UNIVERSITÀ DI PARMA

UNIVERSITA' DEGLI STUDI DI PARMA

DOTTORATO DI RICERCA IN
"Scienze degli Alimenti"

CICLO XXXII

Effects of processing and storage conditions on milk and whey products: a detailed molecular investigation of the induced modifications on whey proteins

Coordinatore:
Chiar.ma Prof.ssa Chiara Dall'Asta

Tutore:
Chiar.ma Prof.ssa Tullia Tedeschi

Co-Tutore:
Dr.ssa Martine P. van Gool

Dottoranda: Alessandra Gasparini

Anni 2016/2019

Abstract

Food processing is required for the safe consumption of products and the extension of the shelf life. These treatments, in particular the thermal ones, may affect protein content inducing aggregation/denaturation and chemical modifications (i.e. the lactosylation, the first step of the Maillard reaction) on proteins. Thus, their properties, such as digestibility and allergenicity may be altered. The aim of this PhD thesis was to study the effects of processing on two of the major allergens present in milk: whey proteins α-lactalbumin and β-lactoglobulin. An UPLC-MS analytical method was developed for the detection of lactosylated whey proteins in milk samples. This method was used to determine that the UV treatment, a possible alternative to pasteurization, does not alter the lactosylation degree significantly. A screening on different pasteurized and UHT milk samples led to the identification of lactosylation sites in the known epitopes reported in literature for the two proteins. Thus, a procedure for the synthesis of the epitopes in the lactosylated form was developed. This study was then focused on whey protein products due to their large use as food additives. The effects of the storage conditions on whey protein concentrates (WPC-35, prepared and provided by FrieslandCampina) were investigated. Storage conditions applied (up to 60°C and up to 14 days) induced some modifications on the protein content (i.e. lactosylation, aggregation/denaturation, chemical modifications) but the nutritional value of the product was preserved. Moreover, the effects of the drying technique were evaluated comparing the spray-drying with the freeze-drying technique and no significant differences were found among them. A recently developed *in vitro* gastrointestinal semi-dynamic digestion protocol was adopted for the evaluation of the effects on protein digestibility. This model allows to simulate gastric emptying and to decrease pH gradually in the gastric phase. Protein degradation and peptides release in the digestion of a whey protein concentrate were investigated. This model was used for the digestion of whey proteins isolates and results were compared with a sample of untreated whey. The allergenicity of the samples and their digested fractions was then evaluated with ELISA inhibition tests. Results showed that the

processing induced a higher digestibility and residual IgE-binding with respect to the untreated sample. The digestion study was completed with transepithelial transport studies with Caco-2 cells. Moreover, the effects of lactosylation on peptide intestinal absorption were evaluated with a synthesised β-lactoglobulin epitope.

Table of Contents

General Introduction

1. Bovine Whey Proteins

1.1 Bovine milk whey

Through the decades milk whey was mostly considered as a by-product obtained during cheese production. In the 20th century the increasing production of dairy products led to corresponding large volumes of whey, resulting in higher disposal costs for dairy industries[1]. It was soon realized that whey, far from being a useless by-product, contains a lot of interesting nutrients. Indeed, whey contains proteins (i.e. α-lactalbumin, β-lactoglobulin, glycomacropeptide, proteose peptone 3, immunoglobulins, bovine serum albumin and low abundance proteins such as lactoferrin, lactoperoxidase and natural growth factors), lactose, minerals, vitamins and some traces of fat. Thus, whey proteins present a high nutritional quality and are involved in a wide range of biological and physiological effects[2].

Moreover, whey proteins are an important source of essential amino acids, more than other food proteins such as soy, meat and egg ones[3]. In particular, they are rich in branched chain amino acids (BCAAs) such as isoleucine, valine and leucine, that, among the other functions, are essential for muscle health[4]. BCAAs are also metabolic regulators of lipid metabolism and glucose and protein homeostasis, and may be involved in weight control[3,5]. With respect to caseins, whey proteins have a higher amount of sulphuric amino acids (SAAs), lysine, threonine and tryptophan. Methionine and cysteine are involved in anti-oxidant functions[6]. Several whey products rich in SAAs and BCAAs are commercially available, such as whey protein isolates, whey protein concentrates, demineralised whey, reduced lactose whey and hydrolysed whey that have a lot of beneficial effects on human health[7]. Moreover they might increase athletics performances and have antimicrobial activity[7].

Thus, in the recent years whey was no more considered a waste but an important source of nutrients[1]. At first whey was used only in feed, in particular as a milk replacer for the young animals (i.e. calf)[8]. With the increased knowledge on its components and its nutritional properties, whey was introduced also in food to increase the

nutritional value (i.e. sports supplements, infant formulas, meat, bakery, dairy, confectionaries, etc.). Whey ingredients are also used as technological agents for thermal stability, emulsification, gelation and foam formation[9]. Up to now, worldwide whey production is estimated to be approximately 190 ton/year but only 50% of this amount is processed being transformed into various food and feed products[10]. It was estimated that the global export of whey ingredients is 1.5 millions of tonnes, with EU and USA as main exporters[11]. The EU exports more than 557[12] thousands tonnes of whey and USA 495 thousands tonnes per year. Only in 2013 the production of dry whey in USA was estimated of 434 thousands tonnes for food and 17 thousands tonnes for feed[11].

Whey proteins are obtained from cheese whey due to membrane-based separation techniques[13]. Whey proteins concentrates (WPC) are usually classified by their protein content: WPC35, WPC50, WPC65 and WPC80 where the number indicates the percentage of proteins (w/w). Whey protein isolates (WPI) have a higher amount of proteins, more than 90%[14]. After being concentrated, these proteins are usually spray-dried in order to have a product more stable for storage and transport[13,15]. In 2013 only in USA were produced 117 thousands tonnes of WPC 25-49.9%, 108 thousands tonnes of WPC 50-89.9%, 40 thousands tonnes of WPI 90%, 4 thousands tonnes of WPC for feed[11]. The production of WPC in USA more than tripled in the 25 years to 2015 and whey protein market is expected to grow to 13.5 billions of dollars by 2020 according to BCC Research[16].

1.2 Whey proteins

The protein content in milk is nearly 30-35 g protein/L and it can be mainly divided in two groups: whey proteins, soluble at pH 4.6, and caseins, not soluble at pH 4.6 (their isoelectric point)[17]. Caseins represents 78% of the whole protein content, and are mainly characterized by four different types: α_{S1}-casein, β-casein, κ-casein and α_{S2}-casein, approximately in this ratio 35:40:12:10[17]. These proteins are organized in micelles: α_S-caseins and β-caseins aggregates with colloidal calcium phosphate, κ-caseins provides steric stabilization due to a surface layer[18]. Whey proteins consist mainly of, immunoglobulin,

β-lactoglobulin, bovine serum albumin (BSA), α-lactalbumin, lactoperoxidase and lactoferrin. Different factors may affect their concentration: milk source, the type of feed, the stagionality, the type of whey (sweet or acid), the quality of processing and the stage of lactation[14]. In minor amount are present also enzymes (i.e. acid and alkaline phosphatases, xanthine oxidases, lipoprotein lipase, superoxide dismutase, lysozyme, catalase, α-amylase, lactoperoxidase etc.), hormones, lactoferrin and growth factors[19].

β-lactoglobulin is a globular protein (58% w/w of total whey proteins[14]) that can act as a carrier of small hydrophobic ligands (i.e. fatty acids, aromatic compounds, retinol, vitamin D, palmitic acid, triacylglycerols, cholesterol and calcium)[9]. Being stable at low pH it can protect these molecules across the gastric phase. It is rich in cysteine residues that are relevant for the glutathione synthesis[9], presenting in the folded state a free thiol group and two disulphide bonds. It has a molecular mass of about 18 kDa at pH nearly 3, while between pH 3 and 7 this protein is present as a dimer (36 kDa)[20]. It is made of 162 amino acids and presents mainly β-sheet motifs[14]. This protein has different genetic variants: A, B, C from jersey dairy cattle breed and D from Montbeliared dairy cow[21]. Isoforms A and B differs only for two amino acids, at position 64 (glycine in isoform B and aspartic acid in A) and 118 (alanine in isoform B and valine in A)[22]. The Isoform C presents an histidine at position 59 where there is a glutamine (from Uniprot)[23].

The α-lactalbumin, a globular protein, is the coenzyme involved in the biosynthesis of lactose[9]. It is the B subunit of the lactose synthetase enzyme[21]. It is the predominant whey protein in human milk, while in bovine milk represents the 20%[20], and it is fully synthesized in the mammary gland[14]. The molecular weight is about 14 kDa, with 123 amino acids, a Ca^{2+} ion and four disulphide bonds that stabilize the globular structure[14]. This protein binds also minerals (i.e. zinc, magnesium, calcium, cobalt) that thus are delivered for the absorption[17]. Alpha-lactalbumin presents two genetic variants, A and B that differs only for one amino acid (residue 10, arginine/glutamine)[21].

2. Processing

2.1 Thermal treatments of milk

During the production of milk and dairy products, technological treatments are necessary to guarantee a product safe for the consumption and with long shelf life. Main technological treatment involves the use of high temperatures: pasteurization, performed at 70-80°C for 15-20 seconds (also called high-temperature short-time process, HTST); ultra high temperature (UHT) treatment, performed at 135-145°C for 0.5-4 seconds; sterilization, performed at 110-120°C for 10-20 minutes[22,24]. After pasteurization heat-resistant spores of microorganism may remain that are able to grow affecting the safety for consumption. With this treatment, shelf life is limited and products require refrigeration (4°C). Pasteurization may also be performed at low temperature, 63-66°C, for 30-32 min (called also low-temperature long-time, LTLT), giving the same destruction of microorganism[25]. Sterilization is a more extreme thermal treatment. It eliminates the entire toxin-forming and pathogenic microorganism and other microorganisms that would cause spoilage of the product during storage. Only some heat resistant non-pathogenic bacterial spores may survive but they cannot multiply under the storage conditions. Products shelf life is nearly 1-2 year[25]. UHT treatment involve temperatures higher that sterilization but for a very short time, thus the effects on product quality and proteins should be minimized[26].

2.2 Whey protein products

Whey obtained after the production of cheese can be classified according to the type of produced cheese. As an example, after the production of semi-hard cheese, such as Gouda, the sweet whey (pH 6.6) is obtained, while from the production of acid-gel-based cheese, like the Cottage cheese, the acid whey (pH 4.6) is obtained[13]. Otherwise, whey can be obtained directly after caseins precipitation at pH 4.6 from skim milk or from chymosin-induced clotting of milk at approximately pH 7[13].

Whey protein concentrates and isolates are commonly obtained concentrating whey with membrane-based techniques such

as the Ultrafiltration (UF), Microfiltration (MF), demineralization and reverse osmosys[17]. The most common is Ultrafiltration, where lactose, minerals and water are removed from fat and proteins through a pressurized solution that flows on a porous membrane. UF use mild pH and temperature conditions[27]. Asymmetric microporous membranes are used with pore diameters that range between 1-20 nm and have different configurations (i.e. spiral-wound, tubular, hollow-fibre, plate and frame) that depend on the application[27]. In this process permeate is a by-product and it could be reused for the productions of galactose, glucose, lactose, cattle feed, alcohol, yeast, single-cell protein, and some farmaceuticals[17]. With UF only is possible to reach a 20-60% of protein concentration[17]. To increase minerals and lactose removal, usually UF is coupled with diafiltration: retentate is diluted with water and again UF is performed. With this operation proteins in the sample are concentrated. Before processing whey may underwent some pre-treatments that aim at dissolving the colloidal calcium phosphate or to remove other components (i.e. calcium lipophosphoprotein complexes, milk fat, casein fines, and insoluble cheese curd). Methods involved in pre-treatments may be addition of calcium or calcium chelating agents, centrifugation, pH adjustments (i.e. pH adjustment to 5.7-6.0 for minerals reduction), microfiltration[17,27]. A heat and hold treatment may also be performed to stabilize calcium phosphate complex. This treatment is achieved cooling whey to 60-65°C for 30-60 min after the pasteurization. Then whey is cooled to 50°C for the UF[17]. Finally evaporation and spray-drying are performed to obtain a powdered product[28].

Spray-drying consists on reducing the liquid in a spray of small droplets that is then exposed to a flow of hot air. The spray is obtained with atomization of the concentrated liquid whey[29]. Transforming the liquid into a spray increases the total surface area, thus the evaporation is faster and it doesn't require high temperatures, minimizing the possible heat damages[17]. Indeed, an advantage of this drying technique is that it does not induce a significant denaturation with respect to the starting whey, and that it is possible to set the conditions to better minimize changes in structure and solubility[17].

2.3 Heat induced modifications

Temperatures involved in the thermal treatments of milk and dairy products may affect their nutritional, sensory and technological quality. Processing affect mainly proteins but also lactose and heat-labile vitamins[30]. Indeed, temperatures applied may affect protein structure inducing chemical modifications and protein aggregation and denaturation. Protein denaturation may be both reversible, occurring when there is only a partial unfolding of the protein and the helical structure is lost, or irreversible, when denatured proteins aggregate through intermolecular interactions (i.e. electrostatic and hydrophobic interactions)[31] or sulphydryl/disulphide interchange reactions[32]. The sulphydryl group that are usually hidden in the native conformation become exposed, after protein denaturation, and may be involved in disulphide interactions[33]. Aggregation may occur between whey proteins or between caseins and whey proteins (most commonly between κ-caseins and β-lactoglobulin)[34,35]. Among whey proteins, β-lactoglobulin is more commonly involved in aggregation phenomena due to the presence of a free sulphydryl group.

The most common chemical modification is the condensation of lactose on lysine residues induced by the Maillard reaction (paragraph 2.3). Other modifications are due to oxidation reactions on tryptophan (i.e. oxidized to hydroxytryptophan, N-formylkynurenine, kynurenine, hydroxykynurenine), cysteine (i.e. oxidized to sulfinic acid) and methionine (i.e. oxidized to methionine sulfoxide). Recently it was found also the oxidation of the N-terminal amine group to N-terminal α-ketoamide[36]. For some of these modifications is still unclear if they are the product of enzymatic reactions or the effect of the thermal treatment (i.e. sulfinic acid, methionine sulfone, N-formylkynurenine, kynurenine). Another chemical reaction promoted by the thermal treatments is the deamidation of acidic amino acids residues, such as the hydrolysis of asparagine to aspartic acid[36]. Thermal treatments may induce modifications mostly on essential amino acids (tryptophan, methionine, lysine, cysteine etc.). Therefore the identification of these modifications is crucial, since losses in the essential amino acid content cause a decrease in the nutritional value of the product[30].

2.4 Maillard Reaction

The Maillard reaction is a non-enzymatic reaction between sugars and proteins. It starts with the condensation of a sugar on the amino group of lysine side chain and ends up in producing brown pigments (melanoidins), volatile compounds (i.e. aroma compounds), flavouring matter (i.e. bitter substances), reductones, potential mutagenic compounds, substances that can promote protein cross-linkage. Moreover it causes losses in essential amino acids[21,37].

The reaction can be summarized in three main steps: the early stage, the advanced stage, and the final stage. In the early step the carbonyl group of a reducing sugar binds amino groups (i.e. in the side chain of lysine residues or the N-terminus). The first intermediate that is generated is a Schiff base product that rearranges into the Amadori product (1-amino-1-deoxy-2-ketose adduct) (Figure 2.4.1).

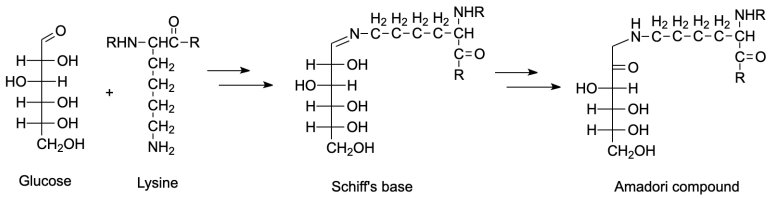


Figure 2.4.1 Early stage of the Maillard reaction. Formation of the Amadori product after the condensation of glucose with a lysine residue.

In the advanced stage of the Maillard reaction α-dicarbonyl compounds are generated. The Amadori compound is degraded to 1-, 3- or 4-deoxysone. At first it is enolized to 2,3-eneaminol or to 1,2-eneaminol (Figure 2.4.2)[21].

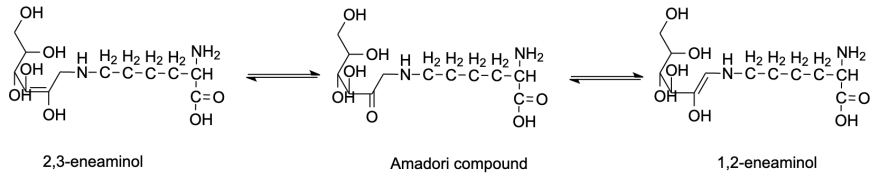


Figure 2.4.2 Enolization of the Amadori compound to 2,3- or 1,2-enaminol.

3-deoxysone is obtained from the 1,2-eneaminol through water elimination and hydrolysis of the imine cation. From the 2,3-eneaminol

with β-elimination is possible to obtain the 1-deoxysone and the 4-deoxysone (Figure 2.4.3). 4-deoxyosone is the only one that remains bound to the lysine residue[21].

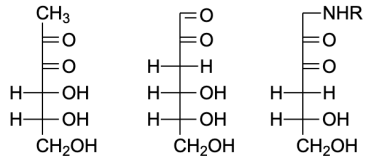


Figure 2.4.3 α-dicarbonyl compounds generated form the Amadori compound.

Then deoxysones are decomposed to give several compounds. The compounds generated are also known as advanced glycation endproducts (AGEs)[37]. In figure 2.4.4 are reported some compounds deriving from 3-deoxysone.

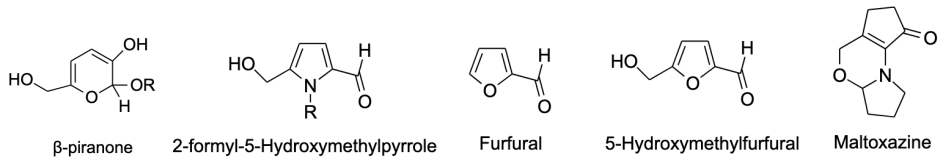


Figure 2.4.4 Examples of compounds deriving from the decomposition of 3-deoxyosone.

From the examples above, 5-hydroxymethylfurfural (HMF) derives from hexoses, while furfural from pentoses. 2-formyl-5-hydroxymethylpyrrole is generated more than HMF when there is a high concentration of amino acids, ammonia or primary amines. Maltoxazine is a secondary product that was identified in malt and beer[21]. In Figure 2.4.5 are reported some compounds deriving from 1-deoxyosone.

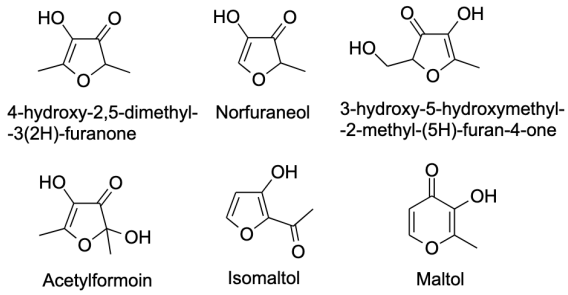


Figure 2.4.5 Examples of compounds deriving from the decomposition of 1-deoxyosone.

Furaneol (4-hydroxy-2,5-dimethyl-3(2H)-furanone) has an intense odour caramel-like and is also biosynthesized in plants (i.e. strawberries, pineapple). Acetylformoin belongs to reductones, a class of substances that have antioxidant properties[21]. In figure 2.4.6 are reported some compounds found after the degradation of 4-deoxyosone[21].

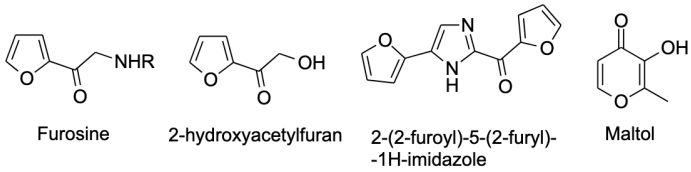


Figure 2.4.6 Examples of compounds deriving from the decomposition of 4-deoxyosone.

From 4-deoxyosone can be formed the 2-hydroxyacetylfuran. This compound is formed mostly in carbohydrate degradation without amine components. When it is formed from the Amadori compound it remains bound to the lysine leading to the formation of furosine.

Then, α-dicarbonyl compounds (deoxysones) react with the amino acids in the Strecker reaction. At the end of this reaction, CO_2, aldehydes and α-aminoketones, from oxidative decarboxylation of α-amino acids, are formed. This reaction occurs under drastic reaction conditions (i.e. under pressure or higher temperatures). From this reaction aldehydes with aroma potential are released, other aroma are cteamine and H_2S, NH_3, 1-pyrroline[21].

In milk and dairy products the Maillard reaction involves lactose, the main sugar present (4-6% of milk[21]). The Amadori

compound is the N^{ε}-(1-deoxy-D-lactulos-1-yl)-lysine, called also lactulosyl-lysine[38] (Figure 2.4.7).

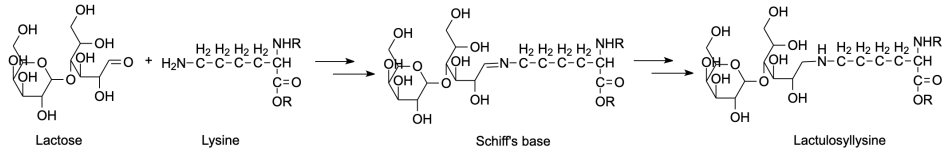


Figure 2.4.7 Formation of the Amadori compound from the condensation of lactose with lysine residues

Due to the Maillard reaction, the side chain of the amino acids could undergo post-translational modifications (PTMs), involving mainly lysine or arginine residues. The arginine can react with the α-dicarbonyl compound generated from carbohydrate degradation[21]. Some PTMs on lysine residues due to the Maillard reaction are showed in figure 2.4.8.

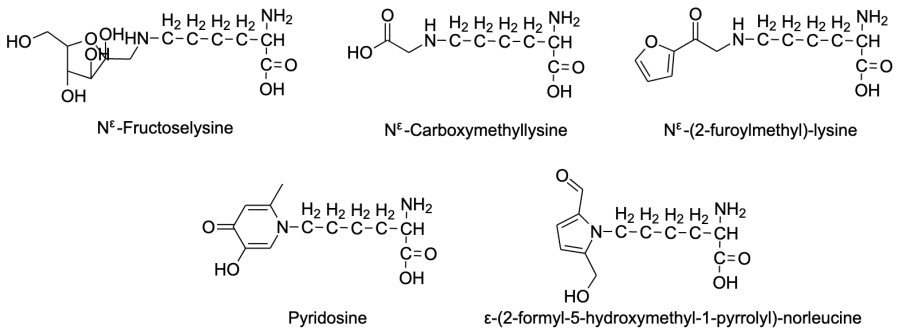


Figure 2.4.8 Some of the PTMs that could be found on the side chain of lysine residues.

N^{ε}-(2-furoylmethyl)-lysine is also known as furosine and is used as indicator of the early stage of the Maillard reaction in food. N^{ε}-carboxymethyllysine (CML) is used as an indicator of the advanced stage of the Maillard reaction. Furosine derives from the acid hydrolysis of lactulosyllysine and it is mainly detected with HPLC techniques. For the detection of carboxymethyllysine different analytical technique may be involved: derivatization with o-phthalaldehyde (OPA) reverse phase HPLC, GC-MS with acetylation of

the amine groups and methylation of the carboxylic groups, UPLC and MALDI-TOF-MS[39,40].

The identification of processing induced modifications may be performed with an enzymatic digestive step before the MS analytical investigation. The digestion may be performed *in gel* after a 2D electrophoresis or *in solution*, using enzymes like endoproteinase GluC, endoproteinase AspN and trypsin[41]. Protein lactoyslation is usually investigated with ESI-MS coupled off-line or on-line with liquid chromatography or with MALDI-TOF-MS, considering a mass shift of +324 Da (the molecular weight of lactose) with respect to the unmodified protein[41].

3 Allergenicity

3.1 Food allergy

Almost 20% of the population in the western countries (i.e. Germany, USA, UK, etc.) has adverse reactions to food. Most of these reactions are non immunological, such as lactose intolerance, that is the most common worldwide[42]. Instead, food allergy is a non toxic adverse reaction to food antigens[43]. It has an incidence of 6-8% in young children (up to 10 years old) and 1-4 % in adults[42,44]. The EU Food Information for Consumers Regulation No 1168/2011 strictly regulated the labelling and safety precautions of 14 main food allergens: cereals containing gluten (rye, barley, wheat, oats), crustaceans (i.e. lobster, crab, shrimp, crayfish, prawn), eggs, fish, peanuts, soya, milk, tree nuts (hazelnut, almond, cashew, pecans, walnuts, brazil, macadamia nuts, pistachio), celery, mustard sesame, sulphur dioxide (sulphites), lupin, molluscs (i.e. oyster, mussel). Most common food allergies are towards cow milk, eggs, peanuts, soy, nuts (wall nuts, almonds, pecans, cashews, pistachios, etc.) shellfish and fish[45]. Food allergy is an immunological reaction, usually Immunoglobulin E (IgE)-mediated. The mechanism of action consists of two main phases, the sensitization and the elicitation. The sensitization occurs with the first contact with the allergen, after which allergen specific IgE are produced, while the elicitation occurs with the following contacts, when symptoms appear[43]. The time between the ingestion and the appearance of symptoms may vary from minutes to

hours[42]. Symptoms may affect different part of the human body: the skin (i.e. angioedema, urticaria, pruritus); the respiratory tract (i.e. hoarseness, asthma); the gastrointestinal tract (vomiting, diarrhoea, contractions); the cardiovascular system (i.e. drop of blood pressure, dizziness, loss of consciousness)[43].

Immunoglobulins are produced by the B cells and are made of two heavy (Fc region) and two light chains. A region of the antibody called Fab region is made of part of the heavy chain and part of the variable light chain. The Fc region binds to the high affinity Fc receptor (FcεRI) of the cells of the immune system, the Fab region binds the antigen. In figure 3.1.1 is reported the scheme of an IgE.

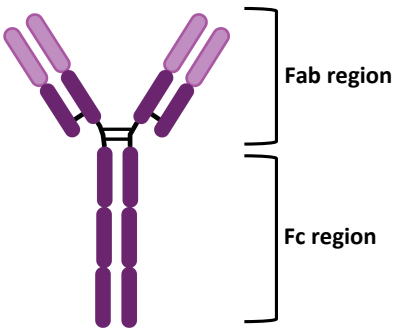


Figure 3.1.1 Immunoglobulin E (IgE) schematic structure

The region of the allergen binding IgE is called epitope[43]. Epitopes may be of two types: conformational epitopes, that depend on the secondary and tertiary structure of the protein, and linear epitopes, consisting of the primary sequence of the protein. When protein denaturation occurs, the conformational epitopes are modified or destroyed, while the linear one are not affected[22]. When two IgEs bound to mast cells or basophils are cross-linked by the epitope, cells degranulate and mediators are released (i.e. prostaglandins, histamine, leukotrienes), eliciting symptoms[42].

Food allergens are divided into two classes: type I, the ones that sensitize through the oral route (i.e. allergens from wheat, milk or peanuts), and type II the ones that indirectly sensitize with cross reactive allergens in the respiratory tract[46]. Diagnosis of food allergy is challenging due to its subjective and variable nature of the symptoms.

The gold standard for the diagnosis is the oral food challenge (OFC), in particular the double blind placebo controlled food challenge (DBPCFC), were subjects are challenged with food or placebo without knowing which they receive (nor the investigators are aware of the difference). But this test is time consuming, costly and difficult to perform. Moreover there is a risk for severe reactions, thus it is sometimes rejected by patient[22]. Most commonly the skin prick test is performed when an IgE-mediated immunological reaction is suspected. It consists on placing a small amount of a solution containing the allergen on the skin and then pricking the skin surface to introduce it in the epidermis. When a wheal appears it indicates the positive reaction and the diameter is measured to determine the grade of the reaction. Negative predicted value is 95%, thus it is possible to exclude and IgE-mediated reaction, while positive predicted values are 50-60%, even if it is quite affordable for strong reactions.

Another possibility for the diagnosis of food allergy is to perform *in vitro* tests, measuring the amount of IgE developed against a particular food allergen in the blood. An example is represented by the ImmunoCAP assay (Thermo Fisher Scientific/Phadia, Uppsala, Sweden). This assay may be divided in singleplex (ImmunoCAP) and multiplex (ImmunoCAP Immuno Solid-phase Allergen Chip or ISAC)[47]. With a singleplex assay it is possible to determine the IgE levels only against one selected allergen, while with the multiplex assay it is possible to test the IgE levels against a fixed array of 112 purified native or recombinant allergens[47]. These tests are useful for the determination of the sensitization to a particular allergen but it is not possible to determine the occurrence and severity of the allergic reaction[22].

However, beside the treatments of the symptoms (i.e. the use of antihistamine) there is still not a treatment of the allergy, apart from avoiding food containing the allergens. Other possibilities are the tolerance induction and the desensitization but their efficacy and safety are still under investigation[22].

3.2 Cow's Milk Allergy

Cow's Milk Allergy (CMA) is one of the most common food allergies, in particular in the first years of life affecting more or less 2-5% of newborns[48]. This incidence decrease to 0.1-0.5% in adulthood[49]. Usually, this allergy disappear at the age of 3-4 years but 15% of infants still present the sensitization[50]. CMA involves different immunoglobulins antibodies (mainly IgE). The reaction occurs in the mucosa or in the blood circulation[51]. It induces skin symptoms in 5 to 90% of cases, gastro intestinal symptoms in 32-60%, or the extremely severe anaphylaxis in 0.8-0.9% of cases[22]. Subjects with transient CMA develop IgE directed to conformational epitopes, while those suffering from permanents CMA develop IgE also directed to linear epitopes that are heat stable[22,52,53]. All proteins present in milk may be allergenic. From studies on large populations of patients allergic to milk proteins it was observed that the majority of them is sensitized to caseins, β-lactoglobulin, α-lactalbumin, bovine serum albumin, lactoferrin and immunoglobulins[22]. Major allergens are β-lactoglobulin, α-lactalbumin and caseins, only 35-50% of patients are sensitized to proteins that are present in a very low amount (i.e. immunoglobulins, lactoferrin and bovine serum albumin). Studies on milk allergenicity reported that for caseins the sensitization occurs prevalently against κ-casein (91.7%) and α-casein (100%)[22]. Concerning α-lactalbumin, the observed prevalence may vary from 0-80% of allergenic subjects, while for β-lactoglobulin may vary from 13-76%[22].

Processing, in particular thermal treatments, may influence milk proteins allergenicity. The effects are mainly related to denaturation. Whey proteins denature progressively, while caseins are more stable to heat due to the absence of secondary, tertiary and quaternary structures, thus heating only partially affects their allergenicity[54]. Denaturation and aggregation of β-lactoglobulin due to thermal treatment up to 90°C increase the antigenicity most likely due to the exposure of the epitopes[55]. As a matter of fact, due to denaturation more linear epitopes are exposed, but with the loss of the native structure the conformational epitopes are destroyed[53]. Indeed, antigenicity of β-lactoglobulin decreases at higher temperatures (90-120°C) probably due to an extensive denaturation and aggregation

that could mask or breakup the conformational epitopes[56]. Similar trend in the antigenicity with increase in temperatures were found for α-lactalbumin, even if some differences were observed probably due to the fact that for this protein the antigenicity is mainly associated to conformational epitopes[56,57]. It has been demonstrated that after pasteurization the IgE binding with the two main whey proteins increases at temperatures from 50 to 90°C with respect to non-heated milk[57]. Sterilization induces 75% of protein denaturation and Maillard reaction affecting the allergenicity but not sufficiently to completely destroy the epitopes[58,59]. There is lack of information on the effects of UHT and spray-drying on the allergenicity. It could be affected by a possible Maillard reaction induced by drying, but it is difficult to evaluate the effects of the spray-drying since the product is processed before[54].

The effects of the lactosylation, as a first step of Maillard reaction on the allergenicity of the protein are still under investigation. It was found that for β-lactoglobulin low or mild glycation produced no effects on the binding with the IgE, while when the glycation had higher rates the binding with the antibodies decreased. This decrease could be related to the masking effect of the sugar on the epitope[59]. Other studies on the effects of the conjugation of sugars on the epitopes confirmed the decrease in antigenicity[60–62], but information on the molecular interaction between the modified epitopes and the antibody are still not known.

4. Protein Digestibility

4.1 Principles of food digestion

During human digestion food undergoes both mechanical and enzymatic transformations. The first reduces the food in particles while the second one hydrolyses macromolecules into smaller compounds that are then absorbed in the small and large intestine[63]. As a first step, in mouth takes place a mastication step followed by the starch degradation due to the amylases present in the salivary fluid. Despite it is a very short step, it has an influence on gastric emptying rate and the whole digestive process[64]. Then, in the stomach and duodenum food compounds are completely digested (i.e. proteins are digested

into medium and small peptides). Briefly, in the proximal part of the stomach food is mixed with gastric juice mostly composed by lipase and pepsin enzymes, mucus for the mucosal surface protection and hydrochloric acid which gradually decreases the pH in the stomach from approximately pH 6.5 to pH 1.5, thus supporting protein hydrolysis (i.e. inducing protein denaturation)[63,65]. In the distal part of the stomach mixing and grinding through peristaltic waves break large particles. At the end of the stomach, the pylorus valve acts as a pump emptying the chyme to the duodenum and as a sieve selecting small particles[63].

Finally, the hydrolysis of large lipids and oligosaccharides and the reduction of peptides size to di- or tri-peptides or to free amino acids take place in the intestine[66] which is organized in three compartments: a first one, where pancreas and liver secretions are delivered; a second one, the duodenum; and a third one characterized by jejunum and ileum[67]. In this phase pH increases, and pancreatic and other digestive enzymes act on the food constituents. Bile is involved in lipid digestion emulsifying them in small droplets and supporting the pancreatic lipase activity[63]. Main processes (i.e. intestinal transit, gastric emptying, motility, digestive fluids and mucus secretion etc.) are regulated by neural and hormonal regulation mechanisms[68,69]. In the digestive tract is present almost the 70% of the immune cells: the intestinal gut presents the lymphoid tissue (known as GALT, Gut-associated lymphoid tissue) that produces the highest amounts of antibodies[66,70].

4.2 Simulated gastrointestinal digestion studies

The fate of food compounds in human gastrointestinal digestion is of great interest in understanding their beneficial or negative influences on human health and for the development of novel food. For instance, digestibility is crucial in the determination of the bioactive properties of proteins, since during protein digestion peptides with known biofunctional properties may be released and the study of their bioavailability becomes very important[94].

Simulated gastrointestinal digestive studies may be performed *in vivo* or *in vitro. In vivo* studies use quite invasive methods, using as an example nasal-gastric and nasal-intestinal probes in human

volunteers[71]. Most commonly, animal models with pigs were used where the stomach and small intestine close resemble the human digestive tract[72]. However, *in vivo* studies are expensive and need ethical justifications, thus *in vitro* digestion models were investigated[73].

A wide number of *in vitro* static gastro intestinal digestion methods were developed but they all use different conditions (i.e. digestion time, pH, substrate:enzyme ratio) making them difficult to compare[73]. A first standardized *in vitro* static gastrointestinal digestion protocol was developed within the INFOGEST[74] COST action. It consists of three main phases: the oral phase, the gastric phase, the intestinal phase (that simulates the small intestine). The oral phase last 2 minutes and the enzyme is amylase (pH 7). For solid and semi-solid food a prior mastication step must be considered. The gastric phase lasts 2 hours and gastric enzyme is usually pepsin (and gastric lipases for lipids) (pH 3). The intestinal phase lasts 2 hours using pancreatin and bile salts (pH 7). In all the cases, digestion is performed at 37°C and all parameters (i.e. digestion times, pH and enzymes activity) are defined on available physiological data[74]. Enzymes inactivation is usually performed differently, depending on the digestive phase: in the gastric phase, pepsin activity is stopped adjusting the pH to 7-8 or adding pepstatin A; in the intestinal phase enzymes are inactivated with heat-shock treatment or using Pefabloc SC of BBI from soybean[75].

