by-product recovery: gelatin and film forming evaluation. 63rd International congress of meat science & technology (ICoMST), Cork (Ireland), 13th-18th August 2017

*__C. Anzani__, B. Prandi, T. Tedeschi, A.Dossena, S. Sforza. Application of Recogniction Tecniques to the Identification of Bovine Flesh Meat in Meat Products. Postgraduate Symposium on Food Fraud, Wageningen (The Netherlands), 23rd-24th June 2016

*__C. Anzani__, B. Prandi, T. Tedeschi, A. Dossena, S. Sforza. Enzymatic protein hydrolysates from fleshing meat. XVIII Eurofoodchem, Madrid (Spain),13th-16th October 2015

*__C. Anzani__, S. Sforza. Enzymatic protein hydrolysates from fleshing meat. XXWorkshop on the Development in the Italian PhD Research on Food Science, Technology and Biotechnology. Perugia (Italy) 23rd-25th September 2015