The enzyme activity plays a fundamental role as an influencing factor in inter-laboratory comparability, and thus standardized assays for the determination of enzymes activity have been suggested[74,76]. Data reproducibility was demonstrated by inter-laboratory trials using different *in vitro* static digestion protocols[76]. This protocol was optimized during the last years and the final version was released recently[75]. The protocol was then validated comparing results with *in vivo* studies[77]. For example, it was found that the protocol's endpoint has a good correlation with *in vivo* human jejunum for milk protein digestion[78]. Nevertheless, with this static procedure pH is constant in each phase, the gastric emptying is not considered and the gastric fluid is not added gradually as it happens in the *in vivo* digestion. Moreover, the intestinal phase is performed as a single phase and not as the sequence of duodenal, jejunal and ileal digestive phases, where the physiological parameters are different. Thus, this protocol is not

suitable for kinetics studies, while it is absolutely suitable for the assessment of digestion endpoints[75]. With the aim of miming the gastrointestinal processes, not only for the biochemical aspects but also for the mechanic ones, dynamic *in vitro* models were developed and their performances and optimization were extensively studied[79].

4.3 Brush Border Membrane enzymes and intestinal absorption

To complete the information on the fate of ingested nutrients, after the gastrointestinal digestion, other parameters that need to be taken into account include either the intestinal absorption as well as the activity of the brush border enzymes.

The intestine has a double role, on one side is related to processing and absorption of nutrients, on the other side it has to protect against pathogens[80]. The internal surface of the small intestine is covered by finger-like protrusions called villi, which are made of a monolayer of epithelial cells[66]. Different types of cells are the constituents of the intestinal barrier, such as cup, M cells and tuft[81]. Enterocytes are the main representative cells in the small intestine, while colonocytes in large intestine, accounting together the 80% of the intestinal epithelial cells (IEC)[82]. These two types of cells are specialized in nutrients absorption. Enterocytes are polarized columnar cells tightly joined each other. The apical surface of these cells is made of microvilli and is called brush border. This structure of villi and microvilli has the purpose of increasing the surface for digestion and absorption[66]. Peptidases and disaccharidases synthesised by the enterocytes are present on the brush border and contribute to food digestion. The second most abundant cells of the IEC are goblet cells, important for their production of mucus that covers the intestinal mucosa[83] and acts as a protection and lubrication of the epithelium and as transport between the luminal content and the epithelium lining[84].

The list of all the Brush Border Membrane (BBM) peptidases is still under definition. Major peptidases identified in literature are reported in Table 4.3.1[66].

Table 4.3.1 Major peptidases present in the intestine that were identified[66].

Peptidases	
Aminopeptidases A, B, N, P and W	γ-glutamyl transferase
Dipeptidylpeptidases IV	Carboxypeptidases G, M and P
Endopeptidases	Neprilisin
(memprin A and B)	
Angiotensins-I converting enzyme (ACE) 1 and 2	Glutamate carboxypeptidases II
Dipeptidase 1	Enteropeptidase

BBM peptidases are distributed differently in the small intestine. As an example, from pylorus to ileum increases the activity of some peptidases, such as dipeptidylpeptidases IV (DPP-IV) and amino peptidases A[85], carboxypeptidases P and aminopeptidases W have an higher activity in the ileum[86]. The activity of aminopeptidases is located in the small intestine, BBM carboxypeptidases support the action of carboxypeptidases B and A of the pancreatic juice, finally there are endopeptidases that can cleave large polypeptides[66]. Moreover, post-translational modifications (PTMs) can affect BBM peptidases activity. Indeed, peptide glycosylation hinders the access to endopeptidases slowing down the activity of the enzymes[87] and phosphorylation can inhibit partially the activity of peptidases[88].

Thus, the latter digestive activity of these peptides should be considered for the *in vitro* digestion studies. The use of BBM hydrolases for the simulation of the intestinal digestion needs further investigation. Several studies were performed on these enzymes but their use in the digestion models is not frequent. Indeed, these enzymes are not easily available (they must be purified from the jejunum of cow, pig and rat) and, moreover, there is not a validated protocol or a consensus on the conditions to be used[66].

An alternative to the use of BBM vesicles is the use of Caco-2 cells, a human colon carcinoma cell line that is commonly used for transepithelial studies. This cell line has the ability to differentiate into a monolayer expressing also many of the brush border hydrolases[89] on the surface that are similar to the ones present in the intestinal

epithelium. Indeed, like the enterocytes, these cells have apical microvilli, a columnar polarization, carrier mediated transport system, tight junction and apical BBM with hydrolases[66].

Transport model system of the small intestine is performed growing the Caco-2 on porous membrane (i.e. Transwell inserts)[90]. Intestinal adsorption could occur in four different ways: passive transcellular route, carrier-mediated route, passive paracellular route, transcytosis[90].

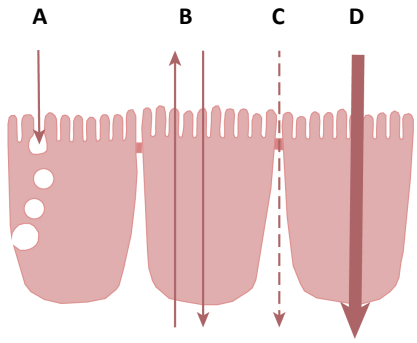


Figure 4.3.1 Scheme of the four possible transport ways through the intestinal barrier: transcytosis (A), carrier-mediated route (B), passive paracellular route (C), passive transcellular route (D).

Several studies on drugs transport investigated all the four possible routes for drug absorption. Lipophilic drugs are usually absorbed completely and rapidly through passive transcellular route. Hydrophilic drugs and peptides are incompletely and slowly transported with passive paracellular via through water filled pores. Some hydrophilic drugs that have a chemical structure similar to some nutrients can be transported through carrier-mediated transport. In some cases the transport may be partly by carrier and partly by passive routes. Transcytosis has low capacity, thus it is considered as a possible route for highly potent drugs (i.e. peptide antigens) that couldn't be transported with other routes due to their size[90]. Recent studies performed on the evaluation of the bioavailability of milk proteins' peptides and their transport trough Caco-2 stressed on the importance of using BBM enzymes for the assessment of peptides bioavailability[91,92].

4.4 Whey proteins gastrointestinal digestion

With respect to caseins, whey proteins are more resistant to gastrointestinal digestion, due to their globular structure[83]. However casein digestion products have a longer retention in the stomach. For this reason casein are called "slow proteins" while whey proteins "fast proteins"[83]. Moreover, during gastric phase β-lactoglobulin may interact with phosphatidylcholine that protect it from the intestinal digestion. This protection depends on the ratio among the two and on the structure of the protein, as the thermal denaturation abolished the protecting effect[93].

Heat treatment may influence also whey proteins digestibility. Indeed, inducing proteins denaturation and aggregation, it influences the action of the digestive enzymes. Protein denaturation influences positively the digestibility, since more cleavage sites are exposed to the action of the enzymes. The aggregation may affect digestion hindering the cleavage sites from the enzymes[34]. Indeed, for β-lactoglobulin it was found that with short heating times it forms dimers that mainly are not digested by pepsin, while with longer heating times aggregates forms also between denatured forms of the proteins that are digested rapidly. Aggregates may be formed also between β-lactoglobulin monomers and peptides, leading to aggregates that are slowly digested[34,95].

Moreover, the presence of protein modifications, for example the first Maillard reaction intermediate with lactose bound to it, could affect the proteolytic reactions promoted during digestion phases. In this light, several studies were performed to understand if the modification has a negative effect on proteolysis. As a matter of facts, the modification alters both the size and the charge of lysine side chains, influencing the possible enzymatic cleavage[96]. For instance, it was demonstrated that plasmin, which is an enzyme specific for lysines and arginine residues, is negatively affected by the presence of the sugar at the cleavage site[97]. For enzymes that are not specific for lysines, it is not easy to make any assumption on the effect of the lactosylation on proteolysis. Nevertheless, it was recently reported that the presence of a sugar does not affect the hydrolysis of not specific enzymes with some exception such as α-chymotripsin[96]. Taking all these findings into consideration, it could be interesting to investigate

if the presence of lactose affects the enzymatic activity of common digestive enzymes[98].

5. Aim and outline of the thesis

Industrial processing, in particular thermal treatments, are required for the microbial safe consumption of milk and dairy products but may induce chemical and structural modification on proteins. These modifications may alter important properties such as protein digestibility and allergenicity.
This PhD thesis aimed at studying, at a molecular level, the effects of the processing on the two main whey proteins in milk, β-lactoglobulin and α-lactalbumin. Here, follows a brief outline of what is discussed in the following Chapters.

In **Chapter 1**, the possibility to use UV irradiation as an alternative to pasteurization for sanitation of cheese whey is described. The product was analysed to assess the microbiological safety and the nutritional value, through determination of the amino acid content. Moreover, the degree of whey proteins lactosylation was determined with a new UPLC-ESI-MS analytical method.

In **Chapter 2**, a screening on different UHT and pasteurized milk samples was performed, using the UPLC-ESI-MS method developed in the previous chapter, to identify whey proteins lactosylation sites and verify their presence in the known epitopes. Synthesis of β-lactoglobulin epitopes is also described. Finally, the development of a protocol for the synthesis of a lactosylated peptide is also reported.

In **Chapter 3**, the effects of storage conditions and different drying techniques (spray-drying vs. freeze-drying) on samples of whey protein concentrates (WPC-35, provided by FrieslandCampina), subjected to controlled conditions, were evaluated, focusing on the chemical modifications (i.e. lactosylation) and the effect on the nutritional value.

In **Chapter 4**, a recently developed semi-dynamic *in vitro* gastrointestinal digestion protocol was applied for the simulated digestion of spray-dried whey protein concentrates. A detailed

molecular characterization of the fractions collected was performed with HPLC-MS/MS.

In **Chapter 5**, the semi-dynamic *in vitro* gastrointestinal digestion protocol was applied in the evaluation of the effects of processing on digestibility of whey protein isolates. Evaluation of the effects of processing and digestion on allergenicity was performed with ELISA test.

In **Chapter 6**, the effects of lactosylation on peptide's intestinal absorption were evaluated with transepithelial transport studies on Caco-2 cells. Intestinal absorption of the digested samples obtained in the study described in Chapter 5 was also evaluated.

References

1. Smithers, G. W. Whey-ing up the options - Yesterday, today and tomorrow. *Int. Dairy J.* **48**, 2–14 (2015).
2. Krissansen, G. W. Emerging Health Properties of Whey Proteins and Their Clinical Implications. *J. Am. Coll. Nutr.* **26**, 713S-723S (2007).
3. Smithers, G. W. Whey and whey proteins-From 'gutter-to-gold'. *Int. Dairy J.* **18**, 695–704 (2008).
4. Smith, K., Wackerhage, H., Greenhaff, P., Rennie, M. J. & Bohe, J. Branched-Chain Amino Acids : Metabolism , Physiological Function , and Application Branched-Chain Amino Acids as Fuels and Anabolic Signals in Human. *J. Nutr.* **22**, 264–268 (2006).
5. Zemel, M. B. Role of calcium and dairy products in energy partitioning and weight management. *Am. J. Clin. Nutr.* **79**, 907–912 (2004).
6. Shoveller, A. K., Stoll, B., Ball, R. O. & Burrin, D. G. Nutritional and Functional Importance of Intestinal Sulfur Amino Acid Metabolism. *J. Nutr.* **135**, 1609–1612 (2005).
7. Pellegrino, L., Masotti, F., Cattaneo, S., Hogenboom, J. A. & de Noni, I. Nutritional Quality of Milk Proteins. in *Advanced Dairy Chemistry: Volume 1A: Proteins: Basic Aspects, 4th Edition* (eds. McSweeney, P. L. H. & Fox, P. F.) **1A**, 1–548 (Springer US, 2013).
8. Whey in animal nutrition,a valuable ingredient, joint pubblication of members of European Dairy Products association.
9. Ramos, O. L. *et al. Whey and Whey Powders: Production and Uses*. *Encyclopedia of Food and Health* (Elsevier Ltd., 2015). doi:10.1016/B978-0-12-384947-2.00747-9
10. Baldasso, C., Barros, T. C. & Tessaro, I. C. Concentration and purification of whey proteins by ultrafiltration. *Desalination* **278**, 381–386 (2011).
11. Lagrange, V., Whitsett, D. & Burris, C. Global Market for Dairy Proteins. *J. Food Sci.* **80**, A16–A22 (2015).
12. *European Dairy Association, Economic Report 2017/2018*.
13. Kelly, P. *Manufacture of Whey Protein Products*. *Whey Proteins* (Elsevier Inc., 2019). doi:10.1016/b978-0-12-812124-5.00003-5
14. Madureira, A. R., Pereira, C. I., Gomes, A. M. P., Pintado, M. E. & Xavier Malcata, F. Bovine whey proteins - Overview on their main biological properties. *Food Res. Int.* **40**, 1197–1211 (2007).
15. Chegini, G. & Taheri, M. Whey powder: Process technology and physical properties: A review. *Middle East J. Sci. Res.* **13**, 1377–1387 (2013).
16. *Horizons, An Exciting Whey Forward, Hoogwegt Group*. **14**, (2017).

17. Schuck, P. Dairy Protein Powders. in *Advances in Dairy Ingredients* (eds. Smithers, G. W. & Augustin, M. A.) (John Wiley &Sons, Inc., 2013).
18. Steinhauer, T., Kulozik, U. & Gebhardt, R. Structure of milk protein deposits formed by casein micelles and β-lactoglobulin during frontal microfiltration. *J. Memb. Sci.* **468**, 126–132 (2014).
19. Alais, C. *Science du lait- principes des tecniques laitières*. (Editions Sepaic, 1984).
20. Chatterton, D. E. W., Smithers, G., Roupas, P. & Brodkorb, A. Bioactivity of β-lactoglobulin and α-lactalbumin-Technological implications for processing. *Int. Dairy J.* **16**, 1229–1240 (2006).
21. Belitz, H.-D., Grosch, W. & Schieberle, P. *Food Chemistry*. (Springer, 2004).
22. EFSA. Scientific Opinion on the evaluation of allergenic foods and food ingredients for labelling purposes. *EFSA J.* 1–277 (2014). doi:10.2903/j.efsa.2014.NNNN
23. Bateman, A. UniProt: A worldwide hub of protein knowledge. *Nucleic Acids Res.* **47**, D506–D515 (2019).
24. Claeys, W. L. *et al.* Raw or heated cow milk consumption: Review of risks and benefits. *Food Control* **31**, 251–262 (2013).
25. Sanguansri, P. *Traditional Thermal Processing*. *Reference Module in Food Science* (Elsevier, 2016). doi:10.1016/b978-0-08-100596-5.03467-3
26. Deeth, H. C. & Datta, N. Heat Treatment of Milk: Ultra-High Temperature Treatment (UHT): Heating Systems. *Encycl. Dairy Sci. Second Ed.* 699–707 (2011). doi:10.1016/B978-0-12-374407-4.00216-8
27. Mulvihill, D. M. & Ennis, M. P. Functional Milk Proteins: Production and Utilization. *Adv. Dairy Chem. Proteins* **1**, 1175–1228 (2003).
28. Luck, P. J. *et al.* Comparison of functional properties of 34% and 80% whey protein and milk serum protein concentrates. *J. Dairy Sci.* **96**, 5522–5531 (2013).
29. Pisecky, I. *Handbook of milk powder*. (2012).
30. Meltretter, J., Becker, C. M. & Pischetsrieder, M. Identification and site-specific relative quantification of β-lactoglobulin modifications in heated milk and dairy products. *J. Agric. Food Chem.* **56**, 5165–5171 (2008).
31. Wijayanti, H. B., Bansal, N. & Deeth, H. C. Stability of Whey Proteins during Thermal Processing: A Review. *Compr. Rev. Food Sci. Food Saf.* **13**, 1235–1251 (2014).
32. Vasbinder, A. J. & De Kruif, C. G. Casein-whey protein interactions in

heated milk: The influence of pH. *Int. Dairy J.* **13**, 669–677 (2003).
33. Pizzano, R., Manzo, C., Adalgisa Nicolai, M. & Addeo, F. Occurrence of major whey proteins in the pH 4.6 insoluble protein fraction from UHT-treated milk. *J. Agric. Food Chem.* **60**, 8044–8050 (2012).
34. van Lieshout, G. A. A., Lambers, T. T., Bragt, M. C. E. & Hettinga, K. A. How processing may affect milk protein digestion and overall physiological outcomes: A systematic review. *Critical Reviews in Food Science and Nutrition* 1–24 (2019). doi:10.1080/10408398.2019.1646703
35. Donato, L. & Guyomarc'h, F. Formation and properties of the whey protein/κ-casein complexes in heated skim milk - A review. *Dairy Sci. Technol.* **89**, 3–29 (2009).
36. Meltretter, J., Wüst, J. & Pischetsrieder, M. Modified peptides as indicators for thermal and nonthermal reactions in processed milk. *J. Agric. Food Chem.* **62**, 10903–10915 (2014).
37. Boekel, M. A. J. S. Van. Effect of heating on Maillard reactions in milk. *Food Chem.* **62**, 403–414 (1998).
38. Arena, S., Renzone, G., D'Ambrosio, C., Salzano, A. M. & Scaloni, A. Dairy products and the Maillard reaction: A promising future for extensive food characterization by integrated proteomics studies. *Food Chem.* **219**, 477–489 (2017).
39. Mehta, B. M. & Deeth, H. C. Blocked Lysine in Dairy Products: Formation, Occurrence, Analysis, and Nutritional Implications. *Compr. Rev. Food Sci. Food Saf.* **15**, 206–218 (2016).
40. Erbersdobler, H. F. & Somoza, V. Forty years of furosine - Forty years of using Maillard reaction products as indicators of the nutritional quality of foods. *Mol. Nutr. Food Res.* **51**, 423–430 (2007).
41. Siciliano, R. A., Mazzeo, M. F., Arena, S., Renzone, G. & Scaloni, A. Mass spectrometry for the analysis of protein lactosylation in milk products. *Food Res. Int.* **54**, 988–1000 (2013).
42. Bischoff, S. & Crowe, S. E. Gastrointestinal food allergy: New insights into pathophysiology and clinical perspectives. *Gastroenterology* **128**, 1089–1113 (2005).
43. Broekman, H. C. H., Eiwegger, T., Upton, J. & Bøgh, K. L. IgE – the main player of food allergy. *Drug Discov. Today Dis. Model.* **17–18**, 37–44 (2015).
44. Sicherer, S. H. & Sampson, H. A. Food allergy: Epidemiology, pathogenesis, diagnosis, and treatment. *J. Allergy Clin. Immunol.* **133**, 291-307.e5 (2014).
45. Husain, Z. & Schwartz, R. A. Food allergy update: more than a peanut of a problem. *Int. J. Dermatol.* **52**, 286–294 (2013).

46. Pekar, J., Ret, D. & Untersmayr, E. Stability of allergens. *Mol. Immunol.* **100**, 14–20 (2018).
47. van Hage, M., Hamsten, C. & Valenta, R. ImmunoCAP assays: Pros and cons in allergology. *J. Allergy Clin. Immunol.* **140**, 974–977 (2017).
48. Schouten, B. *et al.* Contribution of IgE and immunoglobulin free light chain in the allergic reaction to cow's milk proteins. *J. Allergy Clin. Immunol.* **125**, 1308–1314 (2010).
49. Crittenden, R., Little, C., Georgiou, G., Forsyth, S. & Bennett, L. Cow's milk allergy: A complex disorder. *Aust. J. Dairy Technol.* **62**, 62–71 (2007).
50. Järvinen, K. M., Chatchatee, P., Bardina, L., Beyer, K. & Sampson, H. A. IgE and IgG Binding Epitopes on α-Lactalbumin and β-Lactoglobulin in Cow's Milk Allergy. *Int. Arch. Allergy Immunol.* **126**, 111–118 (2001).
51. Cong, Y. J. & Li, L. F. Identification of the Critical Amino Acid Residues of Immunoglobulin E and Immunoglobulin G Epitopes in α-Lactalbumin by Alanine Scanning Analysis. *J. Dairy Sci.* **95**, 6307–6312 (2012).
52. Järvinen, K. M. *et al.* B-cell epitopes as a screening instrument for persistent cow's milk allergy. *J. Allergy Clin. Immunol.* **110**, 293–297 (2002).
53. Vila, L. *et al.* Role of conformational and linear epitopes in the achievement of tolerance in cow's milk allergy. *Clin. Exp. Allergy* **31**, 1599–1606 (2001).
54. Verhoeckx, K. C. M. *et al.* Food processing and allergenicity. *Food Chem. Toxicol.* **80**, 223–240 (2015).
55. Kleber, N., Krause, I., Illgner, S. & Hinrichs, J. The antigenic response of β-lactoglobulin is modulated by thermally induced aggregation. *Eur. Food Res. Technol.* **219**, 105–110 (2004).
56. Bogahawaththa, D., Chandrapala, J. & Vasiljevic, T. Modulation of milk immunogenicity by thermal processing. *Int. Dairy J.* **69**, 23–32 (2017).
57. Bu, G., Luo, Y., Zheng, Z. & Zheng, H. Effect of heat treatment on the antigenicity of bovine α-lactalbumin and β-lactoglobulin in whey protein isolate. *Food Agric. Immunol.* **20**, 195–206 (2009).
58. Ehn, B. M., Ekstrand, B., Bengtsson, U. & Ahlstedt, S. Modification of IgE Binding during Heat Processing of the Cow's Milk Allergen β-Lactoglobulin. *J. Agric. Food Chem.* **52**, 1398–1403 (2004).
59. Taheri-Kafrani, A. *et al.* Effects of heating and glycation of β-lactoglobulin on its recognition by ige of sera from cow milk allergy patients. *J. Agric. Food Chem.* **57**, 4974–4982 (2009).
60. Zhong, J. Z. *et al.* Antigenicity and functional properties of β-

lactoglobulin conjugated with fructo-oligosaccharides in relation to conformational changes. *J. Dairy Sci.* **96**, 2808–2815 (2013).

61. Hattori, M. *et al.* Reduced immunogenicity of β-lactoglobulin by conjugation with acidic oligosaccharides. *J. Agric. Food Chem.* **52**, 4546–4553 (2004).
62. Xu, L., Gong, Y., Gern, J. E., Ikeda, S. & Lucey, J. A. Glycation of whey protein with dextrans of different molar mass: Effect on immunoglobulin E–binding capacity with blood sera obtained from patients with cow milk protein allergy. *J. Dairy Sci.* **101**, 6823–6834 (2018).
63. Guerra, A. *et al.* Relevance and challenges in modeling human gastric and small intestinal digestion. *Trends Biotechnol.* **30**, 591–600 (2012).
64. Woda, A. *et al.* Development and validation of a mastication simulator. *J. Biomech.* **43**, 1667–1673 (2010).
65. Loveday, S. M. Food Proteins: Technological, Nutritional, and Sustainability Attributes of Traditional and Emerging Proteins. *Annu. Rev. Food Sci. Technol.* **10**, 311–339 (2019).
66. Picariello, G., Ferranti, P. & Addeo, F. Use of brush border membrane vesicles to simulate the human intestinal digestion. *Food Res. Int.* **88**, 327–335 (2016).
67. Barrett, K. E. *Gastrointestinal Physiology*. (Lange Medical Books, 2006).
68. Mayer, E. A. Gut feelings: The emerging biology of gut-"brain communication. *Nat. Rev. Neurosci.* **12**, 453–466 (2011).
69. Schubert, M. L. Hormonal regulation of gastric acid secretion. *Curr. Gastroenterol. Rep.* **10**, 523–527 (2008).
70. Mason, K. L., Huffnagle, G. B., Noverr, M. C. & Kao, J. Y. Overview of gut immunology. *Adv. Exp. Med. Biol.* **635**, 1–14 (2008).
71. Boutrou, R. *et al.* Sequential release of milk protein – derived bioactive peptides in. *Am. J. Clin. Nutr.* **97**, 1314–1323 (2013).
72. Roura, E. *et al.* Critical review evaluating the pig as a model for human nutritional physiology. *Nutr. Res. Rev.* **29**, 60–90 (2016).
73. Hur, S. J., Lim, B. O., Decker, E. A. & McClements, D. J. In vitro human digestion models for food applications. *Food Chem.* **125**, 1–12 (2011).
74. Minekus, M. *et al.* A standardised static in vitro digestion method suitable for food-an international consensus. *Food Funct.* **5**, 1113–1124 (2014).
75. Brodkorb, A. *et al.* INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nat. Protoc.* **14**, 991–1014 (2019).
76. Egger, L. *et al.* The harmonized INFOGEST in vitro digestion method:

From knowledge to action. *Food Res. Int.* **88**, 217–225 (2016).
77. Egger, L. *et al.* Physiological comparability of the harmonized INFOGEST in vitro digestion method to in vivo pig digestion. *Food Res. Int.* **102**, 567–574 (2017).
78. Sanchón, J. *et al.* Protein degradation and peptide release from milk proteins in human jejunum. Comparison with in vitro gastrointestinal simulation. *Food Chem.* **239**, 486–494 (2018).
79. Dupont, D. *et al.* Can dynamic in vitro digestion systems mimic the physiological reality? *Crit. Rev. Food Sci. Nutr.* (2018). doi:10.1080710408398.2017.1421900
80. Robert, H. *et al.* Impact of mycotoxins on the intestine: are mucus and microbiota new targets? *J. Toxicol. Environ. Heal. - Part B Crit. Rev.* **20**, 249–275 (2017).
81. Gerbe, F., Legraverend, C. & Jay, P. The intestinal epithelium tuft cells: Specification and function. *Cell. Mol. Life Sci.* **69**, 2907–2917 (2012).
82. Goto, Y. & Kiyono, H. Epithelial barrier: An interface for the cross-communication between gut flora and immune system. *Immunol. Rev.* **245**, 147–163 (2012).
83. Giromini, C., Cheli, F., Rebucci, R. & Baldi, A. Invited review: Dairy proteins and bioactive peptides: Modeling digestion and the intestinal barrier. *J. Dairy Sci.* **102**, 929–942 (2019).
84. Deplancke, B. & Gaskins, H. R. Microbial modulation of innate defense: Goblet cells and the intestinal mucus layer. *Am. J. Clin. Nutr.* **73**, 1131S-1141S (2001).
85. Sterchi, E. E. Letter to the editor: Distribution of the brush border peptidase activities along the small intestine. *Pediatr. Res.* **15**, 884–885 (1981).
86. Bai, J. P. F. Distribution of Brush-Border Membrane Peptidases Along the Rat Intestine. *Pharmaceutical Research: An Official Journal of the American Association of Pharmaceutical Scientists* **11**, 897–900 (1994).
87. Boutrou, R., Jardin, J., Blais, A., Tomé, D. & Léonil, J. Glycosylations of κ-casein-derived caseinomacropeptide reduce its accessibility to endo- but not exointestinal brush border membrane peptidases. *J. Agric. Food Chem.* **56**, 8166–8173 (2008).
88. Boutrou, R., Coirre, E., Jardin, J. & Léonil, J. Phosphorylation and coordination bond of mineral inhibit the hydrolysis of the β-casein (1 - 25) peptide by intestinal brush-border membrane enzymes. *J. Agric. Food Chem.* **58**, 7955–7961 (2010).
89. Bailey, C. A., Bryla, P. & Malick, A. W. The use of the intestinal epithelial cell culture model, Caco-2, in pharmaceutical development.

Adv. Drug Deliv. Rev. **22**, 85–103 (1996).

90. Artursson, P., Palm, K. & Luthman, K. Caco-2 monolayers in experimental and theoretical predictions of drug transport. *Adv. Drug Deliv. Rev.* **64**, 280–289 (2012).
91. Picariello, G. *et al.* Transport across Caco-2 monolayers of peptides arising from in vitro digestion of bovine milk proteins. *Food Chem.* **139**, 203–212 (2013).
92. Picariello, G. *et al.* Role of intestinal brush border peptidases in the simulated digestion of milk proteins. *Mol. Nutr. Food Res.* **59**, 948–956 (2015).
93. Mandalari, G., Mackie, A. M., Rigby, N. M., Wickham, M. S. J. & Mills, E. N. C. Physiological phosphatidylcholine protects bovine β-lactoglobulin from simulated gastrointestinal proteolysis. *Mol. Nutr. Food Res.* **53**, 131–139 (2009).
94. Pihlanto-Leppälä, A. Bioactive peptides derived from bovine whey proteins: Opioid and ace-inhibitory peptides. *Trends Food Sci. Technol.* **11**, 347–356 (2000).
95. Peram, M. R., Loveday, S. M., Ye, A. & Singh, H. In vitro gastric digestion of heat-induced aggregates of β-lactoglobulin. *J. Dairy Sci.* **96**, 63–74 (2013).
96. Deng, Y., Wierenga, P. A., Schols, H. A., Sforza, S. & Gruppen, H. Effect of Maillard induced glycation on protein hydrolysis by lysine/arginine and non-lysine/arginine specific proteases. *Food Hydrocoll.* **69**, 210–219 (2017).
97. Dalsgaard, T. K., Nielsen, J. H. & Larsen, L. B. Proteolysis of milk proteins lactosylated in model systems. *Mol. Nutr. Food Res.* **51**, 404–414 (2007).
98. Moscovici, A. M. *et al.* The impact of the Maillard reaction on the in vitro proteolytic breakdown of bovine lactoferrin in adults and infants. *Food Funct.* **5**, 1898–1908 (2014).

Chapter 1

UV irradiation as a comparable method to thermal treatment for producing high quality stabilized milk whey

Sofie Buhler [a‡], Federico Solari [b‡], Alessandra Gasparini [a], Roberto Montanari [b], Stefano Sforza [a] and Tullia Tedeschi[a*]

[a] Università di Parma, Food and Drug Department, Parco Area delle Scienze 27/A, 43124 Parma.

[b] Università di Parma, Department of Engineering and Architecture, Parco Area delle Scienze 181/A, 43124 Parma.

‡ These two authors (S.B. and F.S.) contributed equally to the work

Abstract

Whey is a valuable by-product of the dairy industry, due to the high nutritional, biochemical and functional properties of the contained proteins. As for other dairy products, to guarantee the microbiological safety of whey, thermal treatments are generally used. However, these processes can have a significant impact on the quality of the product. Thus, in recent years, interest in the use of non-thermal treatments in the dairy industry is significantly grown. In the present work, the use of UV irradiation as stabilizing treatment has been investigated on whey obtained from the Parmigiano-Reggiano cheese production. Two treatments were tested: UV treatment "1" which is characterized by three passages and UV treatment "2" by six ones.

On skimmed whey samples, microbiological tests showed UV treatment 2 and the thermal treatment have the same effectiveness on Enterobacteriaceae, but UV treatment 2 is more effective than thermal treatment on total viable counts. Molecular characterization of the protein and total amino acid content was used as markers for the quality of the product. The results highlight that UV treatment can be equivalent to thermal pasteurization in terms of microbiological stabilization, and better preserving the whey quality.

Keywords: milk whey, whey proteins, pasteurization, non-thermal treatments, UV radiation.

1.1 Introduction

In the dairy industry, whey is the main by-product, remaining inside the cauldrons at the end of the cheese production. For every 100 kg of milk used to produce cheeses, around 90 kg of whey are formed (Parra, 2009). Often considered as a waste, whey is, conversely, a high-value product, especially if raw milk is used and process temperatures are kept low, allowing to preserve intact the nutritional characteristics of its proteins and sugars.

Cheese whey is increasingly being used as an ingredient for the production of animal feed, due to its important content in lactose (0.18

- 60 kg/m^3), proteins (1.4 - 33.5 kg/m^3) and fats (0.08 - 10.58 kg/m^3) (Bacenetti et al., 2018).

Moreover, whey proteins are considered for their functional properties, such as gelation, foaming, and emulsifying activity, which are relevant for their use in various food applications (Kinsella & Whitehead, 1989; Playne, Bennett & Smithers, 2003).

During recent decades, interest has grown in the nutritional efficacy of whey proteins in infant formula and in dietetic and health foods, using either native or pre-digested proteins (De Wit, 1998).

In order to reduce transportation costs, whey is usually subjected to a concentration process which can be a membrane separation process (ultrafiltration, nanofiltration, reverse osmosis and microfiltration) or a drying process (thermal drying or freeze drying) (Ganju & Gogate, 2017). Today membrane separation techniques are the most diffused technique in the dairy industry (Saxena, Tripathi, Kumar, & Shahi, 2009), since the temperature change during the process is kept to a minimum and also various selective mechanisms such as ion exchange and solution diffusion can be used to yield higher selectivity in the membrane separation (Ganju & Gogate, 2017).

Milk can harbor a variety of microorganisms and can be important sources of foodborne pathogens. The presence of foodborne pathogens in milk is due to direct contact with contaminated sources in the dairy farm environment and to excretion from the udder of an infected animal. Many foodborne pathogens commonly found in raw milk can be detected in cheese expecially when the production temperature (55°C for Parmigiano Reggiano) is lower than the minimum lethal temperature of many pathogens.

More recently, the family of Enterobacteriaceae, which includes a number of important foodborne pathogens such as Salmonella, Yersinia enterocolitica, pathogenic Escherichia coli (including E. coli O157:H7), Shigella spp. and Cronobacter spp. have been used as indicators of hygiene and microbial quality of dairy products (Anand and Griffiths, 2011, Baylis, Uyttendaele, Joosten, & Davies, 2011).

Heat treatment processes are generally used to inactivate microorganisms in milk, whey, or whey products (Atamer, Ali, Neve, Heller, & Hinrichs, 2011, 2009; Atamer & Hinrichs, 2010). However, especially to reach sufficient reduction on thermo-resistant

microorganisms, heat treatments might have consequences on the functional properties of the product. The Maillard reaction, usually involving a lysine side chain of a protein and a reducing sugar such as lactose, is one of the most important alterations triggered by heating of dairy products. (Mauron, 1981) A decrease in the nutritional value of dairy products results from protein lactosylation, since it reduces the bioavailability of lysine, an essential amino acid (Dyer et al., 2016).

Interest in non-thermal treatments, such as pulsed electric fields (PEF), high pressure (HP) and ultraviolet (UV) irradiation, is significantly grown in recent years since they inactivate microorganisms with the benefit of better preserving the original quality. In particular, the interest on sterilization by ultraviolet radiation is increasingly grown, with diffusion in many food industrial contexts thanks to its cost effectiveness, its efficiency over a wide range of microorganisms and its ease installation and use.

UV treatments exploit the capabilities of light, at a wavelength of 254 nm (UV-C), to alter the genetic material in cells so that bacteria, viruses, molds, and other microorganisms can no longer reproduce and may be considered inactive (Billmeyer, 1997; Bolton, 2001; Giese, 1997). The germicidal effect is calculated on the basis of the dose, that is the product of the light intensity and the contact time. The light intensity, in every point of the fluid volume, depends on both the power of the lamps, and the penetration effect of UV light through the fluid medium. The penetration effect of UV-C radiation depends on the type of liquid, on its UV-C absorption coefficient, on soluble solutes present in the liquid, and on suspended matter. Whey has a high absorption coefficient to UV-C radiation basically due to a 6% in volume of dry matter and UV light penetrates into the liquid only a few millimeters, instead of a penetration of several centimeters as in the case of water. For that reason, standard UV reactors, designed for clear fluids, fail to achieve satisfactory performances on whey and a dedicated solution should then be designed.

Therefore, this work had a dual objective. The first one is to evaluate the effectiveness of UV treatment in ensuring the microbiological safety of both skimmed and concentrated whey. For this purpose a specific UV reactor has been designed.

The latter objective is determination by LC-ESI-MS of the nutritional value of the treated whey by quantification of whey proteins and amino acids.

1.2 Materials and methods

1.2.1 Whey samples

Two different raw materials were furnished by local Parmigiano-Reggiano producers: Skimmed whey "cold": skimmed whey (6% of dry matter) which has been cooled at a temperature lower than 15°C just after its extraction from the cauldron and kept cool; Skimmed whey "hot": skimmed whey (6% of dry matter) which has been kept at the cheese cooking temperature (55°C) for a time that can vary from four to 8 h.

Skimmed whey "cold" was further used to produce two concentrated whey samples: Concentrated whey "1": Skimmed whey, concentrated with a reverse osmosis system having a concentration ratio of 3.8 (22.8 % of dry matter); Concentrated whey "2": Skimmed whey, concentrated with a reverse osmosis system having a concentration ratio of 4 (24 % of dry matter).

Concentration process was performed twice in order to have a repeatability of the test even on concentrated whey. The two concentration factors are slightly different due to a normal fluctuation of the process.

1.2.2 Computational Fluid Dynamics (CFD) simulation

Ansys®, Release 18.0 (Ansys Fluent Theory Guide, ANSYS, Inc.), was used for the CFD modeling. Reactor geometry doesn't imply the formation of wake region, in which transient phenomena can occur, then a standard k-epislon turbulence model has been used (Liu, Wu, Linden, & Ducoste 2007).

A flow rate of 30 m^3/h, in the case of skimmed whey, and of 7,9 m^3/h, in the case of concentrated whey, has been imposed as boundary condition on the inlet section. A relative static pressure of 0 Pa has been imposed on the outlet section. A no slip wall condition has been set on all wet surfaces inside the reactor.

The UV radiation field was calculated by means of Discrete Ordinates (DO) radiation model, which solves the radiative transfer equation for a finite number of discrete solid angles. The model is described in Supplementary Material Section).

The refractive index has not been considered because the quartz sleeve and the air annulus inside it, have not been modeled. The effect of thermal radiation due to temperature differences between the fluid and the lamp sleeves has also been minimized. Irradiation intensity has been fixed on lamp sleeves surface by dividing the lamp power by the surface area of the sleeve.

In order to evaluate the incidence of the characteristics of the raw material on treatments efficiency, the previously described whey samples have been tested.

Whey absorption coefficients were evaluated at the sterilizing wavelength (253.7 nm) by means of spectrophotometry (Aquamate - Thermo Electron Corporation).

The UV dose distribution was computed by integrating UV intensity over the path of a certain number of water particles (6200 in this study). A Lagrangian model, based on a random-walk algorithm, was used to calculate water particle trajectories (Ducoste et al. 2005; Ducoste & Linden, 2006).

1.2.3 Ultraviolet treatment

Two different treatments have been performed on the four whey samples, each one characterized by a different number of passages through the apparatus represented in Figure 1. UV treatment "1" is characterized by three passages, while UV treatment "2" is characterized by six passages.

Flow rate for both the treatments was 30 m^3/h for skimmed whey and 7.9 m^3/h for concentrated whey.

Samples were collected through appropriate sample taps installed just before the inlet section and just after the exit section of the reactor. Three samples for each treatment were taken to ensure sufficient repeatability in the results.

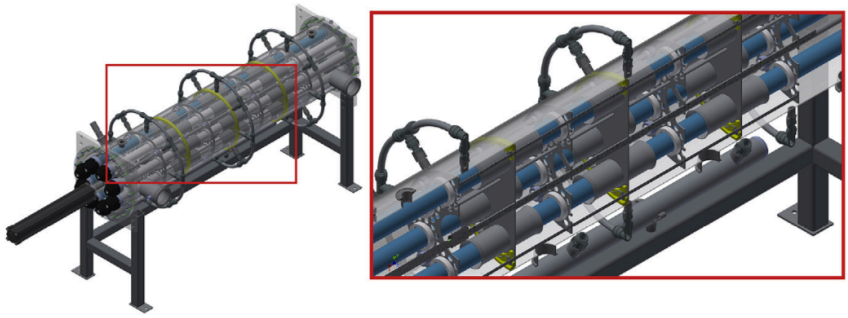


Figure 1. Apparatus used for the UV treatment of the whey samples

1.2.4 Thermal treatment

Whey samples were collected in sterile glass tubes which were then immersed in a hot water bath, inside an insulated cell, whose temperature was verified with a calibrated thermometer (Testo 926, equipped with a Probe 06031293). The above-mentioned thermometer was then used to monitor the temperature of the whey inside a tester tube, during the pasteurization heat treatment.

Heat treatment has been carried out by counting fifteen seconds from the time the thermometer detected a temperature of at least 72.1 ° C within the tester tube. During the entire period, the temperature inside the tester tube was monitored to ensure that it remained above 72.1 ± 0.1 °C.

1.2.5 Microbiological tests

For an assessment of the safety and overall quality of the product, *Enterobacteriaceae (ETB)* and *Total Viable Counts* (TVC) (aerobic mesophilic) have been taken as marker microorganisms as regulated in the standard EN ISO 4833. *ETB* and *TVC* have been evaluated by means of plate count, involving serial dilutions of the samples (1:10, 1:100, 1:1000, 1:10000, 1:100000 and 1:1000000 are the dilutions used in this study) in sterile H_2O and cultivation on nutrient agar in a sealed and incubated dish. The media used to perform plate count was *Plate Count Agar with Skim Milk* (Biotec srl, Via Rubino, 14B - 58100, Grosseto) for TVC, and *Violet Red Bile Glucose Agar* for *ETB* (Biolife, Italiana srl, Viale Monza, 272 – 20128 Milano). For each sample two microbiological tests have been

performed: one before and one after the treatment in order to evaluate microbial changes.
Detailed experimental procedure is described in Supplementary Material Section.

1.2.6 LC/ESI-MS analysis

Cheese whey samples described in section 2.1 were characterized by UPLC-MS analysis, following the experimental conditions depicted in Supplementary Material Section.α-lactalbumin and β-lactoglobulin in the native and lactosylated forms were identified and quantified.

EXtract Ion Chromatograms (XICs) were obtained extracting the characteristic ions for α-lactalbumin and β-lactoglobulin and their lactosylated forms and the areas of the signals were integrated with the MassLynx software (Waters 34 Maple Street Milford, MA 01757, USA). An external calibration curve was obtained analyzing aqueous solutions containing α-lactalbumin and β-lactoglobulin standards.

1.2.7 Total amino acids quantification

Total amino acids quantification was performed on the cheese whey samples described in section 2.1, following the experimental procedure described in Supplementary Material Section.

First the samples were hydrolyzed with HCl or NaOH, then the amino acid residues were mixed with 20 µl of 2.5 mM Norleucine in 0.1 M HCl; 10 µl of the produced solutions were derivatized with the Waters AccQ-Fluor reagent kit, according to the instructions of the manufacturer.

The derivatized samples were analyzed on a UPLC/ESI-MS system, following the experimental conditions described in Supplementary Material section.

1.2.8 Statistical analysis

Analysis of variance (ANOVA) was performed test at a significance level of $\alpha=0.05$. Significant differences among the mean values were calculated using Duncan's ($p\leq0.05$). Correlation coefficients were computed using Pearson's coefficient ($p\leq0.05$). All

experimental data were statistically analyzed using the SPSS version 25.0 (SPSS Inc., Chicago, IL, USA).

1.3 Results and discussion

The stabilizing UV treatment was tested on all the whey samples described in Materials and Method.

1.3.1 Design of the reactor

At the design phase of a reactor, computational fluid dynamics (CFD) helps to predict the UV dose distribution and then to identify the optimal configuration as a function of the operating condition to which the reactor will operate (Ferretti et al., 2011; Solari et al., 2015).

The absorption coefficients of the studied whey samples were measured spectrophotometrically. For the two samples of skimmed whey results the same absorption coefficient (7.98 cm^{-1}), while for the concentrated samples results an higher value for the more concentrated one (41.55 cm-^{1} for *concentrated whey "2"* and 41.40 cm^{-1} for *concentrated whey "1"*). The obtained values were used to estimate the radial distribution of the UV radiation field around a single lamp in case the lamp is immersed in water and in case it is immersed in the whey (Figure 2).

As highlighted by Figure 2, radiation decreases very quickly with radius in whey, having a penetration distance of approximately 5 mm in the case of skimmed whey (both "hot" and "cold") and 1 mm in the case of concentrated whey (both concentration ratios).

A proper configuration was designed and its performance has been evaluated by means of CFD simulation. It is characterized by the insertion of three special diaphragms that force the fluid to pass through very thin sections around each lamp (treatment sections). Consequently, inside the reactor, there are three treatment sections for each lamp; each treatment section is 18 cm long (Figure 3).

The reactor mounts 8 UV-C amalgam lamps with a power of 400 W , emitting radiation mainly at the sterilizing wavelength (253.7 nm). Lamps are inserted inside quartz sleeves having a diameter of 45 mm.

According to the results highlighted in Figure 2, treatment sections have been made as thin as possible, while ensuring the necessary

tolerances (for assembly and operation) and taking into account commercially available tubes diameters. The fluid thickness inside each treatment section is 5,5 mm.

Starting from the flow field computation, it is possible to reconstruct the path traveled by each microorganism that passes through the reactor (Figure 4). By integrating the UV radiation along the trajectory of each microorganism, the dose distribution guaranteed by the reactor can be calculated.

Performances of the reactor were evaluated in terms of *average UV dose [mJ/cm^2]* and in terms of *percentage of volume exposed to a dose higher than 40 mJ/cm^2*. Designed reactor resulted to provide an average UV dose of 61 mJ/cm^2 and a percentage of volume of 45 % exposed to a dose higher than 40 mJ/cm^2, in the case of skimmed whey, and an average UV dose of 39 mJ/cm^2 and a percentage of volume of 26 % exposed to a dose higher than 40 mJ/cm^2, in the case of concentrated whey.

As stated in "Materials and methods" section, in this work, two different UV treatments were tested on the Parmigiano-Reggiano whey samples. These treatments have been chosen based on the results obtained with CFD simulation (Figure 2):

- "UV treatment 1" ensures that at least 90% of the whole volume of skimmed whey is exposed to a fluence of 40 mJ/cm^2.
- "UV treatment 2" ensures that at least 95% of the whole volume of skimmed whey is exposed to a fluence of 40 mJ/cm^2.

The reference value of 40 mJ/cm^2 has been chosen on the basis of the German Gas and Water Association (DGVW 2006) standard and similar standards established in Austria (ÖNorm 2001) which are recognized throughout the world and form the basis of many other national standards. The core ethos of the DVGW (and ÖNorm) standard is that a UV system should be proven to continuously deliver a minimum germicidal fluence of 40mJ/cm^2, under all operational conditions.

Contextually to the UV treatments, a thermal pasteurization has been carried out to compare their performance concerning the qualitative and the microbiological of view.

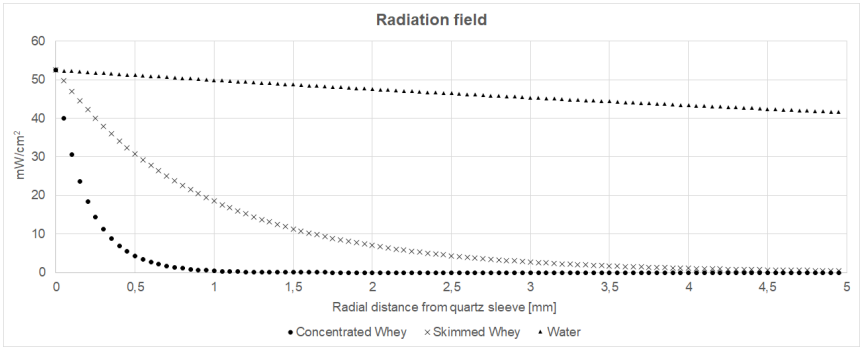


Figure 2. Radial distribution of the UV radiation field around a single lamp in the case of water and the used whey samples (skimmed and concentrated)

1.3.2 Microbiological tests

In the present work, the influence of the UV and thermal treatments on *ETB* and on *TVC* has been evaluated; the obtained results are reported in Table 1 and Figure 3.

Table 1. *Enterobacteriaceae* and total viable count before and after the stabilizing treatments

Eterobacteriaceae (ETB)			
	UV treatment 1	UV treatment 2	Thermal treatment
	Log reduction	Log reduction	Log reduction
Skimmed whey "cold"	2,75^{a} ± 0,13	4,13^{b} ± 0,55	3,84^{b} ± 0,21
Skimmed whey "hot"	1,62^{a}± 0,10	4,41^{b} ± 0,36	3,97^{b} ± 0,58
Concentrated whey "1"	0,73^{a} ± 0,05	1,37^{b} ± 0,16	2,33^{b} ± 0,50
Concentrated whey "2"	0,645^{a} ± 0,08	1,02^{b} ± 0,03	2,08^{c} ± 0,09
Total Viable Count at 30°C (TVC)			
	UV treatment 1	UV treatment 2	Thermal treatment
	Log reduction	Log reduction	Log reduction
Skimmed whey "cold"	1,92^{a} ± 0,05	2,88^{b} ± 0,19	2,15^{a} ± 0,08

Skimmed whey "hot"	1,44[a] ± 0,10	2,91[c] ± 0,40	2,14[a] ± 0,21
Concentrated whey "1"	0,66[a] ± 0,07	1,29[b] ± 0,18	2,12[c] ± 0,20
Concentrated whey "2"	0,51[a] ± 0,07	0,77[a] ± 0,06	2,12[b] ± 0,30

Different letters in the same section of the same row are significantly different (p≤0.05).

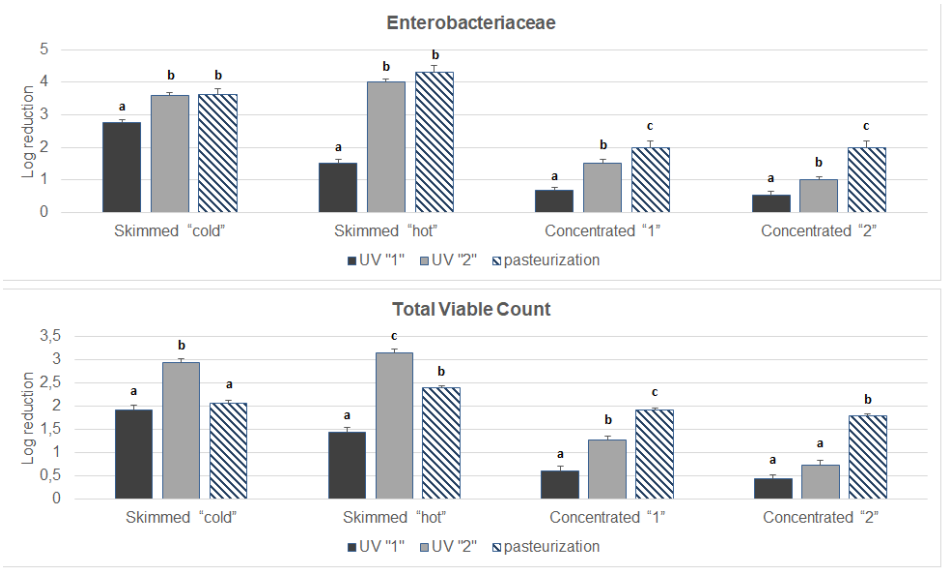


Figure 3. *Enterobacteriaceae* (A) and TVC (B) reduction obtained with the three different stabilizing treatments**.** Different letters in the same group are significantly different (p≤0.05).

The results were analysed by ANOVA test followed by a post-hoc test (Duncan's test), in order to evaluate any significance differences between the three different treatments used: UV1, UV2 and pasterurization.

On ETB, for both the skimmed whey samples, whether "hot" or "cold", "UV treatment 2" was found to have a performance comparable with the thermal treatment. While on the *TVC* "UV treatment 2" results to be more effective on both the samples. "In particular, the hot skimmed whey after the "UV treatment 2" is found under the allowed limits of the law (<10cfu/g) as reported in the Commission Regulation

2073/2005 on microbiological criteria for foodstuffs and subsequent amendments (No. 1441/2007 and 365/2010) for pasteurised milk and other pasteurised liquid dairy products and milk powder and whey powder (Baylis et. al, 2011). (see Table 1)

On concentrated whey, neither "UV treatment 1" nor "UV treatment 2" can guarantee the same performance of the heat treatment. This result is related to the fact that the penetrating effect of the UV radiation in the concentrated whey is less than 1 mm (Figure 2), much less than the radial thickness of the treatment sections (5,5 mm). In order to enhance the performance of UV reactor on concentrated whey, the gap between the diaphragms and the quartz sleeves should be further reduced, but this conflicts with the minimum assembly and machining tolerances. It can therefore be concluded that UV technology represents a valid alternative to heat treatment only for values of absorption coefficients not too high.

1.3.3 Characterization of the effect of heat and UV treatment on soluble whey proteins

The quantitation of the soluble whey proteins and of their lactosylated forms by HPLC-ESI-MS has been proposed as a robust method for the evaluation of the harshness of the thermal treatments performed on milk and dairy products (Losito et al., 2007).

In this work an UPLC-ESI-MS method was set up for analyzing whey proteins in Parmigiano Reggiano whey samples before and after the UV treatments, along with the heat treated samples, to evaluate the possible effects of the stabilizing processes on the amount and the degree of lactosylation of the main whey proteins, β-lactoglobulin and α-lactalbumin.

A typical UPLC–ESI-MS chromatogram obtained from the analyzed whey samples is depicted in Figure 4a. The corresponding ESI-MS spectrum (Figure 4b) shows the presence of the main whey proteins (α-lactalbumin and the two isoforms of β-lactoglobulin) and of their mono-lactosylated forms: multi-charge profiles characterized by m/z ratios compatible with a +324 Da mass increase, corresponding to the initial Maillard reaction product with a lactose molecule, were observed in the spectra.

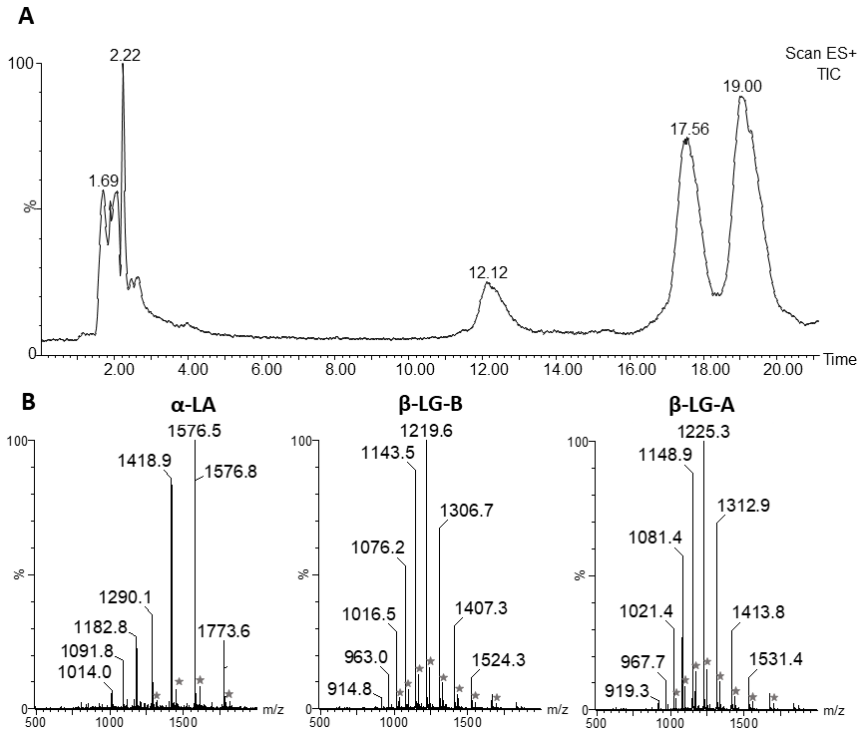


Figure 4. (**A**) UPLC–ESI-MS chromatogram of an analyzed whey; peaks having retention times of 12.12′, 17.56′ and 19.00′ are relative to α-lactalbumin and the isoforms B and A of β-lactoglobulin, respectively. (**B**) ESI-MS spectra corresponding to the three proteins; the signals highlighted by the grey stars are relative to the mono-lactosylated forms

Multiple ion current extractions were performed on sets of m/z ratios corresponding to the three proteins and their mono-lactosylated forms. Total concentration values (free + lactosylated form) for the soluble proteins in the analyzed samples were obtained by using an external calibration curve; the two isoforms of β-lactoglobulin were quantified together assuming an identical response factor.

The results obtained for the "skimmed" whey samples showed that the two UV treatments do not alter the amount and the degree of lactosylation of the soluble whey proteins (Figure 5).

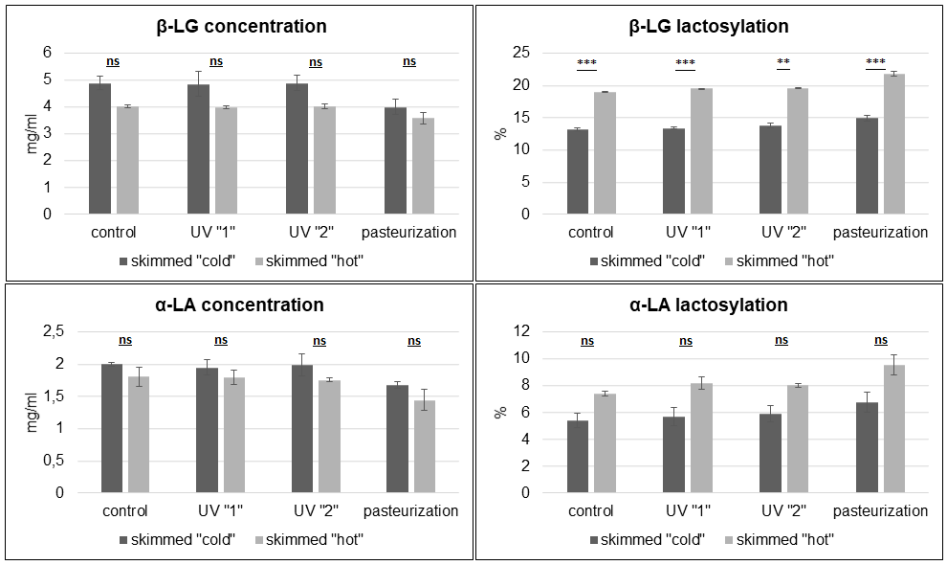


Figure 5. Concentration and percentages of lactosylation of the soluble whey proteins in the skimmed whey samples with different post-production storage conditions. *p ≤ 0.05. **p ≤ 0.01. ***p ≤ 0.001. ns=not significant.

Conversely, the heat treated samples show a slightly lower content in soluble whey proteins and their percentage of lactosylation was found to be higher. Interestingly, one-way ANOVA statistical analysis showed that significant difference (p≤0.05) was present in terms of beta-lactoglobulin lactosylation among the post-production storage conditions. This highlights that the post-production storage has a significant impact on the quality of the product: the whey which has not been cooled after its production (skimmed whey "hot") has a lower content of soluble whey proteins and the degree of lactosylation of the latter is significantly higher.

Concerning the results obtained for the concentrated whey samples the two UV treatments seem to cause a slight decrease in the amount of the soluble whey proteins, but again they both were found to perform better than the traditional thermal process (Figure 6).

The degree of lactosylation was not significantly altered by the UV treatments, as reported by ANOVA, highlighting the fact that the concentration process has a neglectable impact on the quality of the product.

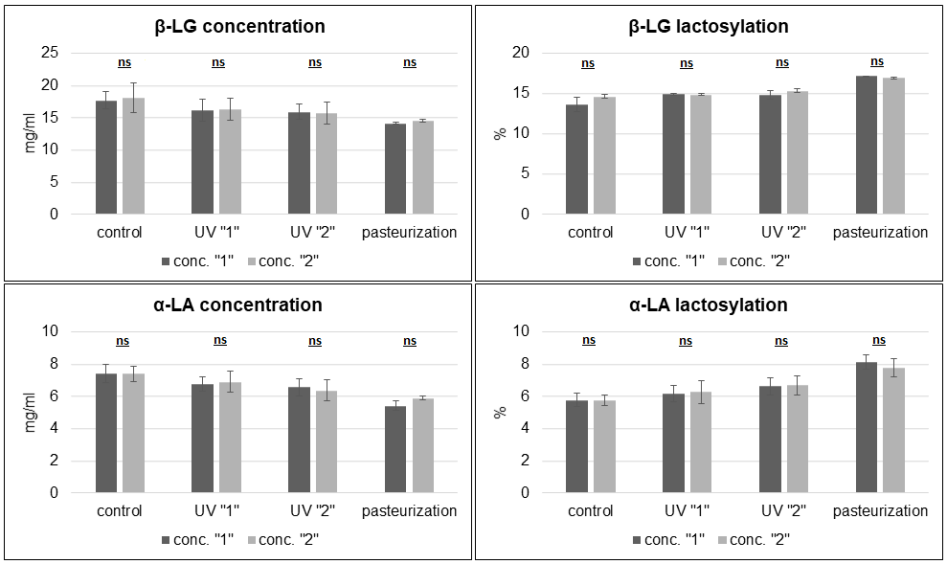


Figure 6. Concentration and percentages of lactosylation of the soluble whey proteins in the concentrated samples. $p \leq 0.05$. $^{**}p \leq 0.01$. $^{***}p \leq 0.001$. ns=not significant.

1.3.4 Total amino acids quantification

Total amino acids were also quantified in the whey samples after standard acidic hydrolysis; alkaline hydrolysis was employed to allow the quantification of tryptophan. The analysis was performed in order to outline if the UV process caused the degradation of some amino acids, in particular the essential ones. Prior to LC-ESI-MS analysis, the amino acids were derivatized with the Waters AccQ-Fluor reagent kit, to enhance the chromatographic separation. Single ions were monitored for each amino acid and for their main possible oxidation products: methionine-sulfoxide (Met-S-ox), methionine-sulphone (Met-S-$(ox)_2$) and N'-formylkynurenine (NFK), a carbonylation product of Trp, but no signals were observed for any of the monitored oxidation products in any of the analyzed samples, indicating that these compounds were likely not being formed during the UV treatment. The obtained results are reported in Figure 7.

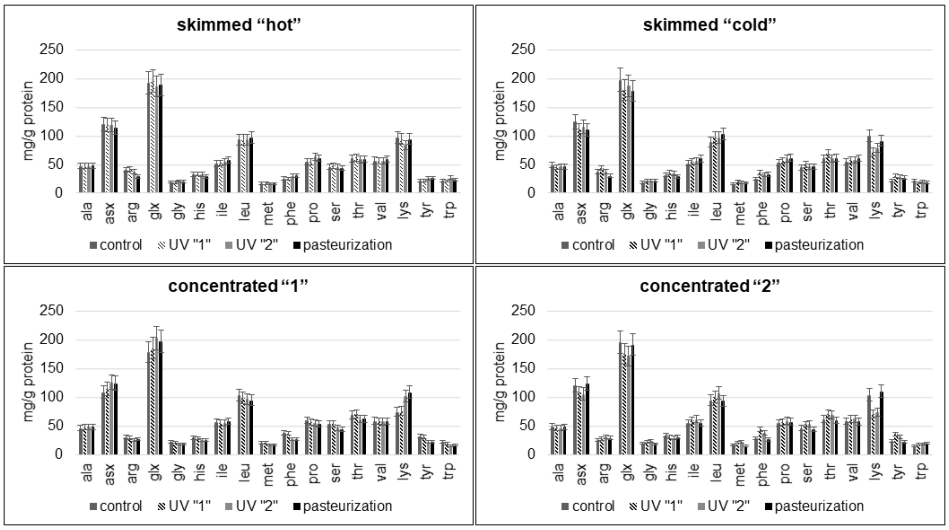


Figure 7. Amino acids quantified after total hydrolysis of the proteins contained in the analyzed whey samples. From ANOVA analysis, not significant differences have been found.

As shown by the reported data, no significant variation in amino acids content was observed after the treatments, as reported by ANOVA statistical analysis. In particular, no loss of aromatic amino acids, that had been described for several complex food matrices after UV irradiation (Viljanen et al., 2005), was found for the different whey samples. Though UV treatments of milk have been reported to possibly induce oxidative changes in proteins (Scheidegger et al., 2010), the amino acids for which the oxidized forms had been monitored (Trp and Met) were also found to be unaffected by the process, thus confirming that no oxidized forms were generated.

1.4 Conclusions

A reactor has been designed for the UV treatment of skimmed whey, to guarantee its microbiological safety, without altering the quality of the contained proteins. Tests were performed on skimmed and concentrated whey samples; the microbiological safety of the products and their quality, in terms of protein quality after the treatment, were assessed and then compared with the performance achieved with a traditional thermal treatment.

In the case of skimmed whey, from a microbiological point of view, the performances were fully comparable and the quality of the product resulted better after the UV treatment than after the thermal treatment. Indeed, a higher amount of soluble whey proteins was detected and their degree of lactosylation was lower.

UV treatment was found not to alter the nutritional value of the whey, since no amino acid loss was observed, even those which can be easily degraded in these conditions.

For the concentrated whey, even if the quality was still preserved, thermal treatment turned out to produce a safer product from the microbiological point of view. In this latter case, the configuration of the reactor needs to be optimized for the UV treatment of this kind of product.

Funding
This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

Anand, S.K. & Griffiths, M. W. (2011). Pathogens in milk | Enterobacteriaceae. In *Encyclopedia of Dairy Sciences* (pp.67-71) G.W. Smithers (Ed.), Elsevier Inc., Amsterdam, the Netherlands.

Atamer, Z., Dietrich, J., Müller Merbach, M., Neve, H., Heller, K. J., & Hinrichs, J. (2009). Screening for and characterization of Lactococcus lactis bacteriophages with high thermal resistance. *International Dairy Journal,* 19, 228–235. https://doi.org/10.1016/j.idairyj.2008.10.012.

Atamer, Z., & Hinrichs, J. (2010). Thermal inactivation of the heatresistant Lactococcus lactis bacteriophage P680 in modern cheese processing. *International Dairy Journal,* 20, 163–168.

Atamer, Z., Ali, Y., Neve, H., Heller, K. J., & Hinrichs, J. (2011). Thermal resistance of bacteriophages attacking flavour-producing dairy Leuconostoc starter cultures. *International Dairy Journal*, 21, 327–334.

Bacenetti, J., Bava, L., Schievano, A., Zucali, M. (2018) Whey protein concentrate (WPC) production: Environmental impact assessment. *Journal of Food Engineering 224 (2018) 139-147*

Baylis C. Uyttendaele M., Joosten H., Davies A. (2011). The Enterobacteriacae and their significance to the food industry. ILSI Europe Report Series. ILSI Europe International Life Sciences Institute.

Billmeyer, F. W. (1997). Ultraviolet lamp. Pages: in McGraw-Hill *Encyclopedia of Science and Technology*. (pp. 19-20, vol. 19). New York 7 McGraw-Hill.

Bolton, J. (2001). What is ultraviolet? In: IUVA website (International Ultraviolet Association). http://www.iuva.org/PublicArea/whatisuv Accessed 10 September 2017.

De Wit, J.N. (1998). Nutritional and functional characteristics of whey proteins in food products. *Journal of Dairy Science,* 81, 597-608.

Ducoste, J., & Linden, K. (2006). Hydrodynamic characterization of UV reactors. Denver: American Water Works Association Research Foundation.

Ducoste, J., Liu, D., & Linden, K. (2005). Alternative approaches to modeling fluence distribution and microbial inactivation in ultraviolet reactors: Lagrangian versus Eulerian. *Journal of Environmental Engineering*, 131, 1393–1403.

DVGW (2006). 'UV Devices for the Disinfection for Drinking water Supply – Parts 1, 2 and 3' Deutsche Vereinigung des Gas und Wassserfaches, Bonn, Germany.

Dyer, J. M., Clerens, S., Grosvenor, A., Thomas, A., Callaghan, C., Deb-Choudhury, S., & Haines, S. (2016). Proteomic tracking of hydrothermal Maillard and redox modification in lactoferrin and β-lactoglobulin: Location of lactosylation, carboxymethylation, and oxidation sites. *Journal of Dairy Science,* 99, 3295-304.

Ferretti, G., Montanari, R., & Solari, F. (2011). A new approach for the optimization of UV-reactor design by mean of CFD simulation. In Proc. 6th CIGR Section VI International Symposium "Towards a Sustainable Food Chain". Nantes, France.

Ganju, S., & Gogate, P. R. (2017). A review on approaches for efficient recovery of whey proteins from dairy industry effluents. *Journal of Food Engineering*, 215, 84-96.

Giese, A. C. (1997). Ultraviolet radiation (biology). in McGraw-Hill *Encyclopedia of Science and Technology*. (pp. 20-22, vol. 19). New York7 McGraw-Hill.

Kinsella, J.E. & Whitehead, D. M. (1989). Proteins in whey: chemical, physical, and functional properties. *Advances in Food and Nutrition Research*, 33, 343-438.

Liu, D., Wu, C., Linden, K. & Ducoste, J. (2007). Numerical simulation of UV disinfection reactors: Evaluation of alternative turbulence models. *Applied Mathematical Modelling,* 31, 1753-1769.

Losito, I., Carbonara, T., Monaci L., & Palmisano F. (2007). Evaluation of the thermal history of bovine milk from the lactosylation of whey proteins: an investigation by liquid chromatography-electrospray ionization mass spectrometry. *Analytical and Bioanalytical Chemistry,* 389, 2065-74. https://doi.org/10.1007/s00216-007-1447-0.

Mauron, J. (1981). The Maillard reaction in food, a critical review from the nutritional standpoint. *Progress in food & nutrition science*, 5, 5-35.

ONORM (2001). Requirements and testing (low pressure mercury lamp plants), Austrian national standard ONORM M 5873-1.

Parra, R. (2009). Whey: importance in the food industry. *Revista Facultad Nacional de Agronomía Medellín*, 62, 4967-4982.

Playne, M, Bennett, L., & Smithers, G. (2003). Functional dairy foods and ingredients. *Australian Journal of Dairy Technology*, 58, 242–64.

Saxena, A., Tripathi, B. P., Kumar, M., & Shahi, V. K. (2009). Membrane-based techniques for the separation and purification of proteins: an overview. *Advances in Colloid and Interface Science*, 145, 1- 22.

Scheidegger, D., Pecora, R. P., Radici, P. M., & Kivatinitz, S. C. (2010). Protein oxidative changes in whole and skim milk after ultraviolet or fluorescent light exposure. *Journal of Dairy Science*, 93, 5101–5109.

Solari, F., Girolimetti, G., Montanari R., & Vignali, G. (2015). A new method for the validation of ultraviolet reactors by means of photochromic materials. Food and Bioprocess Technology, 8, 2192-2211.

Viljanen, K., Kylli, P., Hubbermann, E. M., Schwarz, K., & Heinonen, M. (2005). Anthocyanin antioxidant activity and partition behavior in whey protein emulsion. *Journal of Agricultural and Food Chemistry*, 53, 2022–2027.

Supplementary Material

1) Experimental Procedures

Microbiological Analysis

The proper quantity of agar has been weighed (8.3 g of Violet Rose Bile Glucose Agar (VRBG), for ETB, and 4.9 g of "*Plate Count Agar with Skim Milk*", for *TVC)* in 200 ml of distilled water. Obtained mixture are well stirred and heated for 2 minutes in a microwave oven. Liquid solutions are stirred again and heated for another 2 minutes. At the end of this phase, the obtained soils are dissolved in the microwave until homogeneous liquids are obtained. *Plate Count Agar with Skim Milk* is also autoclaved at 121 ° C for 15'. The obtained liquids are then stirred to optimize mixing and are left in a bain-marie so that they reach a temperature of 45 ° C and solidification is avoided. Under sterile hood, sterile Petri dishes (9 cm diameter) are prepared: for each plate 1 ml of whey sample is taken with a 1 ml pipette and, if necessary, appropriate dilution is prepared using 9 ml tubes of phosphate buffer. For each plate 1 ml of appropriately diluted sample is deposited on the bottom of the Petri dish. A thin layers of previously prepared soil is also deposited into the plate containing the sample, moving it with rotary motion to distribute it over the entire surface of the bottom of the plate and mix it with the sample homogeneously. A new layer of soil is then deposited over it so that it covers the entire surface.

Each Petri dish is then incubated in a stove at 37 ° C for 24 hours, as regards *ETB,* and incubated in the oven at 30 ° C for 72 hours, as regard *TVC*, turning the plate upside down so that the condensation deposits on the lid. At the end, purple-red colonies (*ETB*) and white colonies (*TVC*) are counted and, depending on the dilution, a number of zeros are added to the number of colonies counted (eg dilution -1 one zero is added, dilution to -2 two zeros are added, and so on). The resulting number is expressed in ufc / ml.

LC-ESI-MS analysis

Whey samples were centrifuged (10', 14000 rpm, 5°C), then the clear supernatant was analyzed either directly (unconcentrated samples) or after 1:4 dilution with H_2O (concentrated samples), on a UPLC/ESI-MS system (UPLC Acquity Waters with a single quadrupole mass spectrometer Waters Acquity Ultraperformance, Waters 34 Maple Street Milford, MA 01757, USA) using a RP column (ACQUITY UPLC BEH 300 C4 1.7 µm 2.1 * 150 mm) and a gradient elution. Eluent A was H_2O with 0.1% formic acid, eluent B was acetonitrile with 0.1% formic acid; gradient: 0–7 min 69% A, 7–20 min from 69% A to 64.5% A; flow: 0.2 ml/min; column temperature: 35°C; sample temperature: 18°C; injection volume: 2 µl. The samples were analyzed in the Full Scan mode; ionization type: positive ions; scan range: 100–2000 m/z; capillary voltage: 3.2 kV; cone voltage: 30 V; source temperature: 150 °C; desolvation temperature: 300 °C; cone gas flow: 100 l/h; desolvation gas flow: 650 l/h.

EXtract Ion Chromatograms (XICs) were obtained extracting the characteristic ions for α-lactalbumin (1091.5, 1182.5, 1289.8, 1418.7, 1576.3, 1772.8) and β-lactoglobulin (isoform A: 1021.1, 1081.0, 1148.6, 1225.1, 1312.5, 1413.4; isoform B: 1016.2, 1076.0, 1143.1, 1219.3, 1306.4, 1406.8) and their lactosylated forms (α-LA: 1116.6, 1209.6, 1319.4, 1451.4, 1612.7, 1813.9; β-LG-A: 1039.4, 1100.2, 1169.1, 1247.0, 1335.8, 1438.7; β-LG-B: 1034.3, 1095.1, 1163.5, 1241.1, 1329.7, 1431.8) and the areas of the signals were integrated with the MassLynx software (Waters 34 Maple Street Milford, MA 01757, USA). An external calibration curve was obtained analyzing aqueous solutions containing α-lactalbumin and β-lactoglobulin standards.

Total amino acids quantification

420 µl of the skimmed whey samples and 110 µl of the concentrated whey samples were dried under N_2 flux in pyrex glass tubes, then 3 ml of HCl 6 M were added and the tubes were flushed for 30 s with N_2. The samples were incubated for 23 h at 110°C, then the liquid phase was dried under N_2 flux. H_2O was added to a final volume of 6 ml for each sample.

Alkaline hydrolysis was performed adding 3 ml of NaOH 4 M to the samples dried in pyrex tubes (same amounts used for the acid hydrolysis). The tubes were flushed for 30 s with N_2, then the samples were incubated for 19 h at 110°C. After cooling, 3 ml of HCl 4 M were added to the samples.

180 µl of the solutions obtained after centrifugation of the acid and the alkaline hydrolysates were mixed with 20 µl of 2.5 mM Norleucine in 0.1 M HCl; 10 µl of the produced solutions were derivatized with the Waters AccQ-Fluor reagent kit, according to the instructions of the manufacturer.

The derivatized samples were analyzed on a UPLC/ESI-MS system (UPLC Acquity Waters with a single quadrupole mass spectrometer Waters Acquity Ultraperformance) using a RP column (ACQUITY UPLC BEH 300 C18 1.7 µm 2.1 * 150 mm) and a gradient elution. Eluent A was H_2O with 0.1% formic acid, eluent B was acetonitrile with 0.1% formic acid; gradient: 0–7 min 100% A, 7–30 min from 100% A to 73.3% A; flow: 0.2 ml/min; column temperature: 35°C; sample temperature: 18°C; injection volume: 10 µl. The samples were analyzed in the SIR Scan mode (monitored ions, which corresponds to the derivatized amino acid moieties are reported in Table 1); ionization type: positive ions; capillary voltage: 3.2 kV; cone voltage: 30 V; source temperature: 150 °C; desolvation temperature: 300 °C; cone gas flow: 100 l/h; desolvation gas flow: 650 l/h.

Calibration curves for the amino acids were obtained as follows: Amino acid standard H (Thermo Scientific, Rockford, IL, USA) was mixed with an equal volume of Norleucine 2.5 mM in HCl 0.1 M; 1:2, 1:4, 1:8 and 1:16 dilutions in H_2O were prepared and the produced solutions were derivatized and analyzed as previously mentioned for the samples.

The calibration curve for Tryptophan was obtained in the same way, after mixing equal volumes of Tryptophan 2.5 mM and Norleucine 2.5 mM in HCl 0.1 M.

Table 1S. Monitored ions, which corresponds to the derivatized amino acid moieties, (m/z values), acquired during the UPLC-MS analysis performed for the total amino acids quantification

Compound	m/z
Gly	246.1
Ala	260.1
Ser	276.1
Pro	286.1
Val	288.2
Thr	290.1
Ile/Leu/Norleu	302.2
Asp	304.1
Lys	317.1
Glu	318.1
Met	320.1
His	326.1
Phe/Met-S-ox	336.1
Arg	345.2
Met-S-$(ox)_2$	352.1
Tyr	352.2
Trp	375.2
NFK	407.2

2) Computational Fluid Dynamics (CFD) simulation

Radiative transfer equation, as described in Ansys Fluent Theory Guide, is defined as:

$$\frac{dI(\vec{r},\vec{s})}{ds} + a \cdot I(\vec{r},\vec{s}) = 0$$

Where:

$\vec{r}$: position vector

$\vec{s}$: direction vector

$\frac{dI(\vec{r},\vec{s})}{ds}$: derivative of the light intensity along the direction vector

a: absorption coefficient [m^{-1}]

I: radiation intensity, which depends on position ($\vec{r}$) and direction ($\vec{s}$) [W/m^2]

s: path length

Chapter 2

Identification of lactosylation sites in IgE-binding epitopes of bovine whey proteins

2.1 Introduction

Before commercialization, milk undergoes different technological treatments in order to guarantee its microbiological safety and extended shelf life[1]. Main thermal treatments used are pasteurization (15-20 s at 72-75°C), sterilization (10-30 min more than 110°C) and Ultra High Temperature (UHT) treatment[1]. The UHT treatment may be performed by direct heating, through steam injection (2-4s at 140-145°C), or by indirect heating, via metal plates or tubes (5-8s at 136-138°C)[2,3]. The high temperatures involved in the treatment influence the protein content inducing some relevant structural and chemical modifications such as protein denaturation/aggregation and the Maillard reaction[4]. This reaction affects principally lysine residues that react with lactose to form the first reaction intermediate (the Amadori compound or lactulosyllysine), as depicted in figure 2.1.1.

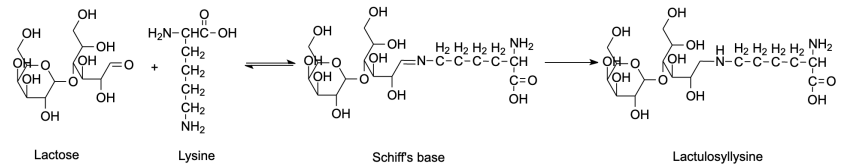


Figure 2.1.1 Conjugation of lactose with lysine residue forming the Amadori compound (lactulosyllysine).

The main effect of the lactosylation is to reduce the available free lysine affecting the nutritional value of the product[5]. Thus, several studies were performed to investigate lysine glycation, in particular in the two main whey proteins β-lactoglobulin and α-lactalbumin. The most common analyses applied for the identification of glycated modifications is the determination of furosine, which is a degradation

product of the Amadori compound by HPLC, or to detect the lactosylated lysine residues in peptides and proteins by LC-MS techniques and MALDI-TOF-MS [2,6–9]. The first lactosylation site, the lysine 100 of β-lactoglobulin, was identified in 1998 with electrospray mass spectrometry[10].

The binding with lactose on the lysine residues may affect not only whey proteins nutritional value but also their allergenicity. Indeed, the two main whey proteins, β-lactoglobulin and α-lactalbumin, are important milk allergens[11] and the presence of lactose bound to the IgE-binding epitope could influence the interaction altering the allergenic response. Nowadays, is still not completely clear how this modification affects allergenicity, in particular regarding the interaction with the IgE antibody. As an example, it was reported that moderate glycation induces little alteration on the IgE-binding of β-lactoglobulin, while extensive glycation decreases the binding due to possible masking effects of the epitope[12]. However, the molecular interaction between the lactosylated epitope and the antibody is still unknown.

The aim of the present work is to identify the effects of the thermal treatments on bovine milk whey proteins integrity. As a first step, the presence of the lactosylation sites in different UHT and pasteurized milk samples was investigated to find a correlation with the harshness of the thermal treatment applied. Then, the identified sites of modification were compared with the know epitopes to verify the presence of lysine residues that could be lactosylated and thus could affect the allergic response. Finally, a protocol for the synthesis of β-lactoglobulin epitopes probes in the lactosylated form was developed for future ELISA studies.

2.2 Materials and methods

2.2.1 Reagents

Diethyl ether and Dimethylformamide (DMF) for peptide synthesis were purchased from Carlo Erba Reagents (Milan, Italy). Dichloromethane (DCM), N,N-diisopropylethylamine (DIPEA), Piperidine, Thioanisole, Triisopropylsilane (Tis), Dithiothreitol (DTT), Iodacetamide, Hydrochloric acid, β-lactoglobulin (98% of purity) and

α-lactalbumin (92% of purity) standards, α-chymotrypsin from bovine pancreas, Hydrazine monohydrate and the amino acid Fmoc-Lys(Dde)-OH were purchased by Sigma Aldrich (St. Luis, MO, USA). All the amino acids (Fmoc-Ala-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Ile-OH, Fmoc-Lys(Boc)-OH, Fmoc-Leu-OH, Fmoc-Asn(Trt)-OH, Fmoc-Val-OH, Fmoc-Cys(trt)-OH, Fmoc-His(trt)-OH, Fmoc-Met-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ser(tBu)-OH), N,N,N′,N′-Tetramethyl-O-(1H-benzotriazol-1-yl)uranium hexafluorophosphate (HBTU) and the resin (Rink Amide MBHA resin 100-200 mesh) used for the synthesis were purchased from Novabiochem (Merk KGaA, Darmstadt, Germany). Trifluoroacetic acid (TFA) was purchased from Acros Organics (Thermo Fisher Scientific). Formic acid was purchased from Fisher Scientific (ThermoFisher Scientific, Waltham, MA, USA). The XT sample buffer, XT reducing agent 20x, Protein Standards, Criterion™ XT 12% Bis-Tris precast gel, XT MES running buffer and Coomassie brilliant blue were purchased from BIO-RAD (Hercules, CA, USA). Quant-iT™ Protein Assay kit was purchased from Invitrogen (ThermoFisher Scientific). Doubly deionized water was obtained using a MilliQ system (Millipore, Bedford, MA, USA). HPLC grade Acetonitrile (ACN) was purchased from VWR International (Milan, Italy). Sep-Pak Plus C18 cartridges were purchased from Waters (Milford, MA, USA).

2.2.2 Whey proteins extraction

Different UHT (17) and pasteurized milk (7) samples were collected from the local retailers. A sample of raw milk was provided from a local farm. The extraction of the soluble whey proteins fraction was performed according to the following procedure. The fat part of the milk sample was removed centrifuging at 3220 g for 15 min at 4 °C and removing manually the fat layer from the upper side, the operation was repeated minimum three times. Then caseins were precipitated from the solution adjusting the pH to 4.6 with 0.5M HCl. After centrifugation at 3220 g for 15 min at 4°C, supernatant whey was collected and filtered on 0.45μm filters. Each sample was prepared in duplicate.

2.2.3 UPLC-MS analysis

Whey protein samples were then analysed with UPLC-MS analysis. The quantification of soluble whey proteins was performed using two calibration curves, one for β-lactoglobulin and one for α-lactalbumin (Table 2.2.1), using standards of the two whey proteins. All the samples were analysed in duplicate.

Table 2.2.2 Calibration curves used for protein quantification

	β-lactoglobulin/ α-lactalbumin
Std 1	2 mM
Std 2	1 mM
Std 3	0.5 mM
Std 4	0.25 mM
Std 5	0.125 mM
Std 6	0,0625 mM

An ACQUITY UPLC separation system with an Acquity UPLC© Protein BEH C4 column (300 Å, 1.7 µm, 2.1mm x 150mm) was used to perform the UPLC-ESI-MS analysis. Eluent A was H_2O + 0.1% HCOOH while eluent B was CH_3CN + 0.1% HCOOH. The following steps were used for the gradient elution: isocratic 69% A for 7 min, from 69% A to 64.5% A by linear gradient in 13 min plus washing step at 100% B and reconditioning. Flow rate was set at 0.20 mL/min, sample temperature 18°C, column temperature 35°C, and injection volume 4 µl. Waters SQ mass spectrometer was used for detection with the following conditions: ESI source in positive ionization mode, cone voltage 30V, capillary voltage 3.2 kV, desolvation temperature 300°C, source temperature 150°C, desolvation gas flow (N2): 650 l/h, cone gas flow (N2): 100 l/h. The software used for data processing was MassLynx™ V4.0 (Waters Corporation, Milford MA, USA).

2.2.4 SDS-PAGE analysis

For the SDS-PAGE analysis, the amount of sample needed was determined with the Quant-iT™ Protein Assay (Invitrogen, Thermo Scientific). Samples (nearly 40 µg of protein) were mixed with the XT sample buffer and the XT reducing agent. The marker was prepared

mixing the protein standard, the XT sample buffer and the XT reducing agent. After 5 min at 95°C and 5 min at -20°C, samples were loaded on a Criterion™ XT Bis-Tris precast gel. Using a XT MES running buffer, gels were run for almost 60 min at constant voltage (150V). After the run, gels were stained with a Coomassie Blu solution (50% water MilliQ, 40% methanol, 10% Coomassie Brilliant Blue) for two hours in order to visualize protein bands. Gels were de-stained with a de-staining solution (50% MilliQ water, 40% Methanol, 10% Acetic Acid) for 20 min, repeating the operation for 3-4 times. Gels were then scanned using a GS-800 calibrated imaging densitometer (BIO-RAD).

2.2.5 In solution proteins' tryptic and chymotryptic digestion

Sample (5 mg) was dissolved in 200 µl of 50 mM NH_4HCO_3 and then 10 µl of DTT 200 mM (prepared in NH_4HCO_3 100 mM) was added. After one our at room temperature 8 µl of iodoacetamide 1M (prepared in NH_4HCO_3 100 mM) were added and the solutions were stored at room temperature for one hour at the dark. Then 40 µl of DTT 200 mM was added. After one hour at room temperature chymotrypsin solution (2 mg of enzyme dissolved in 200 µl of NH_4HCO_3 100 mM) is added at the ratio 1:50 enzyme:substrate. Samples were stored at 37°C overnight and then purified through Sep-Pak C18 cartridges using eluent A (98% water, 2% acetonitrile and 0,1% formic acid) and eluent B (65% acetonitrile, 35% water and 0,1% formic acid).

LTQ-Orbitrap analysis was performed with a Jupiter® 4 µm Proteo 90Å column (Phenomenex, 150mmx0.3mm) and a µ-Precolumn™ Cartridge (Acclaim™ PepMap™ 100 C18, 5 µm, 100 Å, 300 µm x 5 mm). Eluent A was H_2O + 0.2% HCOOH and eluent B was CH_3CN + 0.2% HCOOH. For gradient elution it has proceeded as follows: isocratic 90% A for 4 min, from 90% A to 50% A by linear gradient in 56 min plus washing step at 95% B and reconditioning. Column temperature 35°C. Samples were loaded using an enrichment cartridge with a loading flow of 30 µl/min (50% eluent A, 50% eluent B). The acquisition was performed in 5 steps: the first one was in full scan from 250 to 2000 m/z high resolution; from the second to the last step was in data-dependent scan. Fragmentation was performed in LTQ in CID mode and with collision energy 35. The software used was

Xcalibur™ 2.0.7 (Thermo Fisher Scientific). For protein identification, the software used were Peaks® (Bioinformatics solutions Inc, Waterloo ON, Canada) and Proteome Discoverer™ (Thermo Fisher Scientific). Positive hits were arbitrary set for proteins identified with a score expressed as -10logP>20 by the program. All the samples were prepared and analysed in duplicate.

Samples were also analysed with UPLC-MS analysis. It was performed with an ACQUITY UPLC® separation system using an Acquity UPLC© Protein BEH C18 (300Å, 1.7 µm, 2.1mm x 150mm) column with an ACQUITY UPLC® Peptide CSH™ C18 VanGuard™ Pre-column (130Å, 1.7 µm, 2.mm x 5mm). Eluent A was H_2O + 0.1% HCOOH and eluent B was CH_3CN + 0.1% HCOOH. Gradient elution was performed as follows: isocratic 100% A for 7 min, from 100% A to 53.5% A by linear gradient in 40 min, washing step at 100% B and reconditioning. Flow rate was set at 0.20 mL/min, sample temperature 18°C, column temperature 35°C and injection volume 4 µL. Detection was performed as described in paragraph 2.2.3. The software used for data processing was MassLynx™ V4.0 (Waters Corporation, Milford MA, USA). All the samples were prepared and analysed in duplicate. For the identification of modified peptides in the MS spectra, the software Proteomics Toolkit (developed by the Institute for Systems Biology, Seattle, WA, USA) was also used.

2.2.6 Peptide synthesis

Peptides synthesis was performed with the automated standard Fmoc protocol for the Solid Phase Peptide Synthesis (Fmoc-SPPS protocol). Peptide was synthesized using the Syro I automated synthesizer (Biotage). The peptides were obtained as amides at the C-terminus of the sequence loading manually a Rink Amide resin with Leucine as first amino acid for peptides TKIPAVFKIDALNENKVLVL and CLVRTPEVDDEALEKFDKALKAL, with Asparagine for peptides EVDDEALEKFDKALKALPMHIRLSFN, TKIPAVFKIDALNEN and TK(Dde)IPAVFKIDALNEN. The loading of the first amino acid was performed according to the following protocol. Swelling of the resin in DCM for 30 min. Fmoc deprotection with a 20% piperidine solution in DMF. Both these two steps were repeated twice, followed by DMF washes. The coupling reaction was performed suspending the resin in

a DMF solution containing 5 equivalents of the amino acid, 4.8 equivalents of HBTU and 10 equivalents of DIPEA. It was left overnight under agitation then washed 4 times with DMF, 2 with DCM and dried under vacuum. Capping reaction was carried out using a solution of acetic anhydride 1:9 in DMF and, for 10 min under agitation. This step was repeated 2 times followed by DMF washes. Then resin was washed with 5% DIPEA in DMF (5 min under agitation) and 5% DIPEA in DCM (5 min under agitation). After washes with DCM, the resin was dried under vacuum.

Before the automated synthesis, the resin was swelled in DCM (3 times for 10 min). The automated synthesis was planned according to the manufacturer standard protocol. Fmoc deprotection was performed using piperidine 40% in DMF. Amino acidic couplings were performed in DMF with 8 equivalents of DIPEA and 4 equivalents of amino acids and HBTU to the initial loading of the resin. Coupling lasts for 40 min. After each step of deprotection and coupling, several washes with DMF were introduced. At the end, the resin was washed with DCM (3 times for 10 min) and dried under vacuum. Before peptide cleavage from the resin, a final acetylation step was performed. Dry resin was then suspended in a mixture of TFA (95%), Tis (2,5%) and Thioanisole (2.5%) for the cleavage of the peptide from the resin. After 2 hours the solution was recovered and the resin washed three times with TFA and then with Acetonitrile. The final solution was then dried under nitrogen flux. The dried peptide is stored overnight in diethyl ether at -20°C. The peptide was then recovered by centrifugation and washed twice with diethyl ether and dried under vacuum. A small amount of peptide is dissolved in double deionized water with 0,1% of Formic Acid and analysed with UPLC-MS. The analytical column used was an Acquity BEH C18 (Waters, 300A, 1.7um, 2.1mm x 150mm). Eluent A was H_2O+0.1% HCOOH and CH_3CN + 0.1% HCOOH was eluent B. For gradient elution, the following steps were applied: isocratic 100% A for 7 min, from 100% A to 53.5% A by linear gradient in 40 min and 1 min at 53.5% A plus washing step 100% B and reconditioning. Flow rate was set at 0.20 mL/min, sample temperature 18°C, column temperature 35°C and injection volume 4 µl. Detection was performed as described in paragraph 2.2.5.

2.2.7 Peptide purification and quantification

Synthesized peptides were purified with Semi-preparative HPLC (Waters 1525 Binary HPLC Pump with a 998 detector, Waters). The column used was a Jupiter C18 column (250 mm x 10 mm, 300 Å, Phenomenex, Torrance, CA, USA). Eluent A was H_2O+0.1% of TFA, eluent B was CH_3CN+0.1% TFA. The UV absorption spectrum was set at 214 nm, flow rate at 4 mL/min. Eluents gradients were optimised for each peptide. Fractions, that were manually collected, were dried and analysed with UPLC-MS analysis (as described in paragraph 2.2.5) to verify peptides purity. The quantification was performed with HPLC-UV analysis (Waters Alliance 2695 separation module equipped with a dual λ absorbance detector 2487, Waters) using a Jupiter 5 µm C18 (250 x 2.0 mm, Phenomenex). Eluent A was water with 0.1% TFA, eluent B was acetonitrile with 0.1%. For gradient elution, the following steps were applied: isocratic 90% A for 5 min, from 90% A to 40% A by linear gradient in 50 min plus washing step at 100% B and reconditioning. Flow rate was set at 0.20 mL/min, injection volume 10 µl, column temperature 35°C. Detection was performed at 214 nm. Quantification was obtained applying the Lambert-Beer equation and calculating the ε as reported in literature[13].

2.2.8 Conjugation of peptide TKIPAVFKIDALNEN with lactose

Dried peptide was homogenized with lactose (1:8 lysine:lactose ratio) in 10 mM buffer phosphate pH 8. The mixture was dried under nitrogen and heated at 70°C for 48h. Reaction mixture was then characterized with LTQ-Orbitrap high-resolution mass spectrometry as described in paragraph 2.2.5 and with UHPLC-MS/MS analysis as follow. An UHPLC system (Dionex Ultimate 3000, Thermo Scientific, Waltham, MA, USA) with a reverse phase column (Aeris Peptide 1.7 µm XB-C18, 150×2.10 mm, Phenomenex, Torrance, CA, USA) was used. Eluent A was H_2O + 0.2% CH_3CN + 0.1% HCOOH and eluent B was CH_3CN + 0.2% H_2O + 0.1% HCOOH. Flow was set at 0.2 mL/min and the gradient used was: 0-7 min, 100% A; 7-50 min, from 100% A to 50% A; 50-52.6 min, 50% A; 52.6-53 min, from 50% A to 0% A; 53-58.2 min, 0% A; 58.2-59 min, from 0% A to 100% A; 59-72 min, 100% A.

Column temperature was 35°C and sample temperature was 18°C. Injection volume was 2 µL. A triple quadrupole TSQ Vantage (Thermo Scientific, Waltham, MA, USA) was used for detection applying the following parameters: acquisition time: 7-58.2 min, positive ion mode, acquisition range: 100-1,500 m/z, micro scans: 1, Scan Time: 0.50, Q1 PW: 0.70, capillary temperature: 250 °C, spray voltage: 3,200 V, sheath gas flow: 22 units, vaporizer temperature: 250 °C. Depending on the charge and mass of the ion to be fragmented, different collision energies (CE) were applied.

2.2.9 Conjugation of peptide TK(Dde)IPAVFKIDALNEN with lactose

Dried peptide was dissolved in DMF in a Pyrex glass tube. Lactose (1:50 molar ratio with the peptide) was dissolved in DMF and mixed with the peptide. The mixture was flushed under nitrogen and then heated at 70°C for 40h. After cooling down it was added hydrazine to a final amount of 1% in DMF in the solution for the Dde deprotection. After 3 min under stirring, solvent was removed under vacuum. The reaction mixture was washed with doubly deionized water and purified with Sep-Pak C18 cartridges using eluent A (98% water, 2% acetonitrile and 0,1% formic acid) and eluent B (65% acetonitrile, 35% water and 0,1% formic acid). The obtained residue was then analysed with UPLC-MS analysis as described in paragraph 2.2.5. MS ions of the lactosylated peptide identified after the UPLC-MS analysis are reported in table 2.3.9. Proteomics Toolkit (developed by the Institute for Systems Biology, Seattle, WA, USA) was used for the identification of the MS ions.

2.3 Results and Discussion

2.3.1 Milk samples screening

A screening on different samples of milk was performed for this study, collecting seven samples of pasteurized milk and 17 samples of UHT milk from local retailers. One sample of raw milk was collected from a local farm. These samples belong to different brands, some of them to different country of origin. The first step was to identify possible whey proteins in a lactosylated form in the analysed samples

and see if there were any differences within the three types of product (raw, pasteurized and UHT milk). With this aim, soluble whey proteins were extracted from the samples after manual defatting and casein's precipitation at pH 4.6, their isoelectric point. Obtained whey was filtered and analysed with SDS-PAGE analysis. In figure 2.3.1 is showed a SDS-PAGE image obtained from some representative samples.

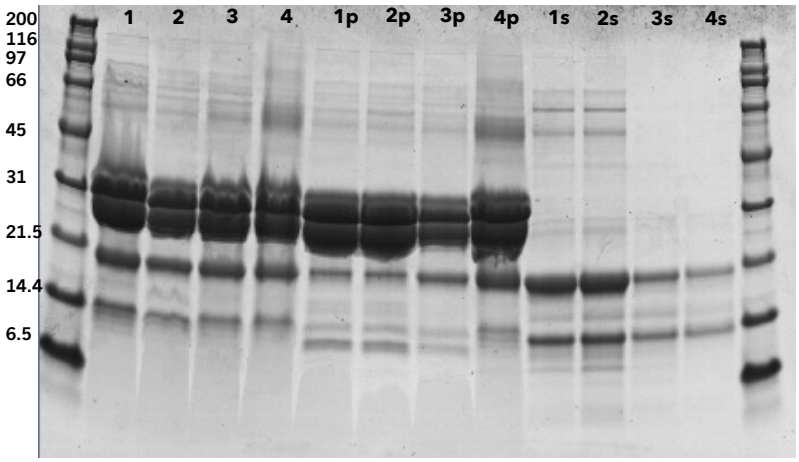


Figure 2.3.1 SDS-PAGE gel image (1,2= pasteurized milk; 3,4=UHT milk; p= precipitated caseins; s= extracted whey). Marker values are expressed as kDa.

The image above shows a comparison between two pasteurized milk samples and two UHT milk samples, the whey extracted and the precipitated fraction. The bands that appear at around 31 kDa correspond to casein's bands. At nearly 18 kDa appears the band of β-lactoglobulin, while at nearly 14 kDa the ones of α-lactalbumin. Comparing the whole milk samples it was not possible to see any differences between them. Looking at the extracted whey it was possible to see that the procedure applied was suitable for the purpose, as caseins seem to be absent. However, in the caseins fractions is possible to observe the presence of a little amount of the two whey proteins.

2.3.2 Whey proteins identification and quantification

In order to identify and quantify whey proteins in the native and lactosylated forms, UPLC-MS analysis was performed applying the

analytical method introduced in Chapter 1[14]. In figure 2.3.2 is represented an example of chromatogram obtained from the analysed whey on a C4 stationary phase.

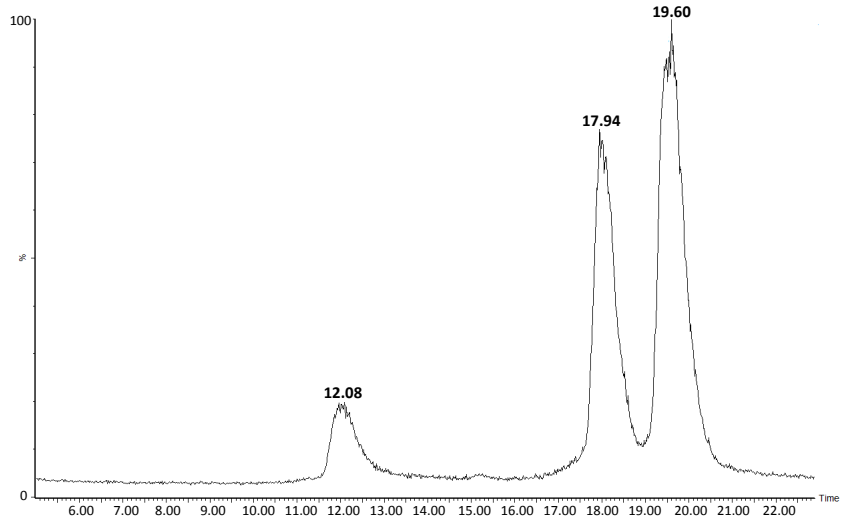
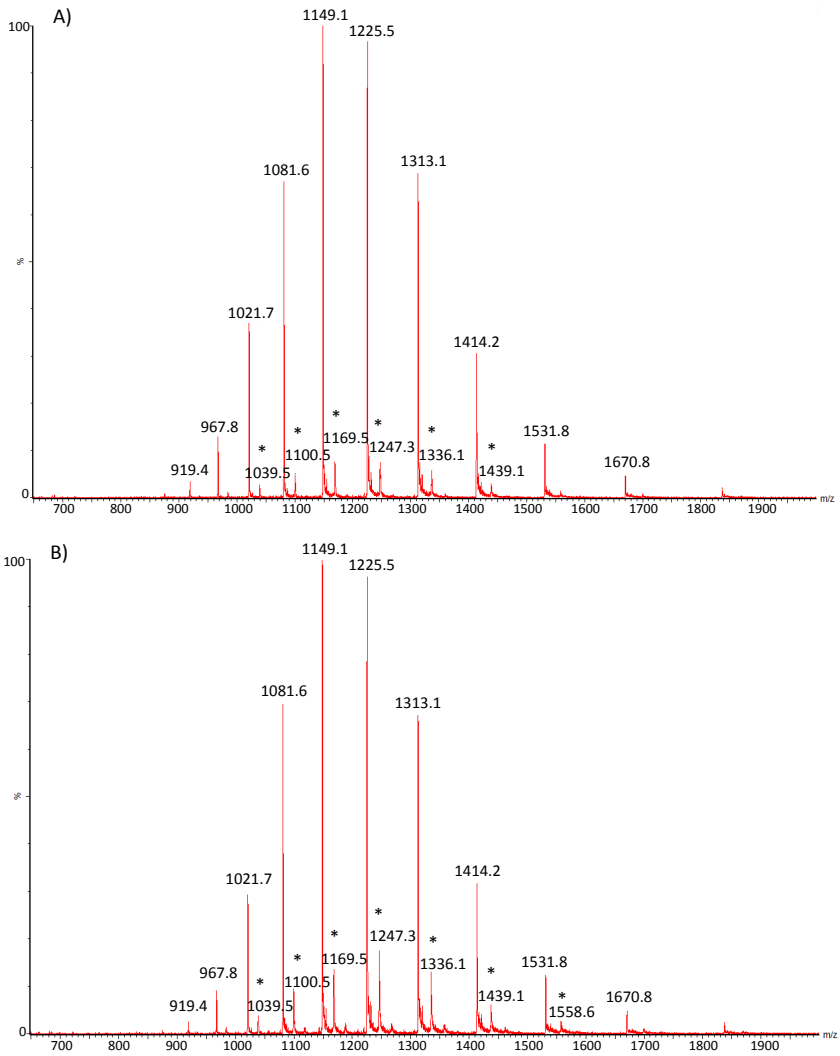


Figure 2.3.2 UPLC-MS chromatogram of extracted whey from pasteurized milk. Intensity is on the y axis, time on the x axis.

From the reported chromatogram is possible to identify the presence of α-lactalbumin at 12.08 min and of β-lactoglobulin isoform B at 17.94 min and of β-lactoglobulin isoform A at 19.60 min. The identification of the two isoforms of β-lactoglobulin was obtained according to the MS spectra. The modified and unmodified forms of these proteins co-elute in the chromatogram, thus it was possible to identify the eventual lactosylated forms of the proteins from the MS spectra. Here follow the MS spectra obtained for β-lactoglobulin isoform A from a pasteurized (B), a UHT (C) and the raw samples (A) (figure 2.3.3).


A)
100
%
0
1149.1
1225.5
1081.6
1313.1
1021.7
1414.2
967.8
1531.8
1169.5
1247.3
1100.5
1336.1
1670.8
919.4
1039.5
1439.1
700
800
900
1000
1100
1200
1300
1400
1500
1600
1700
1800
1900
m/z
B)
100
%
0
1149.1
1225.5
1081.6
1313.1
1021.7
1414.2
1247.3
1169.5
967.8
1336.1
1531.8
1100.5
1439.1
1670.8
919.4
1039.5
1558.6
700
800
900
1000
1100
1200
1300
1400
1500
1600
1700
1800
1900
m/z

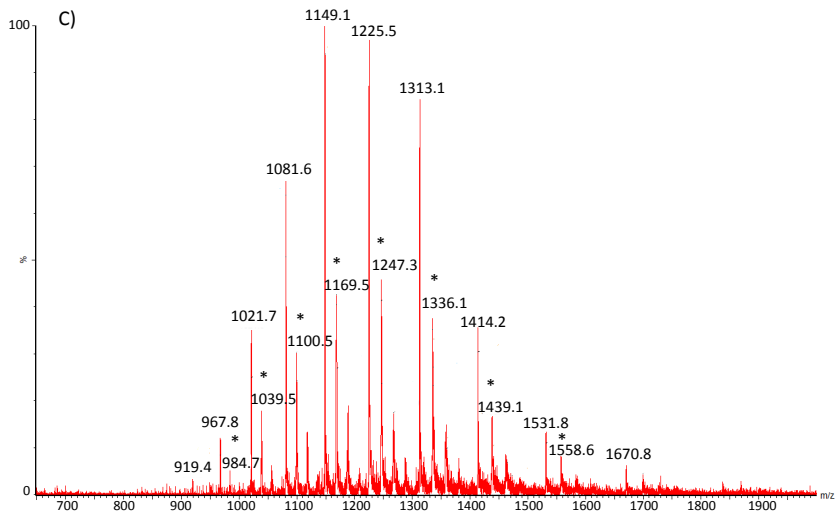


Figure 2.3.3 β-lactoglobulin A MS spectra from the raw sample (A)), the pasteurized sample (B)) and UHT sample (C)). With the star are highlighted the MS ions corresponding to the lactosylated form of the proteins. Intensity is on the y axis, time is on the x axis.

As an example, the MS spectra of the isoform A of β-lactoglobulin are reported. The identification of the lactosylated forms of the whey proteins in the MS spectra was obtained considering an increase of protein's weight of 324 Da (the lactose molecular weight). As it is possible to see in all the three samples the lactosylated form of the protein was identified but the intensity of the MS ion increase with the harshness of the treatment. Indeed, signals are more evident for the UHT treated sample than the pasteurized one. For β-lactoglobulin isoform B and α-lactalbumin the observed trend was the same as the one described. From the images above it's possible to see that a small presence of these modified forms is present in the raw milk too. Indeed, the Maillard reaction normally occurs in food and the temperature only catalyse the reaction. This finding was confirmed from literature, where it was previously reported that in the raw milk some glycated peptides were identified[7,15].

With the external calibration curves for the two whey proteins it was possible to quantify the amount of soluble whey proteins in the samples. By integration of the peaks area were also calculated percentages of native and modified proteins. The areas of the signal

were integrated with the MassLynx™ software from the extracted ions chromatograms (XICs), obtained extracting for each protein the MS ions reported in Table 2.3.1.

Table 2.3.1. List of the whey proteins' MS ions in their unmodified and lactosylated forms (average masses obtained from the MS spectra, molecular weight of the lactosylated form was determined adding 324 Da).

Protein		**MS ions**
β-lactoglobulin	*Isoform B Native form* (18284 Da)	915.2 $[M+20H^+]^{20+}$; 963.2 $[M+19H^+]^{19+}$; 1016.8 $[M+18H^+]^{18+}$; 1076.4 $[M+17H^+]^{17+}$; 1143.8 $[M+16H^+]^{16+}$; 1219.9 $[M+15H^+]^{15+}$; 1306.9 $[M+14H^+]^{14+}$; 1407.2 $[M+13H^+]^{13+}$; 1524.7 $[M+12H^+]^{12+}$; 1663.2 $[M+11H^+]^{11+}$
	Isoform B Lactosylated form (18608 Da)	931.6 $[M+20H^+]^{20+}$; 980.4 $[M+19H^+]^{19+}$; 1034.6 $[M+18H^+]^{18+}$; 1095.5 $[M+17H^+]^{17+}$; 1164.0 $[M+16H^+]^{16+}$; 1241.5 $[M+15H^+]^{15+}$; 1330.3 $[M+14H^+]^{14+}$; 1432.3 $[M+13H^+]^{13+}$; 1551.5 $[M+12H^+]^{12+}$; 1692.7 $[M+11H^+]^{11+}$
	Isoform A Native form (18370 Da)	919.4 $[M+20H^+]^{20+}$; 967.8 $[M+19H^+]^{19+}$; 1021.7 $[M+18H^+]^{18+}$; 1081.6 $[M+17H^+]^{17+}$; 1149.1 $[M+16H^+]^{16+}$; 1225.5 $[M+15H^+]^{15+}$; 1313.1 $[M+14H^+]^{14+}$; 1414.2 $[M+13H^+]^{13+}$; 1531.8 $[M+12H^+]^{12+}$; 1670.8 $[M+11H^+]^{11+}$
	Isoform A Lactosylated form (18695 Da)	935.7 $[M+20H^+]^{20+}$; 984.7 $[M+19H^+]^{19+}$; 1039.5 $[M+18H^+]^{18+}$; 1100.5 $[M+17H^+]^{17+}$; 1169.5 $[M+16H^+]^{16+}$; 1247.3 $[M+15H^+]^{15+}$; 1336.1 $[M+14H^+]^{14+}$; 1439.1 $[M+13H^+]^{13+}$; 1558.6 $[M+12H^+]^{12+}$; 1700.3 $[M+11H^+]^{11+}$
α-lactalbumin	*Native form* (14183 Da)	946.3 $[M+15H^+]^{15+}$; 1013.9 $[M+14H^+]^{14+}$; 1091.9 $[M+13H^+]^{13+}$; 1182.9 $[M+12H^+]^{12+}$; 1290.3 $[M+11H^+]^{11+}$; 1419.4 $[M+10H^+]^{10+}$;

		1576.6 $[M+9H^+]^{9+}$; 1773.7 $[M+8H^+]^{8+}$
	Lactosylated form (14507 Da)	1036.6 $[M+14H^+]^{14+}$; 1116.8 $[M+13H^+]^{13+}$; 1210.0 $[M+12H^+]^{12+}$; 1319.8 $[M+11H^+]^{11+}$; 1451.5 $[M+10H^+]^{10+}$; 1612.9 $[M+9H^+]^{9+}$; 1814.5 $[M+8H^+]^{8+}$

In table 2.3.2 are reported the values obtained after the quantification and percentage determination. Values are reported for each type of milk as ranges of the values obtained in all the samples.

Table 2.3.2 Whey proteins quantification and percentages of lactosylated forms.

	α-lactalbumin		**β-lactoglobulin**	
Milk sample	Total amount of whey proteins (mg/mL)	% of lactosylated forms	Total amount of whey proteins (mg/mL)	% of lactosylated forms
Raw milk	1,2	8	3,04	12
Pasteurized milk (n=7)	0,77-0,88	6-7	1,8-2,1	15-16
UHT milk (n=17)	0,1-0,5	12-36	0,1-0,90	26-52

It was found a higher amount of proteins in the raw milk sample, and it decreases with the intensity of the treatment. This decrease could be due to aggregation and denaturation phenomena that occur catalysed by the temperature applied[16]. Besides, the percentage of lactosylated protein increases with the harshness of the treatment as expected. Indeed, it was previously reported that the glycation degree of proteins increases from raw milk to pasteurized milk and is higher in UHT milk[7,15,17].

2.3.3 Identification of whey proteins' lactosylation sites

In order to identify the lysine residues involved in the binding with lactose an in solution tryptic and chymotryptic digestion was performed. Beside trypsin that is commonly used for this kind of experiments and has specificity for Arginine and Lysine residues, it was decided to use also chymotrypsin that has a different specificity (aromatic amino acids residues) to see if tryptic digestion affects the identification of lactosylated peptides. Since the amount of lactosylated forms was low, it was decided to concentrate the whey through ultrafiltration using a cut-off of 3 kDa. In table 2.3.3 are reported the lysine residues that were found glycated for α-lactalbumin and in table 2.3.4 for β-lactoglobulin.

Table 2.3.3 Glycation sites identified in α-lactalbumin after tryptic and chymotryptic digestion of whey samples from UHT milk.

Residue	Trypsin	Chimotrypsin
K5		Hexose
K13		Lactose/Hexose
K16		Hexose
K58	Lactose	Lactose/Hexose
K62	Lactose	Lactose
K79	Lactose/Hexose	
K93	Hexose	
K94		Lactose
K98	Lactose	
K108		Hexose
K122	Hexose	

Table 2.3.4 Glycation sites identified in β-lactoglobulin after tryptic and chymotryptic digestion of whey samples from UHT samples.

Residue	Trypsin	Chimotrypsin
K14	Lactose	Lactose
K47	Lactose/Hexose	Lactose
K69	Lactose	
K70	Hexose	Lactose
K75	Hexose	
K77	Hexose	
K83	Lactose	Lactose

K91	Lactose	
K100	Hexose	
K101	Hexose	Lactose/Hexose
K135	Lactose	Lactose/Hexose
K138		Lactose/Hexose
K141	Hexose	Lactose

More peptides with a sugar bound to them were found in the digestion performed with chymotrypsin, suggesting that trypsin's activity may be affected by the presence of the sugar on lysine residues. Indeed, from the literature it is expected that the activity of lysine/arginine specific proteases is negatively affected by the presence of the sugar on the side chain that changes the charge and the size of the side chain[18]. From the results, it was possible to see that many of the lysine residues that are present in proteins are involved in the Maillard reaction. This data are in agreement with the literature were the same lysine residues were identified as possible glycation sites[6,7,15]. Concerning α-lactalbumin, in the pasteurized sample it was not possible to identify glycated peptides. This might be ascribed to instrumental limitations due to the low amount of this protein and the low percentage of lactosylation with respect to the UHT samples. Lysine residue K5 was previously reported as a lactosylation site in raw, pasteurized and treated milk[7]. In the present study, it was found glycated in the UHT milk, thus it might be the degradation product of a lactosylated lysine residue were lactose was hydrolysed bound to the protein. With respect to what is reported in literature[6,7,15,19], no peptides with glycated K5 and K114 were found.

Concerning β-lactoglobulin, from the comparison with the literature[7,15,20], it was not observed the glycation of K60 as expected. However, in the present study, it was found K101 glycated in UHT milk, while in literature it was found lactosylated only in raw colostrum or carboxymethylated in traces in heated raw milk[7,20]. From the pasteurized sample, only residues K47, K70, K75 and K101 were found glycated (in particular lactosylated) while in literature are reported more glycation sites in pasteurised milk[7,15]. Even in this case, the identification of fewer glycation sites in the pasteurized sample with respect to what is reported in literature might be ascribed to

instrumental limitations. Moreover, the use of chymotrypsin in the study of lactosylated peptides was demonstrated to be an efficient alternative to other enzymes commonly used.

2.3.4 Identification of lactosylation sites in IgE binding epitopes

Among whey proteins, β-lactoglobulin is the most abundant and it is the first antigen that appears in the human diet, as it is present in bovine milk but absent in human milk[21]. Due to its importance, this study was then focused on this protein. Chymotryptic and tryptic digested samples were analysed with UPLC-MS analysis and results were compared with the ones obtained after the LTQ-Orbitrap analysis. In table 2.3.5 are listed the modified peptides identified from the comparison.

Table 2.3.5 Identified modified peptides deriving from β-lactoglobulin in the chymotryptic and tryptic digestions (in bold is highlighted the modified residue).

Peptide	Modification	MS ions
KIDALNENKVL	Lactosylation	1581 $[M+H]^+$ 791 $[M+2H]^{2+}$ 473.7 $(Y4)^+$ 132.1 $(Y1)^+$
TPEVDDEALE**K**FDK	Lactosylation	980.6 $[M+2H]^{2+}$ 654.1 $[M+3H]^{3+}$
WENGECAQ**K**	Carboxymethyl lysine	1179.5 $[M+H]^+$ 590.4 $[M+2H]^{2+}$
IDALNEN**K**VLVLDTDYK	Lactosylation	1145.0 $[M+2H]^{2+}$ 763.1 $[M+3H]^{3+}$
VLVLDTDY**K**K	Lactosylation	1518.5 $[M+H]^+$ 759.6 $[M+2H]^{2+}$
VRTPEVDDEALE**K**F	Lactosylation	986.8 $[M+2H]^{2+}$ 658.2 $[M+3H]^{3+}$ 166.0 $(Y1)^+$

Carboxymethylation derives from the oxidative cleavage of lactulosyllysine (the Amadori compound)[22]. Obtained data were compared with the known epitopes reported in literature[23]. In Table

2.3.6 are listed the IgE-binding epitopes reported for the two whey proteins.

Table 2.3.6 List of the whey proteins IgE-binding epitopes reported in literature[23].

Protein	IgE-binding epitopes	Reference
α-lactalbumin	**1-19** EQLTKCEVFRELKDLKGYGG **15-34** ELKDLKGYGGVSLPEWVCTAFHTS **45-64** NDSTEYGLFQINNKIWCKDD **60-79** WCKDDQNPHSSNICNISCDK **90-109** MCVKKILDKVGINYWLAHKA **105-123** LAHKALCSEKLDQWLCEKL	Hochwallner et al 2010[24]
	1-16 EQLTKCEVFRELKDLK **13-26** KDLKGYGGVSLPEW **47-58** STEYGLFQINNK **93-102** KKILDKVGIN	Järvinen et al 2001[25]
β-lacto globulin	**1-16** LIVTQTMKGLDIQKVA **56-70** EILLQKWENGECAQKK **76-90** TKIPAVFKIDALNEN **136-150** FDKALKALPMHIRLS	Cong et al. 2012[26]
	58-77 LQKWENGECAQKK **76-95** TKIPAVFKIDALNENKVLVL **121-140** CLVRTPEVDDEALEKFDKAL	Cerecedo et al. 2008[27]
	1-16 LIVTQTMKGLDIQKVA **31-60** LLDAQSAPLRVYVEELKPTPEGDL EILLQK **67-86** AQKKIIAEKTKIPAVFKIDA **127-152** EVDDEALEKFDKALKALPMHIRLSFN	Järvinen et al 2001[25]

From the comparison, it was found that some epitopes, listed in table 2.3.7, present in the sequences lysine residues that could be glycation sites.

Table 2.3.7 β-lactoglobulin IgE-binding epitopes reported in literature[23] that may present lactosylation sites (highlighted in bold).

IgE-binding epitope
TKIPAVF**K**IDALNEN
TKIPAVF**K**IDALNEN**K**VLVL
CLVRTPEVDDEALE**K**FD**K**AL
LE**K**FD**K**ALKALPMHIRLSFN

The presence of lactose bound to IgE-binding epitopes could affect the binding between the antibody and the antigen altering the allergenic response. In this light, it was decided to synthesize the epitopes reported in table 2.3.7 in the unmodified and in the lactosylated form in order to further study the effects on the binding with the IgE with ELISA tests.

2.3.5 Peptide synthesis and lactose conjugation

The four epitopes were synthesised adopting the Fmoc protocol for the Solid Phase Peptides Synthesis described in paragraph 2.2.6. For the synthesis, it was decided to use a Rink Amide resin in order to obtain an amide group at the C-terminal of the sequence. Before cleavage from the resin, it was performed an additional step to acetylate the N-terminal of the sequence. These strategies were adopted in order to obtain peptides that mimic the sequences into the entire protein structure.

Since the cleavage from the resin is performed under acidic conditions that may remove lactose from the peptide, it was decided to perform the lactosylation step after the release from the resin. In literature several procedures for the glycation of whey proteins are reported in studies on the effects of the presence of the sugar on the main protein properties[28–30], while fewer studies are reported for the glycation of peptides[31,32], in particular with lactose. Starting from the literature[18], several attempts were made in order to define the optimal reaction conditions, focusing in particular on the shorter epitope TKIPAVFKIDALNEN, for which the reaction obtained the best yields. Different temperatures were used (50, 60 and 70°C) and the higher yields were obtained at 70°C. Different peptide:lactose ratios were applied (1:4, 1:8, 1:12, 1:16, 1:24) resulting in better yields for the 1:16

ratio. Performing the reaction for 48h seemed to be preferable than 24h. Reaction yields were determined by integration of the chromatographic peaks areas.

Table 2.3.8 Reaction conditions tested for peptide lactosylation

	peptide:lactose molar ratio	**Temperature (°C)**	**Reaction time (hours)**	**Reaction yields***
1	1:4	60	20	27% m.l. / 3% d.l.
2	1:8	60	20	29% m.l. / 3% d.l.
3	1:12	70	48	25% m.l. / 4% d.l.
4	1:16	50	24	31% m.l. / 4 % d.l.
5	1:16	70	48	39% m.l. / 13% d.l.

*m.l.= mono-lactosylated; d.l.= di-lactosylated

Since in the sequence are present two lysine residues that could bind lactose, from the first attempts on this peptide it was always obtained a mixture of the peptide with none, one or two lysine residues glycated (Figure 2.3.4).

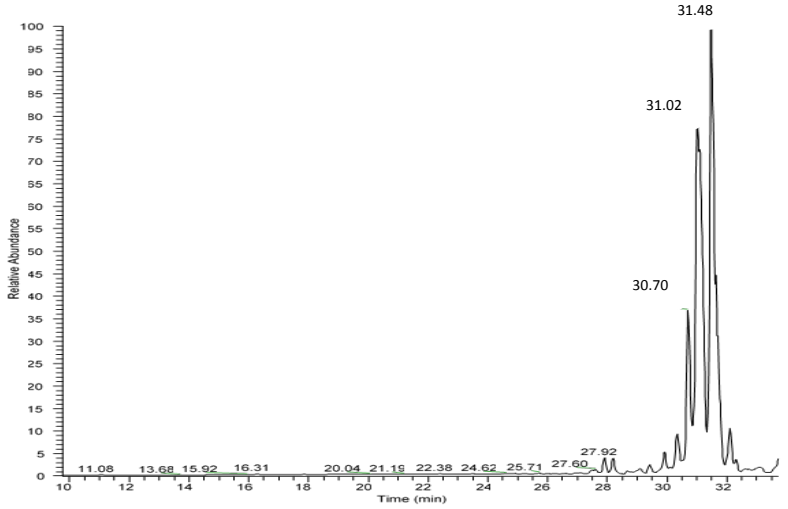


Figure 2.3.4 UHPLC-MS/MS analysis. TIC profile of reaction mixture obtained. The relative abundance is on the y axis, while time is on the x axis.

In the figure above is possible to see a peak at 30.7 min corresponding to the di-lactosylated peptide, the mono-lactosylated peptide at 31.02 min and the unreacted peptide at 31.48 min. Peptide identification

was obtained from the MS spectra. For the modified forms the identification was obtained considering an increase in peptide molecular weight of 324 Da for the mono-lactosylated form and 628 Da for the di-lactosylated form.
The mixture was analysed with LTQ-Orbitrap high resolution mass spectrometry in order to verify which lysine residue was preferably involved in the reaction.

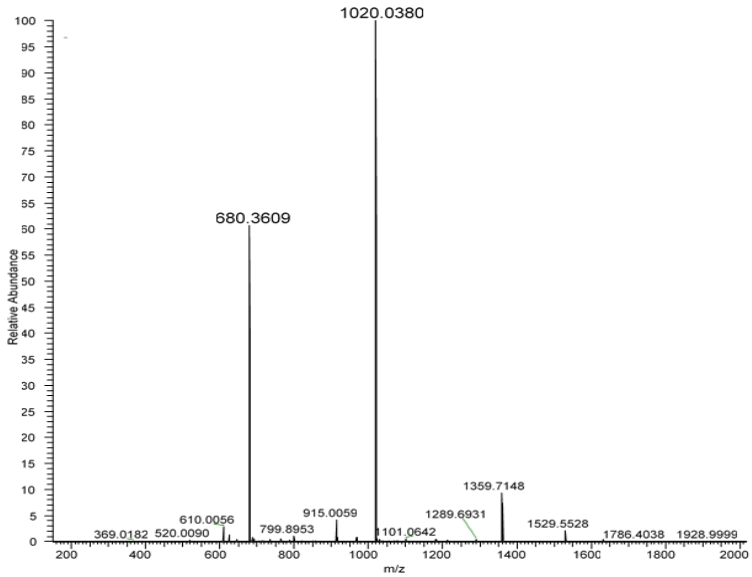


Figure 2.3.5 HRMS full scan spectrum of the mono-lactosylated peptide. Relative abundance is on the y axis, mass-to-charge (m/z) ratio on the x axis.

From the obtained full scan spectrum (Figure 2.3.5) it was possible to identify MS ions corresponding to the mono-lactosylated peptide: 1020.0380 $[M+2H]^{2+}$ and 680.3609 $[M+3H]^{3+}$. The first listed MS ion was then fragmented obtaining the HRMS/MS spectrum in figure 2.3.6.

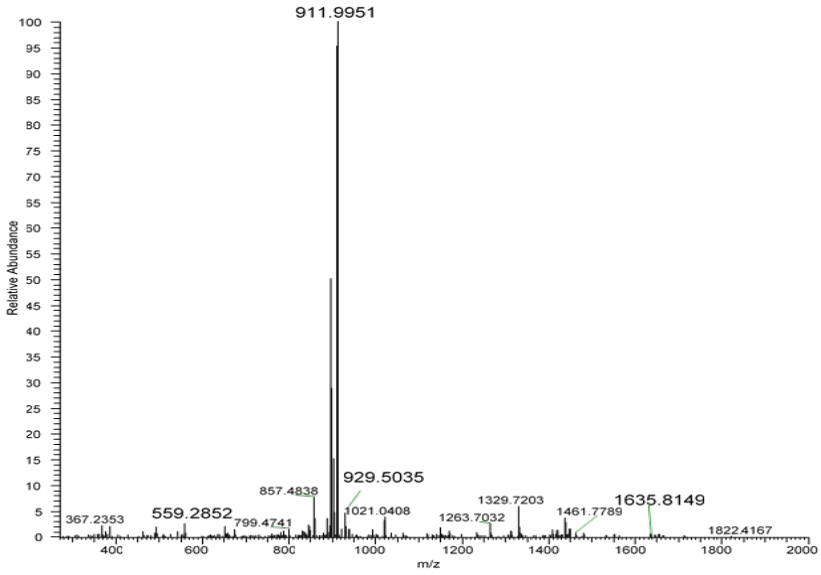


Figure 2.3.6 HRMS/MS spectrum obtained fragmenting the doubly charged ion of the mono-lactosylated peptide. Relative abundance is on the y axis, mass-to-charge (m/z) ratio on the x axis.

From this spectrum, it was possible to identify some fragments with the help of the software Mass Frontier 5.1™ (Thermo Electron Xcalibur®). Here follows the identified fragments and the corresponding MS ion identified (Figure 2.3.7).

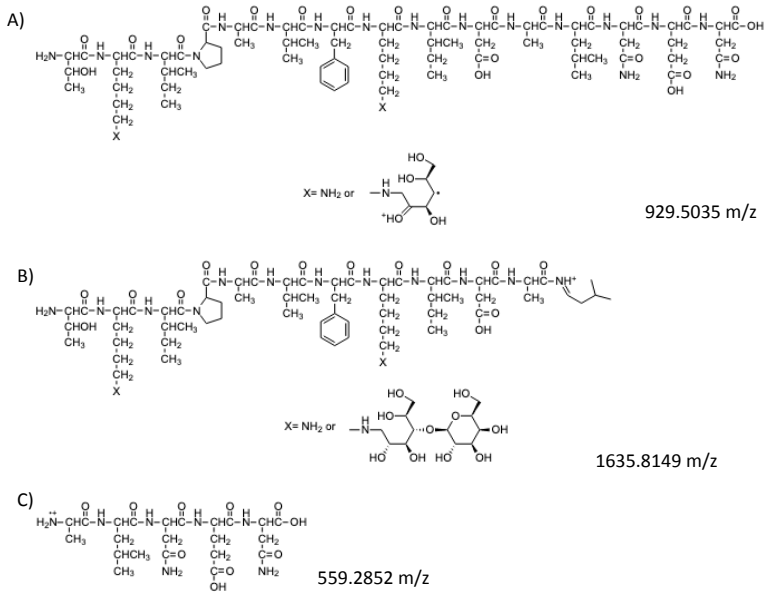


Figure 2.3.7 Peptides fragments identified in the HRMS/MS spectrum.

In the HRMS/MS spectra (figure 2.3.6) the MS ion with higher intensity (911.9951 m/z) was identified as the doubly charged ion of 1635.8149 $[M+H]^+$ ion. The software indicates that this ion correspond to a fragment of the mono-lactosylated peptide (Figure 2.3.8) presenting only one lysine residue.

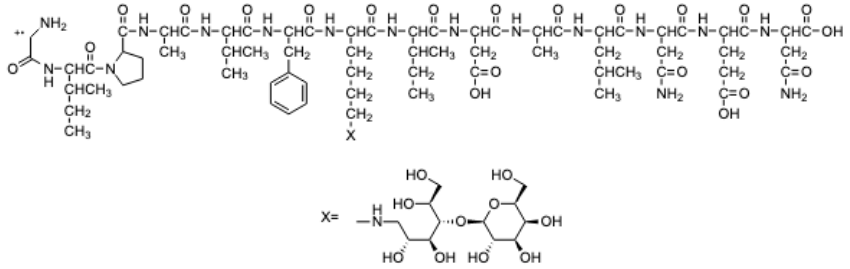


Figure 2.3.8 Peptide fragment identified in the HRMS/MS spectra with only one lysine.

Ion 911.9951 $[M+2H]^{2+}$ was fragmented leading to a spectrum (Figure 2.3.9) in which some fragments (Figure 2.3.10) were identified deriving from the hypothesised peptide (Figure 2.3.8).

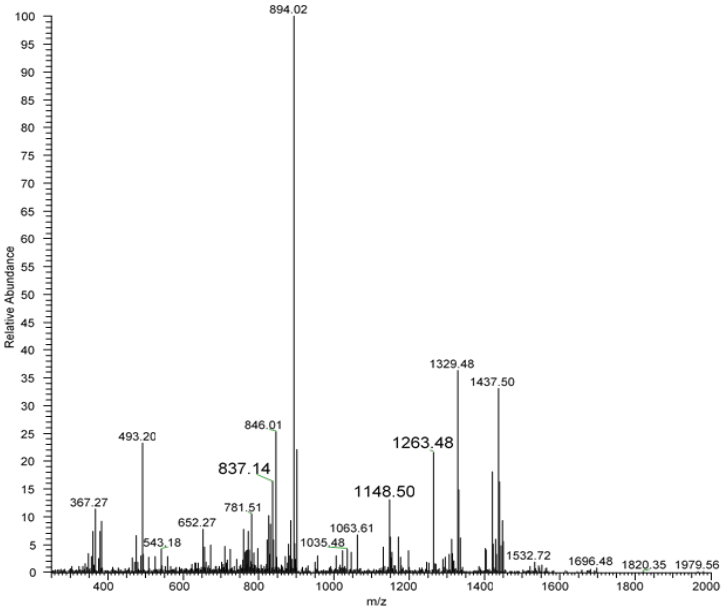


Figure 2.3.9 MS^3 spectrum obtained from the fragmentation of the doubly charged ion identified in the HRMS/MS spectrum. Relative abundance is on the y axis, mass-to-charge (m/z) ratio on the x axis.

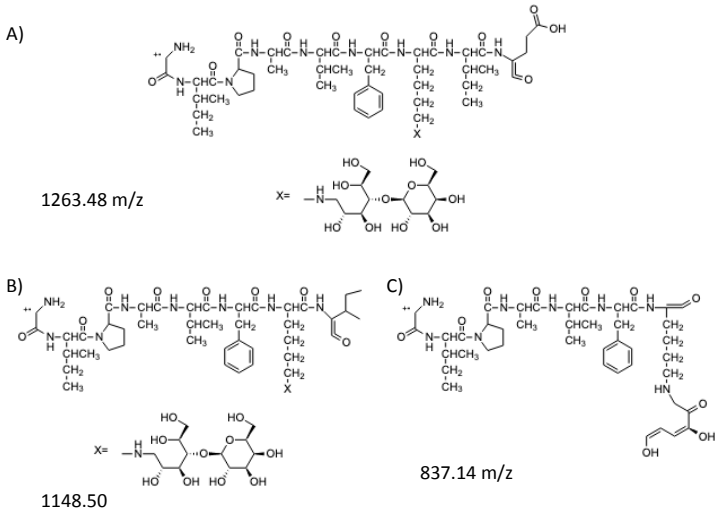
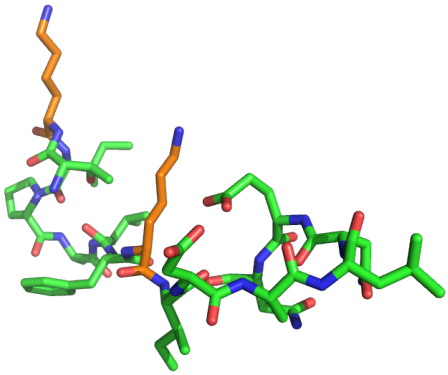


Figure 2.3.10 Fragments of the mono-lactosylated peptide identified in the MS^3 spectrum.

What emerges from these findings is that the lysine that was involved in the binding with lactose was the lysine in the middle of the

sequence, between the residues of phenylalanine and isoleucine. Since the reaction was performed in solution with a large excess of lactose it can be assumed that the above mentioned lysine might be preferred for the lactosylation that the other.
Using the software PyMOL (DeLano Scientific LLC, Schrödinger LLC) it was investigated the 3-D structure of the peptide (Figure 2.3.11).



TKIPAVFKIDALNEN

Figure 2.3.11 3D structure of the peptide in the protein, in orange are highlighted the two lysine residues.

It was found that near to this lysine residue there are hydroxyl and carbonyl groups that might coordinate lactose near to this residue, favouring in this way the binding with this lysine before than with the more external one. This hypothesis suggests a sort of anchimeric assistance in the lactosylation.

With this information then the peptide synthesis was modified in order to obtain only the mono-lactosylated peptide and reactions conditions were optimised. In the SPPS synthesis, the external lysine was protected with a Dde (*N*-[1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl]) protecting group that remains bound to the residue after the cleavage. It was decided to perform the reaction also in DMF[33]. Different reaction conditions were used while optimizing the reaction yields (Table 2.3.10). Reaction yields were determined by integration of the XICs peaks areas before purification (MS ions are reported in table 2.3.9).

Table 2.3.9 MS ion of the synthesised peptide identified from the UPLC-MS spectra.

	MS calculated ions	**MS identified ions**
TK(Dde)IPAVFKIDALNEN *(r. t. 38.9 min)*	1879.15 $[M+H]^{+}$; 940.07 $[M+2H]^{2+}$; 627.05 $[M+3H]^{3+}$; 470.54 $[M+4H]^{4+}$	1878.7 $[M+H]^{+}$; 940.1 $[M+2H]^{2+}$; 627.2 $[M+3H]^{3+}$
TK(Dde)IPAVFK(lact)IDALNEN *(r. t. 38.07 min)*	2203.45 $[M+H]^{+}$; 1102.23 $[M+2H]^{2+}$; 735.16 $[M+3H]^{3+}$; 551.62 $[M+4H]^{4+}$	1102.2 $[M+2H]^{2+}$; 735.2 $[M+3H]^{3+}$; 550.6 $[M+4H]^{4+}$

Table 2.3.10 Reaction conditions tested in the optimization of the site-specific peptide lactosylation.

	peptide/ lactose molar ratio	**Solvent**	**Temperature (°C)**	**Reaction time (hours)**	**Reaction yields***
1	1:12	Dry	70	48	22%
2	1:24	Dry	70	48	19%
3	1:24	Dry	80	48	23%
4	1:50	Dry	70	48	21%
5	1:100	Dry	70	48	42%
6	1:70	Dry	70	48	46%
7	1:25	DMF	70	24	74%
8	1:25	DMF	70	48	61%
9	1:50	DMF	70	4	63%
10	1:50	DMF	70	24	80%
11	1:50	DMF	70	48	90%

Temperatures above 70°C were found too high and induced the deprotection of the peptide. Molar ratio 1:25 peptide lactose in DMF was found too low and molar ratio higher than 1:50 did not induce better yields. Different reaction times were used (4h, 24h, 40h, 48h). After 48h it was observed the presence of the peptide deprotected, thus the best reaction time was found 40h. For the final deprotection of the Dde group, it was used Hydrazine (2% in DMF). At this concentration it was found that lactose may be removed a little, thus it was lowered to 1%. The deprotection was performed in a very short

time to avoid the removal of lactose and just before the removal under vacuum of the solvent from the reaction mixture.

Thus, the mono-lactosylated peptide was obtained with the lactose bound on the desired residue. This optimized procedure for the lactosylation is the one described in paragraph 2.2.8. The developed protocol showed higher yields with respect to what is reported in literature for the on-resin site-specific glycation of peptides with glucose in DMF (35% of yields)[31].

2.4 Conclusions

In this study it was investigated the correlation between the harshness of the treatment with the degree of protein lactosylation. Comparing the data collected after the UPLC-MS analysis from the pasteurized and UHT treated samples, it was possible to confirm the increasing trend in proteins lactosylation due to the harshness of the treatments. After in solution tryptic and chymotryptic digestion, it was possible to identify some lactosylation sites in both α-lactalbumin and β-lactoglobulin. The comparison with the known reported IgE-binding epitopes confirmed the presence of some modified lysine residues in the epitopes. This information is crucial in the study of the effects of this modification on protein allergenicity. Four β-lactoglobulin IgE-binding epitopes were synthesised with Fmoc Solid Phase Peptide Synthesis and a procedure for the *in solution* peptide lactosylation was developed. The analysis of peptide TKIPAVFKIDALNEN lactosylation with LTQ-Orbitrap suggests a sort of preferential site in the sequence for the condensation of lactose with the lysine residue. Thus, it was developed a procedure for the selective lactosylation of a lysine residue. These epitopes synthesised in the unmodified and lactosylated forms will be used for further investigations, with ELISA tests, on the effects of lactose on the binding with human IgE.

References

1. Arena, S., Renzone, G., D'Ambrosio, C., Salzano, A. M. & Scaloni, A. Dairy products and the Maillard reaction: A promising future for extensive food characterization by integrated proteomics studies. *Food Chem.* **219**, 477–489 (2017).
2. Dalabasmaz, S., Dittrich, D., Kellner, I., Drewello, T. & Pischetsrieder, M. Identification of peptides reflecting the storage of UHT milk by MALDI-TOF-MS peptide profiling. *J. Proteomics* **207**, 103444 (2019).
3. Deeth, H. C. & Datta, N. Heat Treatment of Milk: Ultra-High Temperature Treatment (UHT): Heating Systems. *Encycl. Dairy Sci. Second Ed.* 699–707 (2011). doi:10.1016/B978-0-12-374407-4.00216-8
4. van Lieshout, G. A. A., Lambers, T. T., Bragt, M. C. E. & Hettinga, K. A. How processing may affect milk protein digestion and overall physiological outcomes: A systematic review. *Critical Reviews in Food Science and Nutrition* 1–24 (2019). doi:10.1080/10408398.2019.1646703
5. Pellegrino, L., Masotti, F., Cattaneo, S., Hogenboom, J. A. & de Noni, I. Nutritional Quality of Milk Proteins. in *Advanced Dairy Chemistry: Volume 1A: Proteins: Basic Aspects, 4th Edition* (eds. McSweeney, P. L. H. & Fox, P. F.) **1A**, 1–548 (Springer US, 2013).
6. Siciliano, R. A., Mazzeo, M. F., Arena, S., Renzone, G. & Scaloni, A. Mass spectrometry for the analysis of protein lactosylation in milk products. *Food Res. Int.* **54**, 988–1000 (2013).
7. Milkovska-Stamenova, S. & Hoffmann, R. Identification and quantification of bovine protein lactosylation sites in different milk products. *J. Proteomics* **134**, 112–126 (2016).
8. Rutherfurd, S. M. Use of the guanidination reaction for determining reactive lysine, bioavailable lysine and gut endogenous lysine. *Amino Acids* **47**, 1805–1815 (2015).
9. Boekel, M. A. J. S. Van. Effect of heating on Maillard reactions in milk. *Food Chem.* **62**, 403–414 (1998).
10. Fogliano, V. *et al.* Identification of a β-lactoglobulin lactosylation site. *Biochim. Biophys. Acta - Protein Struct. Mol. Enzymol.* **1388**, 295–304 (1998).
11. Villa, C., Costa, J., Oliveira, M. B. P. P. & Mafra, I. Bovine Milk Allergens: A Comprehensive Review. *Compr. Rev. Food Sci. Food Saf.* **17**, 137–164 (2018).
12. Taheri-Kafrani, A. *et al.* Effects of heating and glycation of β-lactoglobulin on its recognition by ige of sera from cow milk allergy

patients. *J. Agric. Food Chem.* **57**, 4974–4982 (2009).
13. Kuipers, B. J. H. & Gruppen, H. Prediction of molar extinction coefficients of proteins and peptides using UV absorption of the constituent amino acids at 214 nm to enable quantitative reverse phase high-performance liquid chromatography-mass spectrometry analysis. *J. Agric. Food Chem.* **55**, 5445–5451 (2007).
14. Buhler, S. *et al.* UV irradiation as a comparable method to thermal treatment for producing high quality stabilized milk whey. *Lwt* **105**, 127–134 (2019).
15. Milkovska-Stamenova, S. & Hoffmann, R. Hexose-derived glycation sites in processed bovine milk. *J. Proteomics* **134**, 102–111 (2016).
16. Qi, P. X., Ren, D., Xiao, Y. & Tomasula, P. M. Effect of homogenization and pasteurization on the structure and stability of whey protein in milk. *J. Dairy Sci.* **98**, 2884–2897 (2015).
17. Wada, Y. & Lönnerdal, B. Effects of Different Industrial Heating Processes of Milk on SIte-Specific Protein Modifications and Their Relationship to in Vitro and in Vivo Digestibility. *J. Agric. Food Chem.* **62**, 4175–4185 (2014).
18. Deng, Y., Wierenga, P. A., Schols, H. A., Sforza, S. & Gruppen, H. Effect of Maillard induced glycation on protein hydrolysis by lysine/arginine and non-lysine/arginine specific proteases. *Food Hydrocoll.* **69**, 210–219 (2017).
19. Meltretter, J., Becker, C. M. & Pischetsrieder, M. Identification and site-specific relative quantification of β-lactoglobulin modifications in heated milk and dairy products. *J. Agric. Food Chem.* **56**, 5165–5171 (2008).
20. Meltretter, J., Wüst, J. & Pischetsrieder, M. Modified peptides as indicators for thermal and nonthermal reactions in processed milk. *J. Agric. Food Chem.* **62**, 10903–10915 (2014).
21. Schuck, P. Dairy Protein Powders. in *Advances in Dairy Ingredients* (eds. Smithers, G. W. & Augustin, M. A.) (John Wiley &Sons, Inc., 2013).
22. Delatour, T. *et al.* Analysis of advanced glycation endproducts in dairy products by isotope dilution liquid chromatography-electrospray tandem mass spectrometry. The particular case of carboxymethyllysine. *J. Chromatogr. A* **1216**, 2371–2381 (2009).
23. Matsuo, H., Yokooji, T. & Taogoshi, T. Common food allergens and their IgE-binding epitopes. *Allergol. Int.* **64**, 332–343 (2015).
24. Hochwallner, H. *et al.* Visualization of clustered IgE epitopes on α-lactalbumin. *J. Allergy Clin. Immunol.* **125**, (2010).
25. Järvinen, K. M., Chatchatee, P., Bardina, L., Beyer, K. & Sampson, H. A.

IgE and IgG Binding Epitopes on α-Lactalbumin and β-Lactoglobulin in Cow's Milk Allergy. *Int. Arch. Allergy Immunol.* **126**, 111–118 (2001).

26. Cong, Y. J. & Li, L. F. Identification of the Critical Amino Acid Residues of Immunoglobulin E and Immunoglobulin G Epitopes in α-Lactalbumin by Alanine Scanning Analysis. *J. Dairy Sci.* **95**, 6307–6312 (2012).
27. Cerecedo, I. *et al.* Mapping of the IgE and IgG4 sequential epitopes of milk allergens with a peptide microarray-based immunoassay. *J. Allergy Clin. Immunol.* **122**, 589–594 (2008).
28. Ter Haar, R., Schols, H. A. & Gruppen, H. Effect of saccharide structure and size on the degree of substitution and product dispersity of α-lactalbumin glycated via the maillard reaction. *J. Agric. Food Chem.* **59**, 9378–9385 (2011).
29. Chevalier, F., Chobert, J. M., Mollé, D. & Haertlé, T. Maillard glycation of β-lactoglobulin with several sugars: Comparative study of the properties of the obtained polymers and of the substituted sites. *Lait* **81**, 651–666 (2001).
30. Chen, Y., Chen, X., Guo, T. L. & Zhou, P. Improving the thermostability of β-lactoglobulin via glycation: The effect of sugar structures. *Food Res. Int.* **69**, 106–113 (2015).
31. Frolov, A., Singer, D. & Hoffmann, R. Sites-specific synthesis of Amadori-modified peptides on solid phase. *J. Pept. Sci.* **12**, 389–395 (2006).
32. Frolov, A., Singer, D. & Hoffmann, R. Solid-phase synthesis of glucose-derived Amadori peptides. *J. Pept. Sci.* **13**, 862–867 (2007).
33. Carganico, S., Rovero, P., Halperin, J. A., Papini, A. M. & Chorev, M. Building blocks for the synthesis of post-translationally modified glycated peptides and proteins. *J. Pept. Sci.* **15**, 67–71 (2009).

Chapter 3

Influence of storage conditions on protein content and integrity of whey protein concentrates

3.1 Introduction

During the food production chain, storage plays an important role in product stability and quality. Trade requires that food must be stored in a suitable way to maintain its quality and shelf life until the final commercialization. Whey protein concentrates and isolates are usually treated to be stored as a dry powder (i.e. through freeze-drying or spray-drying techniques). In the dry state they are more stable in terms of physicochemical reactions, easier to handle and the costs of storage and transportation are lower compared to not dried products[1]. During storage, crucial parameters are temperature, relative humidity, water activity, light and oxygen[2]. For instance, it is known that storage temperature affects protein structure, inducing protein unfolding and denaturation. Aggregation phenomena could occur, both reversible and irreversible, due to intermolecular covalent and non-covalent interaction (i.e. disulphide bonding), affecting protein solubility[3]. Moreover, the presence of the same protein in a denatured, native and aggregated state influences the reaction between lactose and proteins. Elevated storage temperature could induce protein lactosylation, the conjugation between lactose and the proteins' lysine residues, initiating the Maillard reaction. With respect to the native protein, a denatured protein is supposed to have more residues available for lactosylation, while the aggregation reduce them[4]. Protein lactosylation is an important factor relating to nutritional quality. As an example, the nutritional value of the product is affected by lysine lactosylation that reduces the amount of bioavailable lysines[5]. In this context, it is important to determine the right storage conditions to guarantee products' shelf life and quality.

The aim of this chapter is to present the data from a study performed on samples of bovine whey protein concentrates (WPC-35), provided by FrieslandCampina (Amersfoort, The Netherlands). These

samples were stored for different days (up to two weeks) at different temperatures (up to 60°C). The overall aim was to investigate the possible effects of the applied storage conditions on protein content and protein structure. Samples were stored also at 60°C; this temperature was selected as an extreme condition as it is not a normal storage applied temperature. Mass spectrometry and chromatographic techniques were applied to 1) determine the protein content, 2) quantify the soluble protein fraction and 3) identify the possible induced chemical modifications (i.e. lactosylation, oxidation, deamidation, etc). The analysis of the total amino acid profile was then performed to investigate if the modifications induced by the storage conditions affect the nutritional value of the final product.

3.2 Materials and methods

3.2.1 Reagents

Sodium sulfate, Boric acid, Dithiothreitol (DTT), α-chymotrypsin from bovine pancreas, DL-Norleucine, L-Cysteic Acid, Hydroxyproline, Iodacetamide, Hydrobromic acid, 5-methyl tryptophan, Hydrochloric acid, Ammonium hydrogen carbonate (NH_4HCO_3), β-lactoglobulin (98% of purity) and α-lactalbumin (92% of purity) standards were purchased from Sigma–Aldrich (St. Luis, MO, USA). Kjeldahl defoamer was purchased from Merck (Darmstadt, Germany). The XT sample buffer, XT reducing agent 20x, Protein Standards, Criterion™ XT 12% Bis-Tris precast gel, XT MES running buffer and Coomassie Brilliant Blue were purchased from BIO-RAD (Hercules, CA, USA). Quant-iT™ Protein Assay kit was purchased from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA). Doubly deionized water was obtained using a MilliQ system (Millipore, Bedford, MA, USA). Formic acid was purchased from Fisher Scientific (Thermo Fisher Scientific). Amino Acid Standard H was purchased from Thermo Fisher Scientific. SepPack Plus C18 cartridges, AccQ Fluor borate buffer and AccQ Fluor reagent were purchased from Waters (Milford, MA, USA). Sodium hydroxide, HPLC grade Acetonitrile, Copper(II)oxide powder, Sulfuric acid 96%, Hydrochloric acid 0.1N and Methanol were purchased from VWR International (Milan, Italy). Acetic acid and Hydrogen peroxide were purchased from Carlo Erba (Milan, Italy).

3.2.2 Sample preparation

Samples were collected from FrieslandCampina (Amersfoort, The Netherlands), which provided whey protein concentrates that were obtained after bovine cheese production followed by ultrafiltration and freeze-drying. The provided samples were stored for different days (3, 8, 10, 14) at different temperatures (20, 30, 40, 50, 60°C). In addition, also another sample of whey protein concentrates (WPC-35) was obtained, which was spray-dried instead of freeze-dried. All the samples were flushed with nitrogen to minimize oxidation reactions during further storage. To preserve the samples during the whole study, samples were stored at -20°C in nitrogen atmosphere.

Table 3.2.1 Lists of provided samples.

Sample	Storage duration (days)	Storage temperature (°C)	Drying technique
1	3	20	Freeze-drying
2	3	30	Freeze-drying
3	3	40	Freeze-drying
4	3	50	Freeze-drying
5	3	60	Freeze-drying
6	8	20	Freeze-drying
7	8	30	Freeze-drying
8	8	40	Freeze-drying
9	8	50	Freeze-drying
10	8	60	Freeze-drying
11	10	20	Freeze-drying
12	10	30	Freeze-drying
13	10	40	Freeze-drying
14	10	50	Freeze-drying
15	10	60	Freeze-drying
16	14	20	Freeze-drying
17	14	30	Freeze-drying
18	14	40	Freeze-drying
19	14	50	Freeze-drying
20	14	60	Freeze-drying
21	-	-	Spray-drying

3.2.3 Total nitrogen determination

For the total nitrogen determination, and consequently the determination of the protein content, the Kjeldahl instrument was used following the standard protocol, according to the European Regulation EC 152/2009[6]. In short, approximately 300 mg of sample was weighed and poured in digestion tubes among with a catalyst (Sodium Sulfate), a defoamer, a small spoon of Copper(II) oxide, and Sulfuric acid (98%). Tubes were heated for 30 min at 420°C, then cooled to room temperature. Samples were distilled with a solution of sodium hydroxide 35%. A solution of boric acid is used for dissolving the generated ammonia gas into ammonium ions again. After adding 0.1N HCl until the change of colour, the nitrogen content was determined[6]. For these samples a conversion factor of 6.41 was used, representative for whey proteins as reported in literature[7].

3.2.4 Determination of the water content

The water content was determined by loss on drying. Samples were heated at 104°C for 24h to determine the weight loss due to moisture loss. All the analyses were performed in duplicate.

3.2.5 Protein characterization by SDS-PAGE analysis

SDS-PAGE analysis was performed to characterize proteins in the samples. The amount of sample needed was determined with the Quant-iT™ Protein Assay (Invitrogen, Thermo Scientific). Samples (approximately 40 µg of protein) were mixed with the XT sample buffer and the XT reducing agent. The marker was prepared mixing the protein standard, the XT sample buffer and the XT reducing agent. After 5 min at 95°C and 5 min at -20°C, samples were loaded on a Criterion™ XT Bis-Tris precast gel. Using an XT MES running buffer, gels were run for almost 60 min at constant voltage (150V). After the run, gels were stained with a Coomassie Blue solution (50% water MilliQ, 40% methanol, 10% Coomassie Brilliant Blue) for two hours in order to visualize protein bands. Gels were de-stained with a de-staining solution (50% MilliQ water, 40% Methanol, 10% Acetic Acid) for 20 min, repeating the operation for 3-4 times. Gels were then scanned using a GS-800 calibrated imaging densitometer (BIO-RAD).

3.2.6 Whey protein quantification with UPLC-MS analysis

Quantification of whey proteins in the samples was performed with UPLC-MS analysis. For each sample, 2 mg was dissolved in 1 mL of Milli-Q water. The solutions were centrifuged before the analysis. For protein quantification a calibration curve was prepared (Table 3.2.2), using standards of β-lactoglobulin (98% of purity) and α-lactalbumin (92% of purity). All the samples were prepared and analysed in duplicate.

Table 3.2.2 Calibration curve used for protein quantification.

	Concentration β-lactoglobulin (mg/mL)	**Concentration α-lactalbumin (mg/mL)**
Std 1	4	2
Std 2	2	1
Std 3	1	0,5
Std 4	0,5	0,25
Std 5	0,25	0,125
Std 6	0,125	0,0625

UPLC-ESI-MS analysis was performed using an ACQUITY UPLC separation system with an Acquity UPLC© Protein BEH C4 column (300 Å, 1.7 µm, 2.1mm x 150mm). The mobile phase was composed of H_2O + 0.1% HCOOH (eluent A) and CH_3CN + 0.1% HCOOH (eluent B). Gradient elution was performed according to the following steps: isocratic 69% A for 7 min, from 69% A to 64.5% A by linear gradient in 13 min plus washing step at 100% B and reconditioning. Flow rate was set at 0.20 mL/min, injection volume 4 µL, column temperature was 35°C and sample temperature 18°C. Detection was performed using Waters SQ mass spectrometer with the following conditions: ESI source in positive ionization mode, capillary voltage 3.2 kV, cone voltage 30V, source temperature 150°C, desolvation temperature 300°C, cone gas flow (N2): 100 L/h, desolvation gas flow (N2): 650 L/h. The software used for data processing was MassLynx™ V4.0 (Waters Corporation, Milford MA, USA).

3.2.7 In solution protein digestion protocol and mass spectrometry analysis of the obtained peptide mixtures.

For the identification of the possible induced modifications on the amino acid residues due to the storage conditions applied, an in solution chymotryptic digestion was performed. The enzyme was selected due to its specificity for large hydrophobic residues that preserves the lysine residues where the Maillard reaction can occur. 5 mg of sample was dissolved in 400 µL of 50 mM NH_4HCO_3 and then 20 µL of 200 mM DTT (prepared in NH_4HCO_3 100 mM) was added. After one hour at room temperature, 16 µL of 1M iodacetamide (prepared in 100 mM NH_4HCO_3) was added and the solutions were stored at room temperature for one hour in the dark. Then 80 µL of 200 mM DTT was added. After one hour at room temperature 10 µL of chymotrypsin solution (2 mg of enzyme, activity ≥40 units/mg, dissolved in 200 µL of 100 mM NH_4HCO_3, the enzyme is added at the ratio 1:50 enzyme:substrate) was added. Samples were stored at 37°C overnight and then purified through Sep-Pak C18 cartridges using eluent A (98% water, 2% acetonitrile and 0,1% formic acid) and eluent B (65% acetonitrile, 35% water and 0,1% formic acid).

LTQ-Orbitrap analysis was performed with a Jupiter® 4 µm Proteo 90Å column (Phenomenex, 150mmx0.3mm) and a µ-Precolumn™ Cartridge (Acclaim™ PepMap™ 100 C18, 5 µm, 100 Å, 300 µm x 5 mm). The mobile phase was composed by H_2O + 0.2% HCOOH (eluent A) and CH_3CN + 0.2% HCOOH (eluent B). Gradient elution was performed according to the following steps: isocratic 90% A for 4 min, from 90% A to 50% A by linear gradient in 56 min plus washing step at 95% B and reconditioning. Column temperature was 35°C. Samples were loaded using an enrichment cartridge with a loading flow of 30 µL/min (50% eluent A, 50% eluent B). Acquisition was performed in 5 steps: the first one was in full scan from 250 to 2000 m/z high resolution; from the second to the last step was in data-dependent scan. Fragmentation was performed in LTQ in CID mode and with collision energy 35. The software used for data acquisition was Xcalibur™ 2.0.7 (ThermoFisher Scientific). For protein identification, the software used was Peaks® (Bioinformatics solutions Inc, Waterloo ON, Canada). All the samples were prepared and analysed in duplicate.

3.2.8 Total amino acid profile determination

The total amino acid profile was determined by a standard protocol as follows:
Sample (100 mg) was weighed into 18 mL Pyrex glass tubes fitted with Teflon-lined screw caps. 1,2 mL of 6 M HCl was added to each sample and mixed slowly. The tubes were flushed with nitrogen for 1 min in order to remove air. Hydrolysis was then carried out at 110°C for 23 h. After the tubes were allowed to cool at room temperature, the internal standard (1.5 mL of 5 mM Nor-leucine in deionized water) was added. Mixtures were filtered and collected into 50 mL volumetric flasks and brought up to volume with deionized water. Acid hydrolysis was used for the determination of all amino acids except for tryptophan (Trp), cysteine (Cys) and methionine (Met). The calibration curve required was prepared starting from a standard solution obtained mixing in a 1:1 ratio the Amino Acid Standard Mixture 2.5 mM with a mixture of other amino acids (Norleucine and Hydroxyproline in 0.1 N HCl) 2.5 mM, in order to reach the final concentration of 1.25 mM. The calibration curve was obtained preparing the dilutions exposed in Table 3.2.3 in duplicate.

Table 3.2.3 Calibration curve used for protein quantification.

	Concentration (mM)
Std 1	1.25
Std 2	0,625 mM
Std 3	0,3125 mM
Std 4	0,156 mM
Std 5	0,078 mM

According to manufacturer instructions, 10 µL of each hydrolysed sample or standard solution was transferred into a 1.5 mL tube, 70 µL of AccQ Fluor borate buffer were added, in order to keep the optimal pH range for derivatization (8.2–9.7), and the solutions were briefly mixed by vortex. Reconstituted AccQ Fluor reagent (20 µL) was finally added and the mixtures were immediately mixed by vortex. Samples were left for one minute at room temperature, and then heated in a heating bath at 55°C for 10 min. The obtained derivatized solutions

were diluted with 100 µL of deionized water before injecting in the UPLC system. UPLC-ESI-MS analysis was performed using an ACQUITY UPLC® separation system with an Acquity UPLC© Protein BEH C18 (300Å, 1.7 µm, 2.1mm x 150mm) column with an ACQUITY UPLC® Peptide CSH™ C18 VanGuard™ Pre-column (130Å, 1.7 µm, 2.mm x 5mm). Mobile phase was composed by H_2O + 0.1% HCOOH (eluent A) and CH_3CN + 0.1% HCOOH (eluent B). Gradient elution was performed according to the following steps: isocratic 100% A for 7 min, from 100% A to 75.6% A by linear gradient in 21 min plus washing step at 100% B and reconditioning. Flow rate was set at 0.20 mL/min, injection volume 4 µL, column temperature 35°C and sample temperature 18°C. Detection was performed as described in paragraph 3.2.6. The software used for data processing was MassLynx™ V4.0 (Waters Corporation, Milford MA, USA). All the samples were prepared and analysed in duplicate.

The determination of the cysteine and methionine amount was performed as described for the total amino acids determination, but the acid hydrolysis was preceded by performic acid oxidation. Sample (100 mg) was weighed in 18 mL Pyrex glass tubes fitted with Teflon-lined screw cap. After 1 mL of freshly prepared performic acid was added, samples were kept in an ice bath for 16 h. Then 0.15 mL of hydrobromic acid was added in order to remove the excess of performic acid. The bromine formed during the reaction was removed by drying with nitrogen flow. Then the acid hydrolysis using 6 M HCl, all the following steps and the UPLC-MS analysis were performed as described above. The two amino acids were analysed as Cysteic Acid and Methionine Sulfone, their corresponding standards were added to the calibration curve at the final concentration of 1.25mM.

Total Tryptophan amount was determined following a protocol reported in literature[8] with some modifications. Sample (100 mg) was weighed into a 7 mL Pyrex glass tube and dissolved in 3 mL of 4N NaOH. 150 µL of 5-methyl-tryptophan (16 mg/100mL), used as internal standard, were added and mixed. Hydrolysis was then performed at 110°C for 4 h. After letting the tubes cool at room temperature, solutions were carefully acidified to pH 6.5 with 4M HCl. Then, mixtures were diluted to 25 mL with sodium borate buffer (0.1 M, pH 9.0). Samples were centrifuged at 3220 g for 5 min and the

supernatants were filtered through 0.45 μm nylon filter membrane into UPLC vials. Trp losses were corrected by internal standard calculation, where the Trp contents were calculated dividing the area of the peak by the area of the internal standard and multiplying this value by the weight of the internal standard and the response factor of tryptophan. All the samples were prepared and analysed in duplicate. UPLC/ESI-MS analysis was performed using an ACQUITY UPLC separation system with an Acquity UPLC© Protein BEH C18 column (300Å, 1.7 μm, 2.1mm x 150mm). The mobile phase was composed by H_2O + 0.2% CH_3CN + 0.1% HCOOH (eluent A) and CH_3CN + 0.1% HCOOH (eluent B). Gradient elution for Trp determination was performed according to the following steps: isocratic 100% A for 1.8 min, from 100% A to 50% A by linear gradient in 11.4 min and 0.8 min at 50% A plus washing step at 0% A (100% B) and reconditioning. Flow rate was set at 0.25 mL/min, injection volume 4 μL, column temperature 35°C and sample temperature 23°C. Detection was performed as described in paragraph 3.2.6 with SIR acquisition mode at 188,0 and 205,0 for Trp; 202,1 and 219,1 for 5-methyl-tryptophan m/z, scan duration 1s.

3.3 Results and Discussion

The examined whey protein concentrate (WPC-35) is a by-product of cheese manufacturing and is obtained by ultrafiltration. It has a final protein content of 35% (w/w). At the end, it was freeze-dried to reduce the chance of undesired chemical modifications and have an equal starting point for all model heat treatments. Samples were stored for different days at different temperatures (see Table 3.2.1) trying to mimic possible situations affecting them during the steps of storage and transportation, after the production and before commercialization. The only exception is represented by the storage at 60°C. This high value was selected as an extreme storage situation to be used as a reference for exaggerated effect. Also, a sample of whey protein concentrate (WPC-35) was provided that was spray-dried. With the aim of studying if also the drying process influences the final product, this sample was compared with the freeze-dried sample stored for 3 days at 20 °C.

3.3.1 Protein content and composition determination

The bulk analyses performed aimed at verifying the effects of the storage conditions applied mainly on the protein content. The Kjeldahl's data, coupled to the determined moisture content are presented in Table 3.3.1.

Table 3.3.1 Protein content determined with Kjeldahl analysis, based on duplicates with given standard deviation of the duplicate measurements.

Sample	% Protein content	Sample	% Protein content
1	34,57±0,09	**12**	35,76±0,82
2	34,46±0,42	**13**	35,36±0,15
3	35,18±0,04	**14**	35,39±0,11
4	35,26±0,02	**15**	35,82±0,10
5	35,42±0,02	**16**	34,62±0,52
6	35,03±0,19	**17**	35,06±0,35
7	35,09±0,45	**18**	35,36±0,36
8	35,70±0,06	**19**	35,27±0,14
9	35,27±0,04	**20**	35,78±0,10
10	35,15±0,05	**21**	35,08±0,29
11	35,79±0,58		

Results reveal that the protein content was not affected by the storage conditions applied. SDS-PAGE analyses are shown in Figure 3.3.1 and Figure 3.3.2.

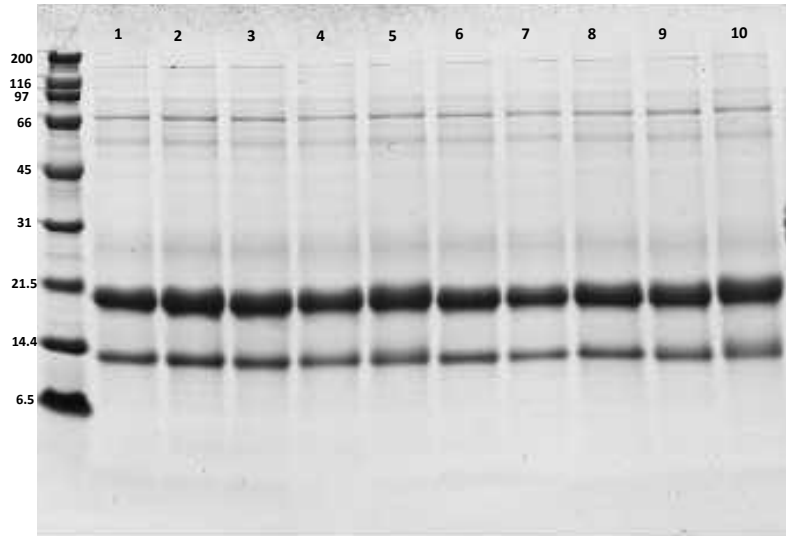


Figure 3.3.1 SDS-PAGE image of the batches of the samples stored for 3 days (samples 1-5, from 20 to 60°C) and 8 days (samples 6-10, from 20 to 60°C). (Marker values are expressed as kDa).

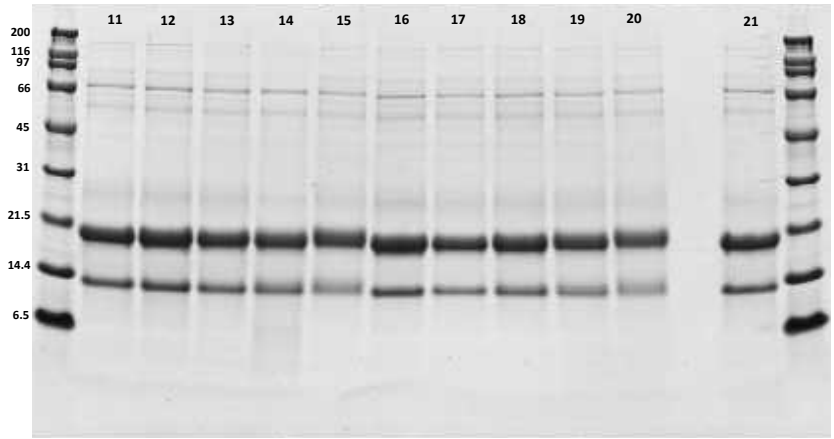


Figure 3.3.2 SDS-PAGE image of the spray-dried sample (sample 21) and the batches of the samples stored for 10 days (samples 11-15, from 20 to 60°C) and 14 days (samples 16-20, from 20 to 60°C). (Marker values are expressed as kDa).

All the samples show the presence of the two main whey proteins, α-lactalbumin at around 14 kDa and β-lactoglobulin at around 18 kDa. While bands corresponding to β-lactoglobulin seem to have no difference in intensity between the gels, it is possible to see a little difference in the α-lactalbumin bands. Looking at the ones relative to

the samples stored for 10 (samples 11-15) and 14 (samples 16-20) days, it is possible to see a decrease in intensity. These could be related to the protein denaturation that occurs due to the increased temperatures applied.

3.3.2 Whey protein quantification

The phenomena of protein aggregation/denaturation and lactosylation, can both affect protein structure influencing their digestibility and allergenicity. UPLC-MS analysis was used to determine the amount of soluble whey proteins and identify their lactosylated forms. The method applied is the one described in Chapter 1[9].

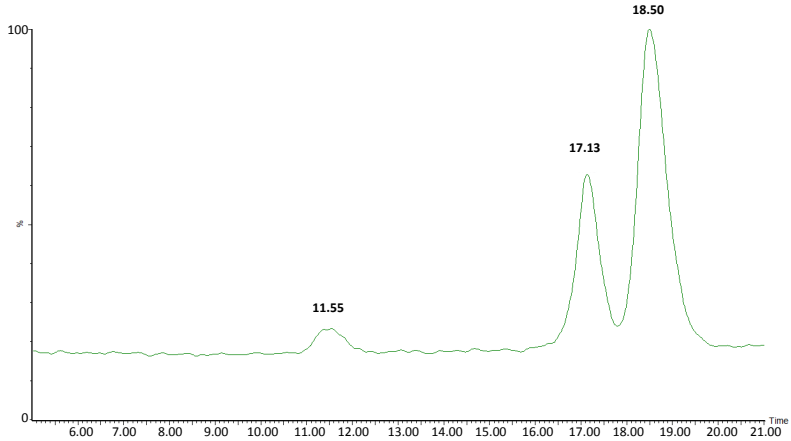


Figure 3.3.3. Example of the UPLC-MS profile of the sample stored for 3 days at 20 degrees. Intensity is shown on the y axis, time on the x axis.

As an example of the obtained UPLC chromatograms, in Figure 3.3.3 is reported one of the samples stored for 3 days at 20°C. As in the other chromatograms, it is possible to identify α-lactalbumin peak at 11,55 min and β-lactoglobulin between 16 and 20 min. Indeed, the peak of β-lactoglobulin is split into two signals relative to the two main isoforms of β-lactoglobulin, isoform A at 18,50 min and isoform B at 17,13 min. The identification of the two isoforms was confirmed with the MS data. These two isoforms differ for two amino acids but they can be well separated with chromatography. On the other side, it is not possible to separate the lactosylated forms of these proteins

chromatographically. The modified forms were identified in the MS spectra. Below are reported the MS spectra of the two whey proteins. Again as an example are shown the ones that belong to the sample stored at 20°C for 3 days.

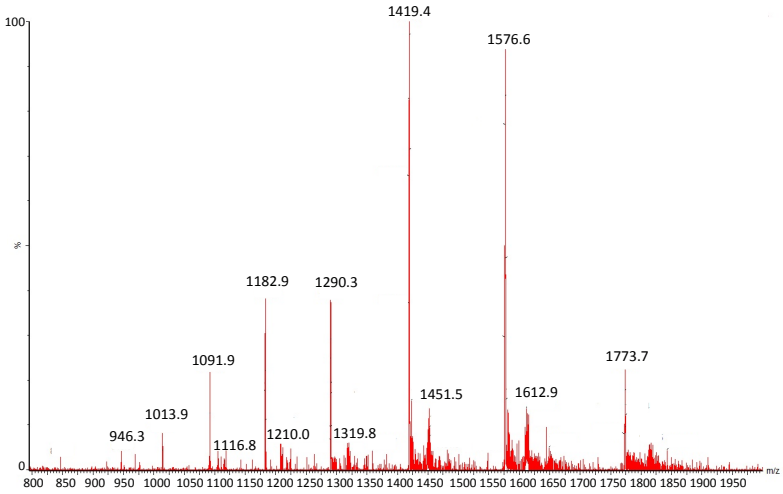
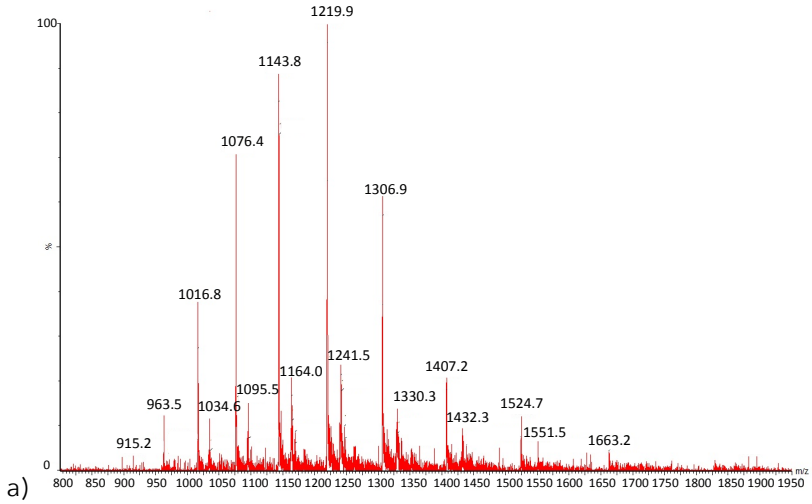


Figure 3.3.4. Example of the UPLC-MS spectrum of α-lactalbumin (corresponding to the peak with retention time 11,55 in the chromatogram). Intensity is shown on the y axis, mass–to-charge ratio (m/z) on the x axis.



a)

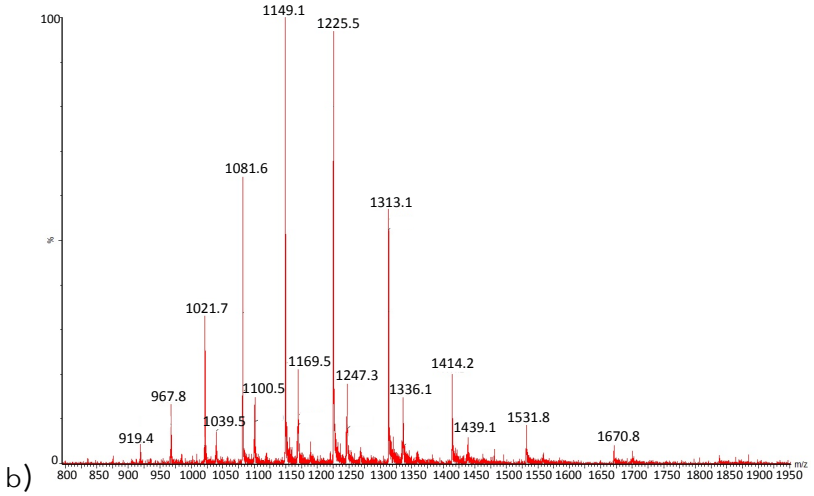


Figure 3.3.5. UPLC-MS spectra of the two isoforms of β-lactoglobulin: β-lactoglobulin B (spectrum a, corresponding to the peak with retention time 17,13 in the chromatogram); β-lactoglobulin A (spectrum b, corresponding to the peak with retention time 18,50 in the chromatogram). Intensity is shown on the y axis, mass–to-charge ratio (m/z) on the x axis.

The identification of the proteins in their different forms was possible according to the MS ions reported in Table 3.3.2.

Table 3.3.2. List of the whey proteins' MS ions in their unmodified and lactosylated forms (average masses calculated from the MS spectra, the molecular weight of the lactosylated forms correspond to the addition of 324 Da for one lactose and 648 Da for two).

Protein		**MS ions**
β-lactoglobulin	*Isoform B Native form* (18284 Da)	915.2 $[M+20H^+]^{20+}$; 963.2 $[M+19H^+]^{19+}$; 1016.8 $[M+18H^+]^{18+}$; 1076.4 $[M+17H^+]^{17+}$; 1143.8 $[M+16H^+]^{16+}$; 1219.9 $[M+15H^+]^{15+}$; 1306.9 $[M+14H^+]^{14+}$; 1407.2 $[M+13H^+]^{13+}$; 1524.7 $[M+12H^+]^{12+}$; 1663.2 $[M+11H^+]^{11+}$
	Isoform B Lactosylated form (18608 Da)	931.6 $[M+20H^+]^{20+}$; 980.4 $[M+19H^+]^{19+}$; 1034.6 $[M+18H^+]^{18+}$; 1095.5 $[M+17H^+]^{17+}$; 1164.0 $[M+16H^+]^{16+}$; 1241.5 $[M+15H^+]^{15+}$; 1330.3 $[M+14H^+]^{14+}$; 1432.3

		[M+13H$^+$]$^{13+}$; 1551.5 [M+12H$^+$]$^{12+}$; 1692.7 [M+11H$^+$]$^{11+}$
	Isoform B Di-lactosylated form (18932 Da)	947.7 [M+20H$^+$]$^{20+}$; 1052.6 [M+18H$^+$]$^{18+}$; 1114.8 [M+17H$^+$]$^{17+}$; 1184.2 [M+16H$^+$]$^{16+}$; 1263.2 [M+15H$^+$]$^{15+}$; 1353.2 [M+14H$^+$]$^{14+}$; 1457.2 [M+13H$^+$]$^{13+}$; 1578.7[M+12H$^+$]$^{12+}$
	Isoform A Native form (18370 Da)	919.4 [M+20H$^+$]$^{20+}$; 967.8 [M+19H$^+$]$^{19+}$; 1021.7 [M+18H$^+$]$^{18+}$; 1081.6 [M+17H$^+$]$^{17+}$; 1149.1 [M+16H$^+$]$^{16+}$; 1225.5 [M+15H$^+$]$^{15+}$; 1313.1 [M+14H$^+$]$^{14+}$; 1414.2 [M+13H$^+$]$^{13+}$; 1531.8 [M+12H$^+$]$^{12+}$; 1670.8 [M+11H$^+$]$^{11+}$
	Isoform A Lactosylated form (18695 Da)	935.7 [M+20H$^+$]$^{20+}$; 984.7 [M+19H$^+$]$^{19+}$; 1039.5 [M+18H$^+$]$^{18+}$; 1100.5 [M+17H$^+$]$^{17+}$; 1169.5 [M+16H$^+$]$^{16+}$; 1247.3 [M+15H$^+$]$^{15+}$; 1336.1 [M+14H$^+$]$^{14+}$; 1439.1 [M+13H$^+$]$^{13+}$; 1558.6 [M+12H$^+$]$^{12+}$; 1700.3 [M+11H$^+$]$^{11+}$
	Isoform A Di-lactosylated form (19019 Da)	951.9 [M+20H$^+$]$^{20+}$; 1002.0 [M+19H$^+$]$^{19+}$; 1057.5 [M+18H$^+$]$^{18+}$; 1119.7 [M+17H$^+$]$^{17+}$; 1189.6 [M+16H$^+$]$^{16+}$; 1268.8 [M+15H$^+$]$^{15+}$; 1359.3 [M+14H$^+$]$^{14+}$; 1464.0 [M+13H$^+$]$^{13+}$; 1585.8 [M+12H$^+$]$^{12+}$; 1730.4 [M+11H$^+$]$^{11+}$
α-lactalbumin	*Native form* (14183 Da)	946.3 [M+15H$^+$]$^{15+}$; 1013.9 [M+14H$^+$]$^{14+}$; 1091.9 [M+13H$^+$]$^{13+}$; 1182.9 [M+12H$^+$]$^{12+}$; 1290.3 [M+11H$^+$]$^{11+}$; 1419.4 [M+10H$^+$]$^{10+}$; 1576.6 [M+9H$^+$]$^{9+}$; 1773.7 [M+8H$^+$]$^{8+}$
	Lactosylated form (14507 Da)	1036.6 [M+14H$^+$]$^{14+}$; 1116.8 [M+13H$^+$]$^{13+}$; 1210.0 [M+12H$^+$]$^{12+}$; 1319.8 [M+11H$^+$]$^{11+}$; 1451.5 [M+10H$^+$]$^{10+}$; 1612.9 [M+9H$^+$]$^{9+}$;

		1814.5 $[M+8H^+]^{8+}$

After the identification, from the total ion chromatograms (TICs) it was possible to determine the total amount of whey protein in the samples, the amount of unmodified proteins and by difference the amount of lactosylated forms. The areas of the signal were integrated with the MassLynx™ software. For the unmodified and lactosylated proteins**,** extract-ion chromatograms (XICs) were obtained extracting the characteristic MS ions (Table 3.3.2). For the extraction of the XICs eight ions of the sequences were used, for the proteins for which we were able to identify more MS ions the eight ions in the middle of the sequence were chosen. As an example, in figure 3.3.6 is reported the XICs for β-lactoglobulin B in Sample 1.

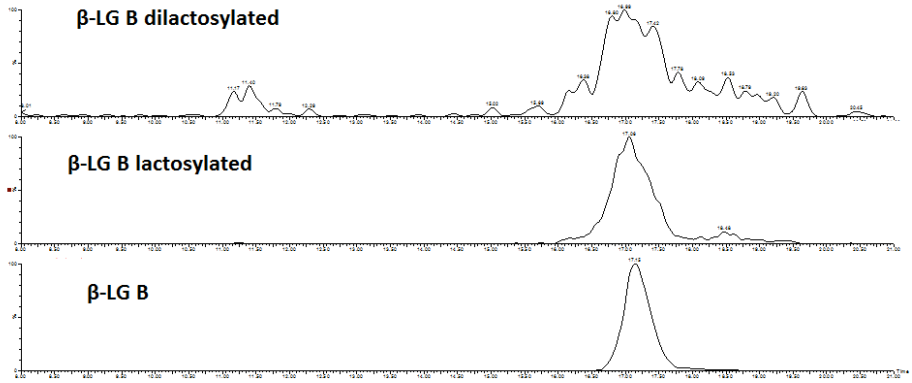


Figure 3.3.6 XICs of the native and modified forms of β-lactoglobulin B in Sample 1.

The quantification was performed using an external calibration curve (Table 3.2.2) and setting the value of the intercept as zero. The two isoforms of β-lactoglobulin were quantified together assuming an identical response factor. In Figures 3.3.7-9 the amount of protein is expressed as percentage on sample concentration.

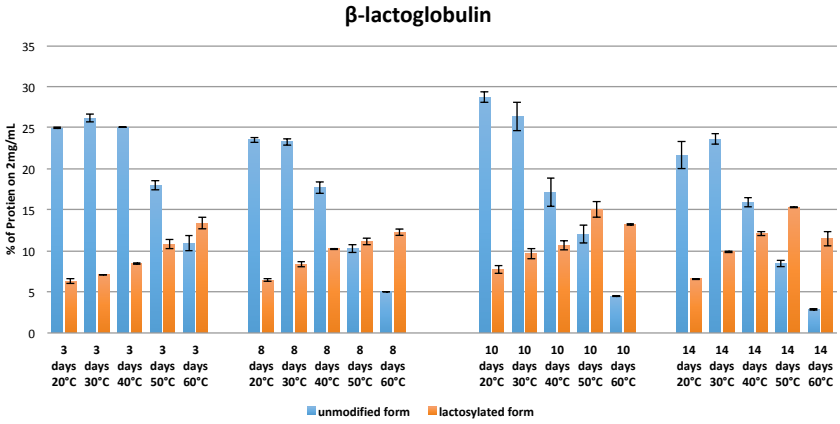


Figure 3.3.7 Percentages of β-lactoglobulin in the native (blue bars) and lactosylated forms (orange bars) based on starting concentration.

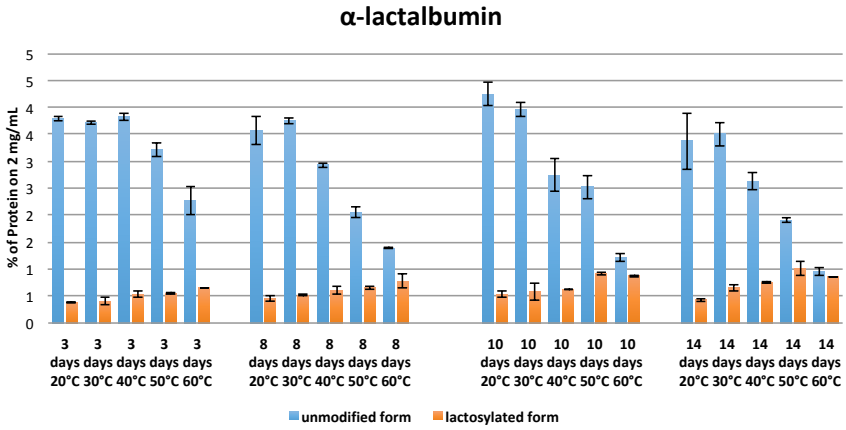


Figure 3.3.8 Percentages of α-lactalbumin in the native (blue bars) and modified forms (orange bars) based on starting concentration.

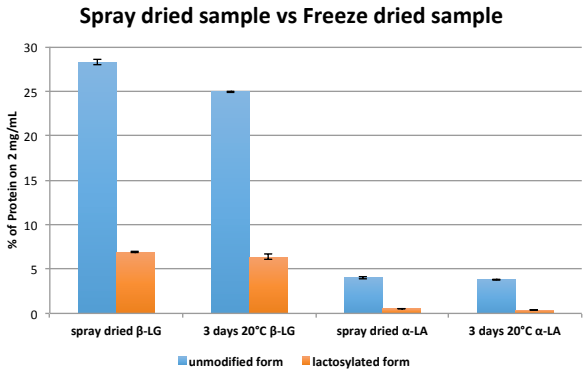


Figure 3.3.9 Comparison between the spray-dried sample and the freeze-dried one stored for 3 days at 20°C. The blue bars represent the native forms, the orange bars the modified forms.

Temperature plays a crucial role during storage; increased temperatures can induce aggregation/denaturation phenomena and catalyse the Maillard reaction favouring the binding between lactose and lysine residues in proteins[10,11]. Both these effects are present in the examined samples. Looking at figure 3.3.7 and figure 3.3.8, it is possible to see a decrease in the total amount of whey proteins due to the high temperatures applied, suggesting the formation of aggregates and protein denaturation that decrease the soluble proteins content. The catalytic effect of temperature on aggregation/denaturation phenomena was previously demonstrated by several authors. As an example, in the storage of whey protein isolates (WPI), it was observed that powders are stable up to 15 months at 20°C while storing at 40°C induced a decrease in the native content after 3 months[11]. An additional example is the storage on four different types of whey which showed a dependence of the aggregation with the temperature applied, with the highest effect at 45°C[3].

In the presents results, the major effects were obtained for samples stored at 50 and 60°C, observing effects for short storage times (up to 14 days). These findings are in agreement with the data demonstrating that the amount of native protein was half decreased in 7 days of storage at 80°C[12].

Moreover, unmodified protein content decreases with temperature while the lactosylated protein content increases, as

expected due to the catalytic action of elevated temperature on the Maillard reaction. Indeed, it was previously reported that storing WPIs for 15 months at 20 °C induced an increase of 27% in the lactosylated protein, while the storage at 40°C induced an increase of 30% in 15 days of storage[11]. Furthermore, storage of skim milk powders at +37°C showed a higher extent of lactosylation in two weeks with respect to powders stored at room temperature, while an advanced state of the Maillard reaction was observed when storing samples at +52°C for 3.5 weeks[13].

Defining the effects of drying is more difficult as the product in the liquid state usually undergoes other treatments that may induce changes in proteins. As an example, lower rates of denaturation were observed in dried samples with respect to liquid products[10,14]. In this study, a very little difference in the total amount of soluble protein was observed comparing products processed with freeze-drying or spray-drying (Figure 3.3.9), meaning that the different technique applied does not influence much protein aggregation/denaturation. Indeed, it was found that dry heating on whey proteins induces little changes in the secondary and tertiary structure and the aggregation was limited to nearly 30% of proteins. The size of the aggregates was small (mainly oligomers and dimers), probably due to the proteins' low translational motion in powders[15]. Heating has a higher influence on the induction of chemical modifications on the amino acids (i.e. oxidation and glycation)[10,16]. As an example, it was found that drying induced a decrease in available lysine in raw milk samples (11.77%) and in skim milk powder (14.27%), due to the effects of the Maillard reaction[17].

To better understand the effect on lactosylation, its degree (in percentage) was determined by integration of the peak areas. All the modified forms were identified in the spectra by the sequences of MS ions reported in Table 3.3.2.

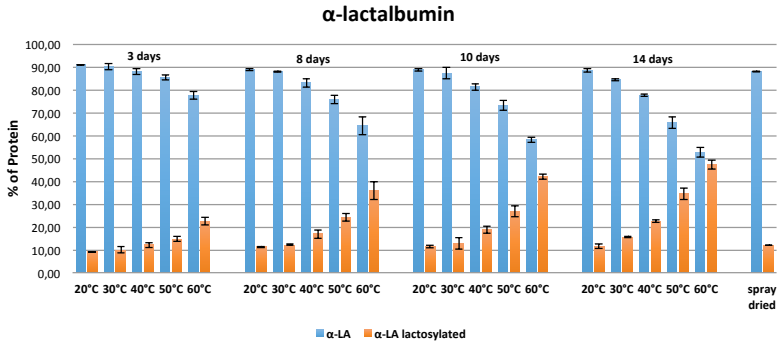


Figure 3.3.10 Relative amount of native (blue bars) and lactosylated proteins (orange bars). Samples are grouped by days of storage.

In figure 3.3.10 and 3.3.11 it's possible to visualize the expected increase in the amount of lactosylated forms due to the temperatures applied.

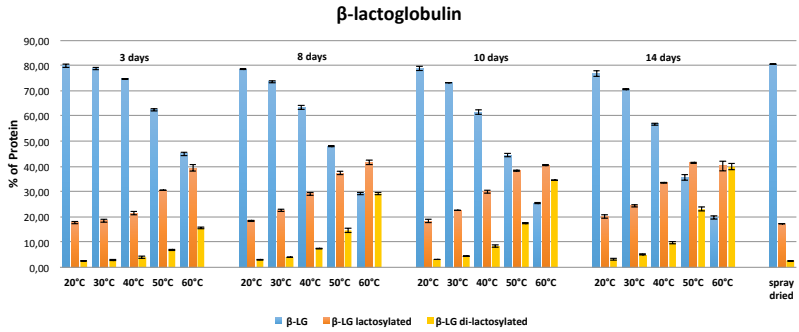


Figure 3.3.11 Relative amount of native (blue bars), lactosylated (orange bars) and di-lactosylated proteins (yellow bars). Samples are grouped by days of storage.

For β-lactoglobulin it was possible to identify also a di-lactosylated form that shows the same increasing trend with higher temperatures of the lactosylated one. Looking singularly to the two isoforms of this whey protein, A and B, they showed the same trend with no differences among them, meaning that there is no difference in the lactosylation degree between the two isoforms.
Comparing the spray-dried sample with the freeze-dried one (3 days 20°C) there is very little difference, meaning that the spray dry technique applied does not alter much the sample with respect to the

freeze-drying technique concerning the lactosylation. Indeed, the extent of lactosylation depends on the parameters that were set for the spray-drying. Critical parameters are the inlet and outlet temperatures of the spray dryer and the combination of the two influences the extent of lactosylation. Currently, it has been demonstrated that the temperature of the spray dryer has a catalytic effect on protein lactosylation[13]. The extent of lactosylation might be reduced changing these temperatures, with low outlet one and inlet one low enough to reduce lactosylation and high enough to obtain a high drying rate[13]. In this light, a possible explanation for present results is that the parameters used for the spray-drying of the WPC-35 were low enough to induce a protein lactosylation comparable with the one observed in the freeze-dried samples.

In figure 3.3.12 percentages of proteins are grouped by temperatures to see the effects of storage duration.

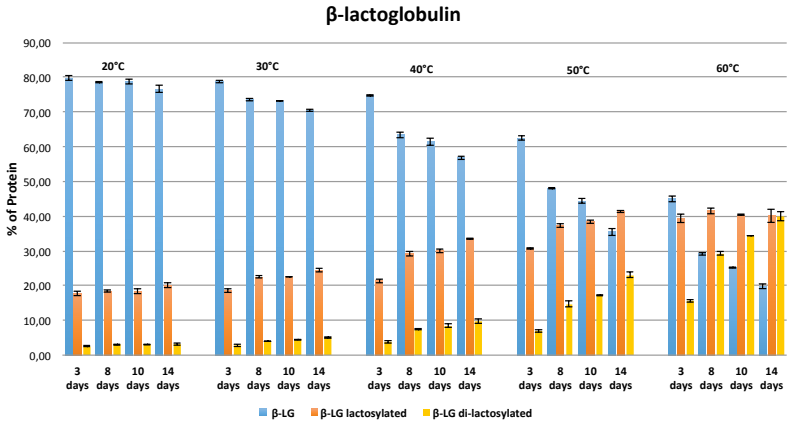


Figure 3.3.12 Relative amount of native (blue bars), lactosylated (orange bars) and di-lactosylated (yellow bars) proteins. Samples are grouped by temperature of storage.

From the figure, it is possible to observe a little effect of the storage duration on protein lactosylation with increased lactosylation upon longer storage. Similar results were obtained for both α-lactalbumin and β-lactoglobulin. The Maillard reaction naturally occurs in food and temperature plays only a catalytic role. Indeed studies on storage of

skim milk powders at room temperature observed a little increase of protein lactosylation in particular after 8 weeks and, as mentioned above, the storage at 20°C of WPIs induces a 27% of lactosylation in 15 months[11,13]. Present findings demonstrate that when samples are stored at higher temperatures it is possible to see a higher decrease in native protein with the days of storage. This finding is mainly caused by the accelerating effect of temperatures on the lactosylation mentioned above.

3.3.3 Identification of the post-translational modifications

Beside lactosylation, the storage conditions could induce also some chemical modifications that could affect the nutritional value of the product. The main chemical modifications that can be induced are well studied, in particular the ones induced by the high temperatures[18]. Among them are the ones depicted in figure 3.3.13.

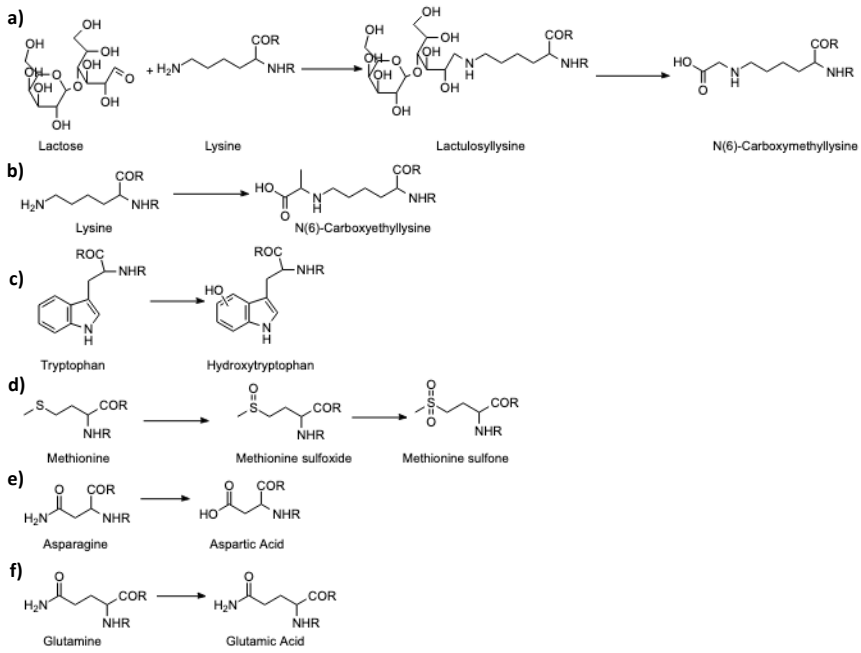


Figure 3.3.13 Possible chemical modifications induced by heating: a) Lysine lactosylation that leads first to the formation of lactulosyllysine then to AGE products such as Carboxymethyllysine; b) Carboxyethyl lysine formation, trough thermal or enzymatic activity; c) Tryptophan oxidation; d) Methionine oxidation; e) Asparagine deamidation; f) Glutamine deamidation.

The modification principally occurs on residues of Trp, Met, asparagine (Asp), glutamine (Gln), lysine (Lys). In solution chymotryptic digestion was performed on all the samples following the protocol described in paragraph 3.2.7. Positive hits for protein identification was arbitrary set for all those proteins identified by the program with a score expressed as -10logP>50.

In table 3.3.3 is reported, as an example, the list of the most abundant proteins identified in Sample 1. Same results were observed for all the samples with little differences in the coverage and number of peptides. With this analysis, it was possible to identify some proteins in the samples, which apparently were absent in the previous SDS-PAGE analysis (i.e. caseins). These analyses are qualitative, so it was possible only to determine their presence.

Table 3.3.3 List of the most abundant bovine milk proteins identified in Sample 1.

Proteins	Uniprot ID	Sequence Coverage (%)	Number of peptides
β-lactoglobulin A	B5B0D4	76	83
β-lactoglobulin B	G5E5H7	76	84
α-lactalbumin	P00711	85	39
β-casein	P02666	82	44
A-S_1-casein	P02662	43	12
k-casein	A0A140T8A9	48	13
BSA	A0A140T897	48	29
Glycam 1	P80195	51	14

With the same software, it was possible to investigate the presence of post-translational modifications (PTMs) in the β-lactoglobulin and α-lactalbumin generated peptides. The peptides with PTMs identified in the samples are reported in Table 3.3.4 and Table 3.3.5.

Table 3.3.4 List of β-lactoglobulin's modified peptides identified in all the samples after the chymotryptic digestion (in bold is underlined the modified residue).

Modification	**Peptide**
Tryptophan oxidation (+15.99 Da)	KGLDIQKVAGT**w**Y
	KGLDIQKVAGT**w**
	DIQKVAGT**w**
	DIQKVAGT**w**Y
Methionine oxidation (+15.99 Da)	SLA**m**AASDISLL
	LIVTQT**m**
	A**m**AASDISLL
	C**m**ENSAEPEQSL
	LIVTQT**m**KGL
Asparagine deamidation (+0.98 Da)	KIDAL**n**ENKVL
	SF**n**PTQLEEQCHI
Glutamine deamidation (+0.98 Da)	SFNPTQLEE**q**CHI
	VEELKPTPEGDLEILL**q**
Carboxy ethyl lysine (+72.02 Da)	VRTPEVDDEALE**k**
Hexose (+162.05 Da)	KIDALNEN**k**VL
Carboxy methyl lysine (+58.01 Da)	ENGECAQK**k**IIAEKTKIPAVF
	VEEL**k**PTPEGDLEILLQ
	VEEL**k**PTPEGDLEILL
	VRTPEVDDEALE**k**
	VEEL**k**PTPEGDLEIL

Table 3.3.5 List of α-lactalbumin's modified peptides identified in the sample after the chymotryptic digestion (in bold is underlined the modified residue).

Modification	**Peptide**
Tryptophan oxidation (+15.99 Da)	KILDKVGINY**w**
	ILDKVGINY**w**
	QINNKI**w**
Methionine oxidation (+15.99 Da)	NICNISCDKFLDDDLTDDI**m**
	NISCDKFLDDDLTDDI**m**
	ISCDKFLDDDLTDDI**m**
Asparagine deamidation (+0.98 Da)	QI**n**NKIW
	QIN**n**KIW
	SSNIC**n**ISCDKFLDDDLTDDIM

	nISCDKFLDDDLTDDIM
Glutamine deamidation (+0.98 Da)	**q**INNKIW

The PTMs identified in the samples are reported below. Results are shown separately for the two whey proteins. PTMs that were found only in one sample or replicate were not considered. Figure 3.3.14 shows bovine β-lactoglobulin isoform B sequence with underlined the amino acids where modifications were found.

```
  4          14          24          34          44          54
LIVT QTMKGLDIQK VAGTWYSLAM AASDISLLDA QSAPLRVYVE ELKPTPEGDL
        64          74          84          94          104         114
EILLQKWENG ECAQKKIIAE KTKIPAVFKI DALNENKVLV LDTDYKKYLL FCMENSAEPE
      124         134         144         154
QSLVCQCLVR TPEVDDEALE KFDKALKALP MHIRLSFNPT QLEEQCHI
```

Figure 3.3.14 Amino acid sequence of bovine β-Lactoglobulin B (Uniprot code P02754, signal peptide sequence was removed). Amino acid residues modified identified in all the samples (**M**= methionine oxidation; **W**= tryptophan oxidation, **K**= carboxymethyl lysine; **Q**= glutamine deamidation; **N**= asparagine deamidation; **K**= carboxyethyl lysine; **K**= hexose).

From the obtained results some consideration can be drawn:
- Methionine oxidation at residues M7 and M24 were found in all the samples suggesting that this modification cannot be related to the storage conditions applied. Indeed, methionine oxidation to methionine sulfoxide may be an enzymatic post-translational modification[18]. Thus, the identification of this modification in all the samples may be the result of enzymatic activity prior processing. M107 was found oxidized in samples stored for 10 days at 50°C and 14 days at 40°C, suggesting the effect of storage at elevated temperatures. Since this modification was detected only in this sample it is not possible to use it as a thermal indicator.
- Tryptophan oxidation is known to be an indicator of heating but it was found in all the samples suggesting that it could derive from enzymatic processes[18].
- Asparagine and Glutamine deamidations were identified in the samples but it was not possible to see a trend in the samples that could indicate the effects of temperature or duration of storage as

expected. In previous research, storage up to two months of UHT milk showed that the deamidation of asparagine is influenced by the storage conditions applied[19].

- Some modifications due to the Maillard reaction (carboxyethylation, carboxymethylation and glycation) were identified in the samples and seem to be related to the temperature applied, as expected.

Carboxymethylation is the product of the oxidative cleavage of the lactulosyllysine (the Amadori compound, first intermediate of the Maillard reaction) and is commonly used as a marker of the Maillard reaction[20]. Carboxymethylation of lysine 47 was identified in all the samples stored at 50 and 60°C. This confirms that the Maillard reaction occurred on this residue catalysed by temperatures of storage.

Carboxyethylation is the product of the modification of lysines with methylglyoxal (lactose degradation product) and it can be formed due to temperatures applied or to enzymatic activity.[18,21] Carboxyethylation of lysine 135 was found in all the samples suggesting that even in this case the modification cannot be related to the storage conditions applied and that this modification is more likely to be due to enzymatic activity.

In the end, it was observed the glycation with a hexose of lysine 91 in all the samples except for Samples 1,2,3 and 4. Lysine's hexose glycation may be observed due to degradation of the lactose bound or to the binding of hexoses that are present in the sample. Indeed, even though lactose is the main sugar present in milk, it was observed the presence of other sugars (i.e. hexoses) in milk that could be involved in the Maillard reaction (i.e. galactose, glucose, etc.).[21,22]. Even for the second hypothesis, the glycation of hexoses, the degree of glycation is related to the temperature applied[23]. Moreover, this last finding shows that the sugar moiety remained stable in the samples stored for different days and at higher temperatures without oxidative degradation phenomena. Unexpectedly, peptides with lactose bound were not found. This might be related to instrumental limitations.

Figure 3.3.15 shows bovine α-lactalbumin sequence with underlined the amino acids where PTMs were identified.

10 20 30 40 50 60
EQLTKCEVFR ELKDLKGYGG VSLPEWVCTT FHTSGYDTQA IVQNNDSTEY GLFQINNKIW
70 80 90 100 110 120
CKDDQNPHSS NICNISCDKF LDDDLTDDIM CVKKILDKVG INYWLAHKAL CSEKLDQWLC EKL

Figure 3.3.15 Amino acid sequence of bovine α-Lactalbumin[24] (Uniprot code P00711, signal peptide sequence was removed). Amino acid residues modified identified in all the samples (**M**= methionine oxidation; **W**= tryptophan oxidation; **Q**= glutamine deamidation; **N**= asparagine deamidation).

From this protein, few modifications were identified and PTMs due to the Maillard reaction were not observed. This could be related to instrumental limits and the lower abundance of this protein with respect to β-lactoglobulin. Indeed, it was previously reported that this protein has some lysine residues that can be lactosylated due to heating[25]. The region 54-60 amino acids residues seem to be the one more affected by PTMs. Deamidation of asparagine 56 was found in all the samples while deamidation of asparagine 57, deamidation of glutamine 54 and oxidation of tryptophan 60 seem to be influenced by the temperatures applied.

Figure 3.3.16 and 3.3.17 show the sequences of the two whey proteins with underlined the amino acids were PTMs were found in the spray-dried sample. Results were compared with the ones obtained for Sample 1.

4 14 24 34 44 54
LIVT QTMKGLDIQK VAGTWYSLAM AASDISLLDA QSAPLRVYVE ELKPTPEGDL
64 74 84 94 104 114
EILLQKWENG ECAQKKIIAE KTKIPAVFKI DALNENKVLV LDTDYKKYLL FCMENSAEPE
124 134 144 154
QSLVCQCLVR TPEVDDEALE KFDKALKALP MHIRLSFNPT QLEEQCHI

Figure 3.3.16 Amino acid sequence of bovine β-Lactoglobulin[24] (Uniprot code P02754, signal peptide sequence was removed). Amino acid residues modified identified in the spray-dried sample (**M**= methionine oxidation; **W**= tryptophan oxidation; **N**= asparagine deamidation; **K**= carboxyethyl lysine).

10 20 30 40 50 60
EQLTKCEVFR ELKDLKGYGG VSLPEWVCTT FHTSGYDTQA IVQNNDSTEY GLFQI**NN**KIW
70 80 90 100 110 120
CKDDQNPHSS NICNISCDKF LDDDLTDDI**M** CVKKILDKVG INY**W**LAHKAL CSEKLDQWLC EKL

Figure 3.3.17 Amino acid sequence of bovine α-Lactalbumin[24] (Uniprot code P00711, signal peptide sequence was removed). Amino acid residues modified identified in the spray-dried sample (**M**= methionine oxidation; **W**= tryptophan oxidation; **N**= asparagine deamidation).

Results obtained for both the whey proteins in this sample confirmed the similarity with Sample 1, suggesting that the drying conditions do not influence the PTMs formation. From β-lactoglobulin no PTMs were identified caused by the Maillard reaction, with the only exception of carboxyethylation of lysine 135 that was found in all the other freeze-dried samples and could be probably the product of enzymatic activity. From α-lactalbumin the deamidation of asparagine 57 was found in the spray-dried sample but not in Sample 1. As above discussed, these findings are in agreement with the previous observation that the drying technique does not influence protein lactosylation.
In all the samples, the modifications found in the β-lactoglobulin sequence were found in both the two isoforms (A and B) of this whey protein.

3.3.4 Total amino acid profile determination

With the aim of knowing the nutritional value of the examined products and the influences of the selected storage conditions on it, the total amino acid profile was determined. Amino acids were quantified using the software MassLynx: by the integration of the peak areas and the external calibration curve it was possible to determine the amount of amino acids in all the samples. During the acid hydrolysis, the amide group in the side chain of Asparagine (Asn) hydrolyse to carboxylic acid, so this amino acid was quantified together with Aspartic Acid (Asp). The same occurs with Glutamine (Gln) that is quantified together with Glutamic Acid (Glu). The obtained results are here presented (Figure 3.3.18). Data are reported on dry matter (per 100g).

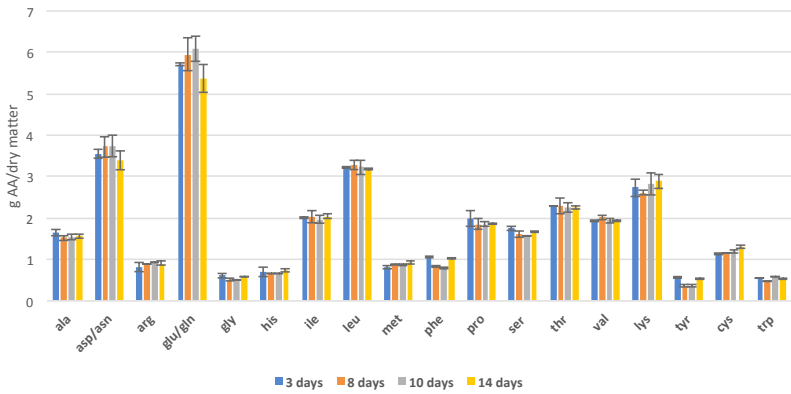


Figure 3.3.18 Example of the total amino acid profile obtained for the analysed samples. In figure is reported the profile obtained for the samples stored at 20°C for 3 days (blue bars), 8 days (orange bars), 10 days (grey bars) and 14 days (yellow bars).

For the other groups of storage temperature, the profile obtained had a similar trend. In particular, it was observed a decrease in the amount of free lysine due to the higher temperatures applied and the time of storage. This finding can be related to protein lactosylation[5]. Indeed, lysine residues involved in the lactosylation are blocked in the Amadori compound and their biological availability is reduced[16]. Moreover, a little decrease in the content of Phe, Tyr and His was also observed and may be due to the increase of days and temperature of storage. Evidences of the decrease in content of these amino acids due to storage conditions were previously not reported in literature. Oxidation phenomena occurring at high temperatures might degrade these amino acids reducing their amounts. The other differences observed are less than 10-15% of variation. After quantification, the obtained values were summed for each sample obtaining results (Table 3.3.6) that are in agreement with the Kjeldahl analysis performed (Table 3.3.1). It was possible, indeed, to estimate a real protein content around 25%, as with the Kjeldahl analysis all the nitrogen content is determined. Indeed, the obtained low value can be due to a possible degradation of the amino acids during the acid

hydrolysis. Moreover, it was reported that in cow's milk the non protein nitrogen content is about 5% of the total nitrogen[7].

Table 3.3.6 Real protein content calculated by the sum of the total amino acids (values are adjusted by the dry matter).

Sample	Protein content (%w/w)	Sample	Protein content (%w/w)
1	25,54	**11**	25,15
2	25,04	**12**	23,38
3	26,34	**13**	23,15
4	24,56	**14**	23,54
5	23,75	**15**	23,86
6	24,74	**16**	24,74
7	24,12	**17**	25,34
8	24,77	**18**	25,14
9	24,89	**19**	25,46
10	25,36	**20**	23,78

In figure 3.3.19 is reported the total amino acid profile of the spray-dried sample compared to Sample 1.

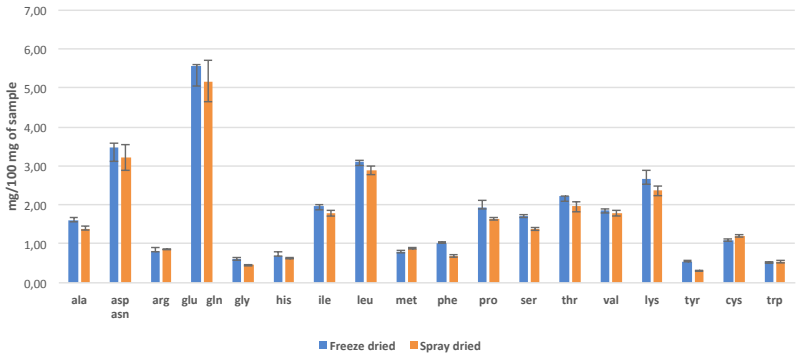


Figure 3.3.19 Comparison between the total amino acids profile of the spray-dried sample (orange bars) with the freeze-dried sample stored for 3 days at 20 °C (blue bars).

As shown in the figure above, very little differences were detected in the two samples, confirming the assumption made before that the temperatures applied for the spray-drying produce similar effects as the freeze-drying[13]. Minor losses in Phe and Tyr content were

observed, even if in the spray-drying the exposure at high temperature for drying is very short[26]. As already discussed above, these findings could be the results of oxidation reactions.

3.3.5 Nutritional value

The amino acid content was also evaluated in terms of quality, considering the relative amount of the nine essential amino acids (phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, lysine and histidine) on the total amino acids[27]. These amounts were calculated comparing the amounts of amino acids for each sample with the protein content determined with the Kjeldahl analysis (Table 3.3.1). Data are reported in Table 3.3.7.

Table 3.3.7 Percentages of essential amino acids in each sample.

Sample	% Essential amino Acids	Sample	% Essential amino Acids
1	43	**12**	44
2	44	**13**	45
3	44	**14**	45
4	44	**15**	44
5	44	**16**	44
6	44	**17**	43
7	44	**18**	43
8	43	**19**	43
9	44	**20**	44
10	44	**21**	44
11	43		

The amount of essential amino acids does not seem to be affected by the storage and temperature applied.

Finally, the chemical scores for essential amino acids were calculated according to Mitchell and Block (1946) in each sample and using a reference protein[28]. The chemical score is a parameter commonly used for the evaluation of the nutritional quality of proteins. It is obtained from the calculation of the content of each essential amino acid as a percentage of the amino acids in a reference protein that is known to be well balanced in the amino acid content related to human requirements[5]. Results are shown in Table 3.3.8.

Table 3.3.8 Chemical scores calculated for the essential amino acids (in bold are highlighted the lower values for each sample).

Sample	His	Ile	Leu	Thr	Val	Lys	Trp	Met/ Cys	Phe/ Tyr
1	119	243	176	256	182	162	234	235	**104**
2	110	238	178	263	195	164	227	239	**77**
3	116	252	178	258	187	168	216	223	**77**
4	118	241	181	265	185	155	251	253	**80**
5	118	237	185	252	194	160	250	240	**71**
6	112	250	183	259	193	157	199	251	**78**
7	126	243	175	259	188	170	231	274	**106**
8	120	253	186	260	197	133	208	274	**90**
9	114	248	184	255	194	166	213	262	**73**
10	111	248	176	262	192	170	197	239	**85**
11	113	238	178	253	184	168	249	250	**75**
12	131	248	186	265	193	135	273	276	**93**
13	128	250	182	262	195	148	287	290	**87**
14	129	250	185	259	195	157	292	258	**83**
15	124	242	180	255	189	158	265	253	**77**
16	125	250	177	256	185	173	233	272	**101**
17	**90**	244	174	249	186	172	231	244	100
18	123	244	178	253	192	146	263	258	**77**
19	129	244	172	252	183	165	247	244	**108**
20	123	244	178	265	196	146	283	249	**80**
21	120	244	179	248	192	159	268	286	**73**

In all the samples Phe and Tyr are the limiting amino acids (with one exception for the Sample 17 where they are the second limiting amino acids and the first one is His). Results are in agreement with data reported in literature for whey protein concentrates.[29,30] In this light, it is possible to assume that even if the modifications induced by storage temperature and time affected the amino acid content, the nutritional value of the product was still adequate.

3.4 Conclusions

With this study it was possible to observe that the selected storage conditions have an effect on protein composition, inducing proteins' denaturation/aggregation and favouring lysine lactosylation. From the UPLC-MS quantification can be concluded that elevated temperature has a denaturing effect on proteins, as expected, in particular for temperatures above 30°C. In all the samples we identified lactose bound to whey proteins. For both whey proteins, the same effect was observed: the lactosylation degree rise with an increased storage temperature and with increased number of storage days. That is true also for the di-lactosylated forms that were identified for both β-lactoglobulin isoform A and isoform B, with the same trend. Thus, the obtained results suggest that storing samples for a short period of time at low temperatures avoids the formation of the lactosylated forms and denatured proteins. Both denaturation and lactosylation may have positive and negative aspects on whey protein concentrates: denaturation on one side makes proteins more digestible but could affect allergenicity [31] (this topic will be discussed in Chapter 5), on the other side lactosylation could mask the epitope to the binding of these proteins with human IgE but reduces the amount of available free lysines reducing the nutritional value of the product.

Through chymotryptic digestion, it was possible to identify some modified amino acids in proteins' sequences but it was not possible to confirm any trend related to the increasing storage temperature or storage days. Some of the identified PTMs can be related to the storage conditions applied. Results here presented could be useful for: the assessment of milk protein damages and milk nutritional value after storage; the determination of shelf life markers and the harshness of protein treatments; the determination of treatments influences on allergenic proteins. This analysis needs a deeper investigation, using different proteomics tools (proteolytic enzymes) and analysing techniques.

The impact of the storage conditions on the whole amino acid content is very little. Losses were found only on phenylalanine, tyrosine and more interestingly on lysine residues, confirming that this amino

acid bound lactose in the sample and reduce its availability. However, the nutritional value of the samples seems not to be affected.

Concerning the drying technique, minimal differences between the spray-dried sample and the freeze-dried one were observed, regarding also the nutritional value. These evidences suggest that the different drying technique applied is not so relevant.

References

1. Norwood, E.-A., Croguennec, T., Le Floch-Fouéré, C., Schuck, P. & Jeantet, R. *Changes in Whey Protein Powders During Storage*. *Whey Proteins* (Elsevier Inc., 2018). doi:10.1016/b978-0-12-812124-5.00004-7
2. Moschopoulou, E., Moatsou, G., Syrokou, M. K., Paramithiotis, S. & Drosinos, E. H. *Food quality changes during shelf life*. *Food Quality and Shelf Life* (Elsevier Inc., 2019). doi:10.1016/b978-0-12-817190-5.00001-x
3. Nishanthi, M., Chandrapala, J. & Vasiljevic, T. Properties of whey protein concentrate powders obtained by spray drying of sweet, salty and acid whey under varying storage conditions. *J. Food Eng.* **214**, 137–146 (2017).
4. O'Mahony, J. A., Drapala, K. P., Mulcahy, E. M. & Mulvihill, D. M. *Whey Protein–Carbohydrate Conjugates*. *Whey Proteins* (Elsevier Inc., 2018). doi:10.1016/b978-0-12-812124-5.00008-4
5. Pellegrino, L., Masotti, F., Cattaneo, S., Hogenboom, J. A. & de Noni, I. Nutritional Quality of Milk Proteins. in *Advanced Dairy Chemistry: Volume 1A: Proteins: Basic Aspects, 4th Edition* (eds. McSweeney, P. L. H. & Fox, P. F.) **1A**, 1–548 (Springer US, 2013).
6. *Commission Regulation (CE) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed*. *Official journal of the European Union* (2009).
7. Maubois, J. L. & Lorient, D. Dairy proteins and soy proteins in infant foods nitrogen-to-protein conversion factors. *Dairy Sci. Technol.* **96**, 15–25 (2016).
8. Delgado-Andrade, C., Rufián-Henares, J. A., Jiménez-Pérez, S. & Morales, F. J. Tryptophan determination in milk-based ingredients and dried sport supplements by liquid chromatography with fluorescence detection. *Food Chem.* **98**, 580–585 (2006).
9. Buhler, S. *et al.* UV irradiation as a comparable method to thermal treatment for producing high quality stabilized milk whey. *Lwt* **105**, 127–134 (2019).
10. van Lieshout, G. A. A., Lambers, T. T., Bragt, M. C. E. & Hettinga, K. A. How processing may affect milk protein digestion and overall physiological outcomes: A systematic review. *Critical Reviews in Food Science and Nutrition* 1–24 (2019). doi:10.1080/10408398.2019.1646703
11. Norwood, E. A. *et al.* Heat-Induced Aggregation Properties of Whey Proteins as Affected by Storage Conditions of Whey Protein Isolate

Powders. *Food Bioprocess Technol.* **9**, 993–1001 (2016).
12. Morr, C. V & Ha, E. Y. W. Whey protein concentrates and isolates: Processing and functional properties. *Crit. Rev. Food Sci. Nutr.* **33**, 431–76 (1993).
13. Guyomarc'h, F., Warin, F., Donald Muir, D. & Leaver, J. Lactosylation of milk proteins during the manufacture and storage of skim milk powders. *Int. Dairy J.* **10**, 863–872 (2000).
14. Singh, H. & Creamer, L. K. Denaturation, aggregation and heat stability of milk protein during the manufacture of skim milk powder. *J. Dairy Res.* **58**, 269–283 (1991).
15. Gulzar, M., Bouhallab, S., Jardin, J., Briard-Bion, V. & Croguennec, T. Structural consequences of dry heating on alpha-lactalbumin and beta-lactoglobulin at pH 6.5. *Food Res. Int.* **51**, 899–906 (2013).
16. Mehta, B. M. & Deeth, H. C. Blocked Lysine in Dairy Products: Formation, Occurrence, Analysis, and Nutritional Implications. *Compr. Rev. Food Sci. Food Saf.* **15**, 206–218 (2016).
17. El, S. N. & Kavas, A. Available lysine in dried milk after processing. *Int. J. Food Sci. Nutr.* **48**, 109–111 (1997).
18. Meltretter, J., Wüst, J. & Pischetsrieder, M. Modified peptides as indicators for thermal and nonthermal reactions in processed milk. *J. Agric. Food Chem.* **62**, 10903–10915 (2014).
19. Holland, J. W., Gupta, R., Deeth, H. C. & Alewood, P. F. Proteomic analysis of temperature-dependent changes in stored UHT milk. *J. Agric. Food Chem.* **59**, 1837–1846 (2011).
20. Delatour, T. *et al.* Analysis of advanced glycation endproducts in dairy products by isotope dilution liquid chromatography-electrospray tandem mass spectrometry. The particular case of carboxymethyllysine. *J. Chromatogr. A* **1216**, 2371–2381 (2009).
21. Arena, S., Renzone, G., D'Ambrosio, C., Salzano, A. M. & Scaloni, A. Dairy products and the Maillard reaction: A promising future for extensive food characterization by integrated proteomics studies. *Food Chem.* **219**, 477–489 (2017).
22. Sundekilde, U., Larsen, L. & Bertram, H. NMR-Based Milk Metabolomics. *Metabolites* **3**, 204–222 (2013).
23. Milkovska-Stamenova, S. & Hoffmann, R. Hexose-derived glycation sites in processed bovine milk. *J. Proteomics* **134**, 102–111 (2016).
24. Bateman, A. UniProt: A worldwide hub of protein knowledge. *Nucleic Acids Res.* **47**, D506–D515 (2019).
25. Milkovska-Stamenova, S. & Hoffmann, R. Identification and quantification of bovine protein lactosylation sites in different milk products. *J. Proteomics* **134**, 112–126 (2016).

26. Chegini, G. & Taheri, M. Whey powder: Process technology and physical properties: A review. *Middle East J. Sci. Res.* **13**, 1377–1387 (2013).
27. Loveday, S. M. Food Proteins: Technological, Nutritional, and Sustainability Attributes of Traditional and Emerging Proteins. *Annu. Rev. Food Sci. Technol.* **10**, 311–339 (2019).
28. *Dietary reference intakes for energy, carbohydrate, fiber fat, fatty acids, cholesterol, protein and amino acids*. *Institute of Medicine* (National Academies Press, 2005). doi:https://doi.org/10.17226/10490
29. Forsum, E. Nutrition Evaluation of Whey Protein Concentrates and Their Fractions. *J. Dairy Sci.* **57**, 665–670 (1974).
30. Sindayikengera, S. & Xia, W. shui. Nutritional evaluation of caseins and whey proteins and their hydrolysates from Protamex. *J. Zhejiang Univ. Sci. B.* **7**, 90–98 (2006).
31. Verhoeckx, K. C. M. *et al.* Food processing and allergenicity. *Food Chem. Toxicol.* **80**, 223–240 (2015).

Chapter 4

In vitro simulated semi-dynamic gastrointestinal digestion: Molecular characterization of whey proteins in spray-dried sample

4.1 Introduction

Human gastrointestinal digestion is a topic of great interest for studies on food components (i.e. nutrients, toxins, drugs, allergens, etc) and their fate and effects on human health. *In vivo* studies (i.e. on animals) are the best ones to investigate gastrointestinal digestions, because they give the closest results to the human situation. Of course, these studies raise a series of ethical issues that limit their use. In this light research was oriented in developing *in vitro* models able to replicate the human situation. Currently, existing models can be divided into static and dynamic ones. The easiest to perform are the static models. They do not require any particular instrumentation and are very cheap. Usually, they are designed to simulate the physiological conditions of the oral, gastric and small intestinal phases principally[1], while the large intestinal digestion usually is not performed as most of the absorption of nutrients mainly occurs in the small intestine[2]. In an *in vitro* simulated digestion crucial parameters are: the type and concentration of enzymes, concentration of the sample, food matrix and pH[3]. The temperature is set at 37°C and the digestion time is based on classical timings of human digestion[3]. Enzymes are usually added consecutively depending on the food components of interest. Usually, proteases are added for protein digestion, while lipases and amylases for lipid or starch respectively[3]. Enzyme activity depends on different factors such as concentration, pH, stability, temperature, activators, incubation time or inhibitors[4].

Dynamic gastrointestinal digestion models include mechanical and physicochemical processes and temporal changes in luminal conditions, resembling the *in vivo* digestion[5]. The first dynamic model proposed was the TIM (TNO Gastro-Intestinal Model)[6], developed at the beginning of the 90s. Initially, TIM-1 was developed which

simulates the stomach and small intestine, then the TIM-2 able to simulate the large intestine. This model was broadly validated during these years, also by comparison with *in vivo* studies[7]. Another example of dynamic model is the SHIME® (Simulator of the Human Intestinal Microbial Ecosystem) that was developed in the same years[8]. This model is made of five reactors that simulate the stomach, the small intestine and 3 regions of the large intestine (ascending, transverse and descending colon). It has been continuously improved to better resemble the *in vivo* situation. For example, the M-SHIME was developed to mimic the mucosal microbial colonization[7]. More recently, other models were developed such as SIMGI® (SIMulator of the GastroIntestinal tract), ESIN (Engineered Stomach and small INtestinal) and DIDGI®[7]. As compared to the static models the dynamic ones better resemble the *in vivo* situation, but they are more complex and expensive.

The need to design a static model for the gastrointestinal simulated digestion able to harmonize existing procedures led to the development, within the COST action FA1005 INFOGEST action, of a first harmonized protocol[9]. As a next step, a semi-dynamic model[10] was then developed, which is a compromise between the dynamic and static models. The gastric phase is performed in a heated vessel from which it is possible to remove samples from the digestion mimicking the gastric emptying and to control the addition of the enzyme. Moreover, it is possible to control the gradual addition of the gastric solution. The intestinal phase is still performed in a static way. This model is currently under revision within the INFOGEST action and its protocol will be soon published[1].

The aim of research activities described in this chapter is to perform a semi-dynamic *in vitro* gastrointestinal digestion to run kinetics studies focused on evaluating protein degradation and peptides release. These data are crucial in studies on peptides bioactivity and for the assessment of protein allergenicity. The *in vitro* simulated gastrointestinal digestion was performed at the Institute of Food Science Research (CIAL, CSIC-UAM, Madrid), in collaboration with the BIOPEP research group where it was possible to use the semi-dynamic model.

4.2 Materials and methods

4.2.1 Reagents

Spray-dried whey protein concentrate (WPC-35) was provided by FrieslandCampina (Amersfoort, The Netherlands). Pepsin from porcine gastric mucosa 3,200-4,500U/mg, Pancreatin from porcine pancreas, Potassium chloride (KCl), Potassium dihydrogenphosphate (KH_2PO_4), Sodium hydrogen carbonate ($NaHCO_3$), Trizma® Base, Bile salts, Dithiothreitol (DTT), Haemoglobin, Tris-HCl, β-Mercaptoethanol, Trichloroacetic acid (TCA), Sodium hydroxide (NaOH), Formic Acid 98-100% (FA), Urea, β-lactoglobulin (86,35% of purity) and α-lactalbumin (87,12% of purity) standards were all purchased from Sigma Aldrich (St. Luis, MO, USA). Magnesium chloride ($MgCl_2$), Sodium dodecyl sulphate (SDS), Trifluoroacetic Acid (TFA) and Bromophenol Blue were purchased from Merk (Darmstadt, Germany). Sodium chloride (NaCl), Ammonium carbonate ($(NH_4)_2CO_3$), Calcium chloride ($CaCl_2$), Ammonium hydrogen carbonate (NH_4HCO_3), Hydrochloric acid (HCl) and glycerol were purchased from Panreac (ITW reagents, Barcelona, Spain). Acetonitrile was purchased from VWR (Milford, MA, USA). Criterion™ XT precast gel 12% Bis-tris, Running Buffer 20x, Precision Plus Protein™ Unstained protein standards were purchased from BIO-RAD (Hercules, CA, USA). Instant blue™ protein stain was purchased from Expedeon (Cambridge, United Kingdom). With a MilliQ system (Millipore, Bedford, MA, USA) doubly deionized water was obtained.

4.2.2 Determination of pepsin activity

Pepsin activity was determined following the protocol[1] reported in literature. A solution of haemoglobin 2% was prepared at pH 2. In 2 mL Eppendorfs were introduced 500 µL of haemoglobin and warmed at 37°C for 4 min. One mg of pepsin was weighted and diluted in 1 mL of buffer (10 mM Tris and 150 mM NaCl, pH 6.5). From this stock solution we prepared 5 different concentration of enzyme in 10 mM HCl: 15, 20, 25, 30, 35 µg/mL of pepsin. Pepsin (100 µL) in the corresponding concentration was added to the haemoglobin at 37°C and 300 rpm in a Thermomixer (Eppendorf, Hamburg, Germany). Then 1 mL of cold TCA 5% was added to the samples to stop the reaction.

Blanks are performed adding to 500 µL of haemoglobin firstly 1 mL of cold TCA 5% was added at 37 °C and 300 rpm, secondly 100 µL of pepsin in the corresponding concentration. The test was performed in triplicate. Samples are then centrifuged at 6000 g for 30 min. Absorbance of the samples was measured at 280 nm with a UV spectrometer (Specord 210 plus, Analytik Jena, Germany).

4.2.3 Semi-dynamic in vitro gastric digestion

Following the consensus INFOGEST[1] protocol the simulated fluids were prepared. For the Simulated Gastric Fluid (SGF) the amount of HCl required to reach pH 2 was determined previously in a test experiment. The SGF contained the 79,4% of SGF 1.25x at pH 7 prepared following the mentioned protocol, 1,12 % of 6M HCl, 0,05% of 0.3M $CaCl_2(H_2O)_2$, 0,62% of pepsin, 18,8% of MilliQ water. The digestion was performed at 37 °C and under stirring with a Hei-TORQUE precision 100 (Heidolph Instruments GmbH, Germany) at 15 rpm. All the solutions were previously warmed at 37°C. Sixty mL of sample (1,63 g of spray-dried WPC-35 diluted in MilliQ water, 0,95% of protein) was introduced in a glass vessel controlled at 37°C with 4,06 mL of the Simulated Salivary Fluid (SSF). After 2 min, 5,08 mL of gastric solution (SGF) was added to simulate the gastric fluid residue in the stomach. At the same time it was started the gradual addition at a constant rate of: gastric solution (64,06 mL) using a pH-stat (TIM856 titration manager, TitraLab®, Radiometer Analytical, Hach) dosing device at 0,63 mL/min; pepsin (87,4 mg in 0,4 mL of SGF1.25x) from porcine gastric mucosa (2932 U/mg solid, activity determined using haemoglobin as a substrate) at 0,0044 mL/min. Pepsin solution was added using a syringe pump. The simulation of the gastric emptying was made considering the caloric density. Five aliquots were collected from the digestion at 18 min, 36 min, 72 min, 54 min and 90 min (nearly 25 mL) in order to mimic the gastric emptying. The pH of these aliquots was measured and increased to pH 7 with 2M NaOH in order to inactivate pepsin activity. Samples were stored at -20°C. Digestion was performed in duplicate.

4.2.4 Static in vitro intestinal digestion

From the fractions collected at 18, 54 and 90 min after the semi-dynamic gastric digestion 10 mL underwent the intestinal digestion in a static way, following the INFOGEST protocol[1]. According to the protocol, the Simulated Intestinal Fluid (SIF) was prepared. 8 mL of pancreatin (365,6 mg in SIF 1.25x) were added to the sample among with 1,26 mL of bile salts (added to reach the final concentration of 2.5 mM) and 0,02mL of $CaCl_2(H_2O)_2$ 0.3M. pH was adjusted to 7 with 6M HCl and MilliQ water was then added to reach the final volume of 20 mL. Digestion was performed for 2 hours at 37°C in a shaking incubator at 120 rpm. Samples were horizontally placed in the shaker to obtain a better mixing. The pH was monitored every 30 min and eventually increased at 7 with 2M NaOH. At the end the enzymatic activity was stopped heating samples at 85°C and centrifuging at 4000g for 30 min. The supernatant was removed and freeze-dried. Digestion was performed in duplicate.

4.2.5 HPLC-MS/MS analysis

Before HPLC-MS analysis, a reduction step with DTT was performed: samples were diluted 1:1 with 146 mM DDT in 25 mM NH_4HCO_3 and left for an hour at 37°C; then they were diluted 1:1 with water+0,1%FA and centrifuged for 10 min at 7000 rpm. HPLC-MS/MS analyses were performed on an Agilent 110 series HPLC separation system (Agilent, CA, USA). The analytical column used was a Mediterranea Sea 18 (5µm, 15cm x 0.21cm, Teknokroma, Barcelona, Spain). The mobile phase was composed of H_2O+0.1% HCOOH (eluent A) and CH_3CN + 0.1% HCOOH (eluent B). Gradient elution was performed according to the following steps: isocratic 100% A for 5 min, from 100% to 70% eluent A by linear gradient in 77 min, then to 55% eluent A in other 20 min, washing step at 70% eluent B and reconditioning. Flow rate was set at 0.20 mL/min, injection volume 50 µL. Detection was performed with a Bruker Daltonics Esquire 3000 Ion Trap mass spectrometer (Bruker, MA, USA) operating in the following conditions: ESI source in positive ionization mode, dry temperature 350°C, nebulizer 60 psi, dry gas 8mL/min, full scan acquisition from 100 to 2300 m/z, target mass 450, 750 and 1200. The software used

for the analysis was Compass Hystar (Bruker). Data were processed with Data Analysis (Bruker), while BioTools and MASCOT (Matrix Science, Boston MA, USA) were used for interpretation and matched MS/MS spectra. Homemade database for whey proteins was used in MASCOT software for peptide sequencing. For peptide visualization, the web application Peptigram was used[11]. Uniprot[12] whey proteins sequences were used (code P00711 for α-lactalbumin and P02754 for β-lactoglobulin). For Peptigram's graphs signal peptide sequence was maintained, while for the discussion and in the tables it was removed from the amino acids numbering.

4.2.6 SDS-PAGE analysis

Samples were diluted to the concentration of 1 mg/mL with the sample buffer (0,05M Tris-HCl, 1.6% w/v SDS, glycerol 8% v/v, β-mercaptoethanol 2% v/v, bromophenol blue indicator 0,002% w/v). After mixing, samples were heated at 95°C for 5 min. The Criterion XT gel (18 wells) was introduced in the vessel with the running buffer and samples were loaded on it (30 μL for the samples and 15 μL for the marker). After running 5 min at 100V, it was left running at 150V for 1,5 hour. Then the gel was washed three times with double deionized water for 10 min each. It was covered with the instant blue and after half an hour was washed and left in double deionized water for one hour. It was washed again and left in water overnight. Then it was scanned with the VersaDoc™ imaging system model 4000 (Bio-Rad) using the software QuantityOne® 1-D (Bio-Rad).

4.2.7 UPLC-UV analysis

Samples are diluted first 1:4 in water deionized, then 1:1 with eluent A +Urea 6M and centrifuged 3 min at 12.800 rpm. UPLC-UV analyses were performed on an ACQUITY UPLC separation system. The analytical column used was an Aeris WIDEPORE XB-C18 LC column (Phenomenex, 200Å, 3.6 μm, 2.1mm x 150mm). Eluent A was 80% water+20% Acetonitrile+0.1% Formic acid (eluent A) and eluent B was 80% Acetonitrile+ 20% water + 0.1% Formic acid. Next steps were followed for gradient elution: isocratic 74% A for 3 min; from 74% to 69% eluent A by linear gradient in 4.3 min; from 69% to 65% eluent A

in 12.5 min; from 65% to 63% eluent A in 1,2 min; an isocratic step at 63% eluent A for 4,8 min; a linear gradient from 37 to 45% eluent B for 7,5 min; washing step at 70% eluent B and reconditioning. Flow rate was set at 0.20 mL/min, injection volume 3 µL. Detection was performed with an Acquity Tunable UV detector operating at 214 nm. For protein quantification two external calibration curves were used, one for α-lactalbumin (87,12% of purity) and one for β-lactoglobulin (86,35% of purity). The dilutions used for the calibration are reported in Table 4.2.1 and 4.2.2. For data acquisition was used the Acquity software, while for data processing was used Data Analysis (version, 4.0 Bruker Daltonics).

Table 4.2.1 β-lactoglobulin standard dilution for the external calibration curve.

	α-lactalbumin
Std 1	0,1 mg/mL
Std 2	0,2 mg/mL
Std 3	0,4 mg/mL
Std 4	0,8 mg/mL
Std 5	1 mg/mL

Table 4.2.2 α-lactalbumin standard dilution for the external calibration curve.

	β-lactoglobulin
Std 1	0,25 mg/mL
Std 2	0,5 mg/mL
Std 3	1 mg/mL
Std 4	2 mg/mL
Std 5	4 mg/mL

4.3 Results and Discussion

4.3.1 Spray-dried sample

Spray-dried whey protein (WPC-35) was provided by FrieslandCampina (Amersfoort, The Netherlands). These whey proteins were obtained collecting whey after the production of cheese and ultrafiltration. In order to be stored in a powdered state, this product was then spray-dried. As declared by the producer it contains a 50,5% (w/w) of lactose, 35% (w/w) of protein, 6.5% (w/w) of minerals,

2,5% (w/w) of fats, 2% (w/w) organic milk salts and 3.5% (w/w) moisture. Complete protein characterization of this sample is described in Chapter 3. Through UPLC-MS analysis (paragraph 3.2.6), whey protein concentration was determined in the sample: 0,703±0,007 mg/mL for β-lactoglobulin and 0,91±0,003 mg/mL for α-lactalbumin. The total amount of β-lactoglobulin is composed of an 80,4% of unmodified protein, 17,1% of lactosylated protein and a 2,5% of di-lactosylated protein was found too (percentages determined from the XIC areas). On the total amount of α-lactalbumin, instead, only 12% is represented by the lactosylated form.

4.3.2 In vitro semi dynamic gastric phase

The *in vitro* gastrointestinal digestion was performed following the INFOGEST[9] consensus protocol. All the amounts and rate of infusion of the fluids were calculated based on the caloric amount of the sample. During the experiment, fractions of the digested samples were collected simulating the gastric emptying to study protein degradation and the peptide profile. Five fractions were collected from the gastric digestion (Tab 4.3.1).

Table 4.3.1 List of the fractions collected during the digestion.

	Digestion time (min)	**Volume (mL)**
Fraction 1 (F1)	18,08	25
Fraction 2 (F2)	36,15	25
Fraction 3 (F3)	54,23	25
Fraction4 (F4)	72,30	25
Fraction 5 (F5)	90,38	25

Total digestion time was 90,38 min. During the digestion, the pH was gradually decreased to pH 2 adding the simulated gastric fluid. The graph in Figure 4.3.2 shows the pH profile during the digestion.

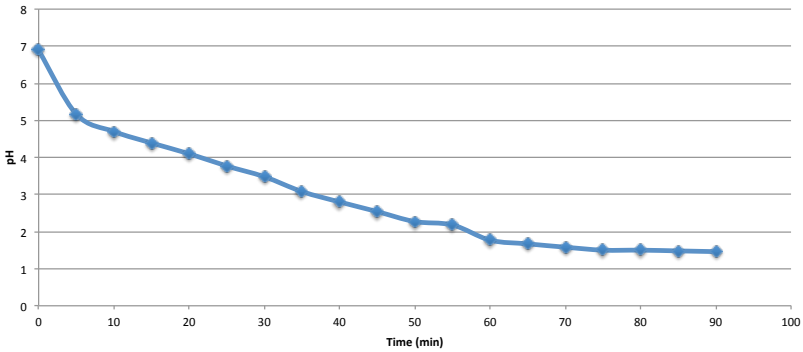


Figure 4.3.2 pH decrease during gastric digestion measured with the pH meter of the titration unit.

The pH of the undigested sample in water was 6,82. Immediately after adding the gastric solution it reached values of 6.6±0,4 and then it gradually decreased to values of pH 1,4±0,1. The obtained pH profile showed that the addition of the gastric phase was gradual and continuous and that gastric emptying reduced the buffering capacity of digested food. This gradual pH decrease from nearly 6 to below 2 was previously reported also in other semi-dynamic gastric digestions performed on bovine milk and dairy products[10,13]. As a control, an external pH measurement was performed measuring the pH of the collected fractions with another pH meter (Figure 4.3.3). The external control confirms the decreasing trend of the pH in the gastric digestion.

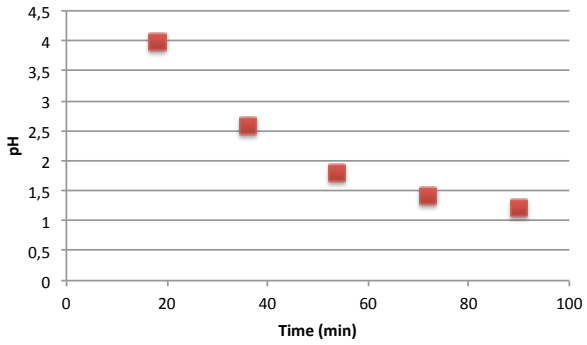


Figure 4.3.3 pH profile obtained from the pH values measured for the five collected fractions that simulate the gastric emptying.

4.3.3 Protein degradation during semi-dynamic gastric digestion

The fractions collected from the gastric phase were characterized by SDS-PAGE (Figure 4.3.4).

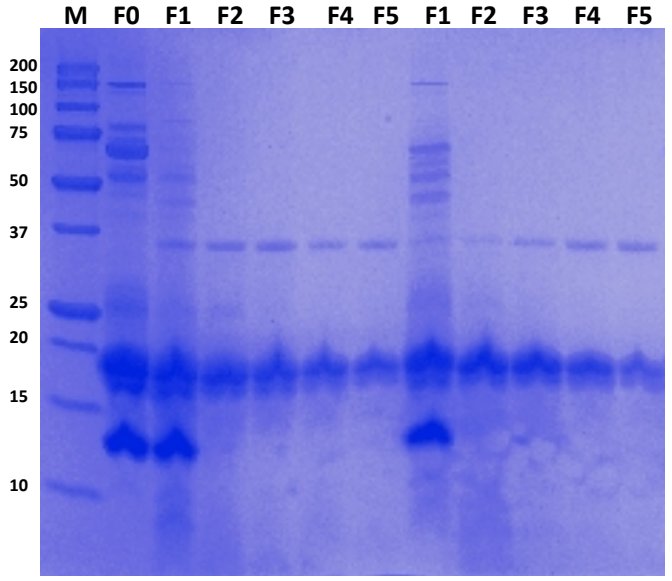


Figure 4.3.4 SDS-PAGE gel image of the gastric digestion performed (F0= undigested protein; F1= Fraction 1; F2= Fraction 2; F3= Fraction 3; F4= Fraction 4; F5= Fraction 5). Marker values are expressed as kDa.

Figure 4.3.4 shows the five fractions collected during the digestion (F1, Fraction 1; F2, Fraction 2; F3, Fraction 3; F4, Fraction 4; F5, Fraction 5) and the undigested sample, used as a control (F0). From this image, it is possible to see the presence of β-lactoglobulin around 18 kDa in all the fractions collected with only a small decrease in intensity from the first to the last collected fraction. This could be due both to the different dilution of the five samples and to a partial digestion of the protein in the gastric phase. Alpha-lactalbumin, instead, was found only in the first fraction around 14 kDa, suggesting that this protein is digested in the first 36 min of the gastric phase. Indeed, after 36 min of digestion the digestion pH is below 4. In these pH conditions α-lactalbumin underwent changes in conformation, thus is more susceptible to pepsin hydrolysis[13,14]. This finding is in agreement with the literature where α-lactalbumin was not detected when pH

decreased below 4 in a semi-dynamic digestion[13]. The bands relative to the two whey proteins show an unusual form. This could be due to the presence of a high amount of lactose in the sample that interferes with the SDS-PAGE run. The digested whey protein concentrates have a high amount of lactose, 50% as declared from the producer (paragraph 4.3.1). At around 35 kDa is possible to see the presence of pepsin in all the fractions.
The collected fractions were then analysed with UPLC-UV analysis. Figure 4.3.5 shows the chromatograms of the five fractions collected from the digestion.

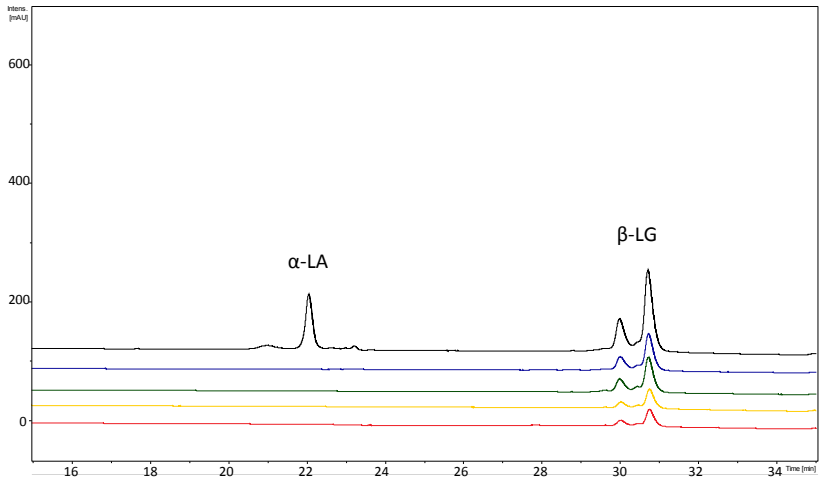


Figure 4.3.5 UPLC-UV chromatograms of the five fractions collected during digestion (Fraction 1 in black, Fraction 2 in blue, Fraction 3 in green, Fraction 4 in yellow and Fraction 5 in red). Intensity is on the y axis while time on the x axis.

From the comparison, it is possible to see the disappearance of α-lactalbumin from the second collected fraction, as noticed in the SDS-PAGE. Again, β-lactoglobulin is present in all the samples but its intensity decreases in the last fractions. Since the detection was performed with UV it was possible to quantify whey proteins in the samples using an external calibration curve for each whey protein. Results expressed as percentages on the starting material are reported in figure 4.3.6.

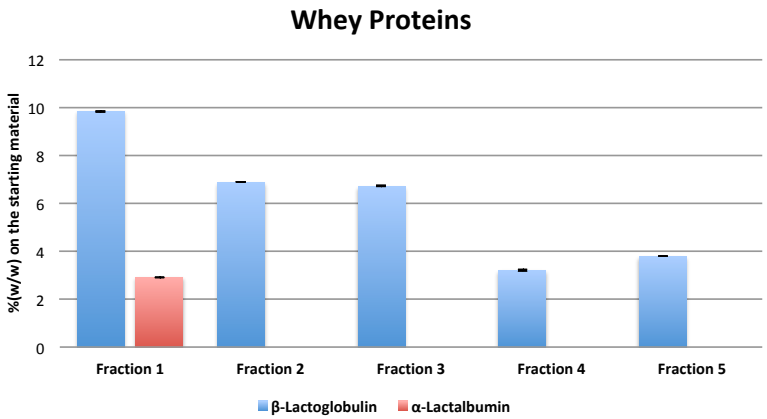


Figure 4.3.6 Whey proteins quantification in the five fractions collected (Fraction 1=18 min, Fraction 2= 36 min, Fraction 3= 54 min, Fraction 4= 72 min, Fraction 5= 90 min).

For the quantification, a dilution factor was calculated considering the gastric emptying and the rate of gastric solution addition. This factor was applied to see protein degradation. It is possible to observe that the amount of β-lactoglobulin slowly decreases during digestion. In the last fraction, there is a little increase of protein with respect to Fraction 4. In the last part of the gastric digestion, the volume was reduced and the sample was not completely homogenous creating some difficulties in sample collection. Thus, it might be assumed that in the last part of the digestion (Fractions 4 and 5) this protein reached a constant amount and the observed differences are related to technical issues. As previously reported, this protein is known to be resistant to gastric digestion[14–17]. However, studies on the dynamic gastric digestion of skim milk powder observed a decrease in the content with the advancement of the gastric digestion[18]. This could be related to the processing that may cause changes in the native structure of the protein[16,18]. Moreover, α-lactalbumin was quantified only in the first fraction collected. Further investigation with OPA analysis will be considered for the determination of the degree of hydrolysis in the five fractions.

4.3.4 Peptide profile after digestion

In order to study peptides released during the digestion, samples were analysed with HPLC-MS/MS analysis. From the MS/MS spectra were identified peptides in the digested fractions. Figure 4.3.7 and figure 4.3.8 show the peptides identified in Fraction 1, 3 and 5 of the gastric phase of the two whey proteins.

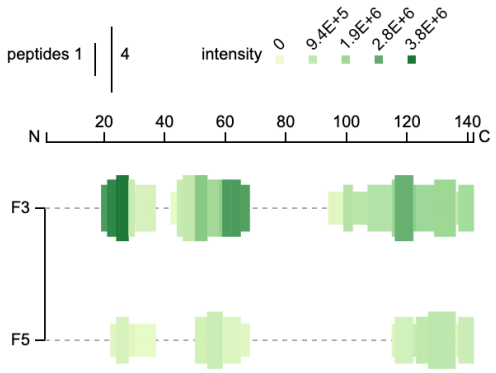


Figure 4.3.7 α-lactalbumin peptides identified in fraction 3 and fraction 5 of the gastric phase. In the graph, the amino acids that were identified in peptide sequences are represented with vertical bars. The intensity of the colour is proportional to the sum of identified peptides' intensities. The dimension is proportional to the number of peptides in which was identified that amino acid in that position. The first 19 amino acids of the graph are the ones belonging to the signal peptide.

Figure 4.3.7 shows the α-lactalbumin peptides identified in Fraction 3 (54 min) and 5 (90 min). In Fraction 1 (18 min) it was not possible to identify peptides deriving from this protein. This finding may be related to instrumental limitations in peptides detection, as from the quantification, a decrease in the %(w/w) on the starting material was observed. Indeed, in the undigested sample was estimated a 4% (w/w) of this protein based on starting material. In Fraction 3 the number of identified peptides is higher than in Fraction 5. Peptides identified derived from three main regions of protein sequence: 1-18, 24-49, 76-123 amino acids residues. Findings were compared with literature where it was reported that few peptides were found in the static *in vitro* gastric phase, mainly deriving from the region 4-35[19]. Table 4.3.2

reports the list of identified peptides, for some of them reported[20] bioactivity was found.

Table 4.3.2 List of all the α-lactalbumin peptides identified in the samples and their eventual reported bioactivity (the amino acids residues of the signal peptide were subtracted).

Peptide sequence	Amino acids residues	Reported Bioactivity
AIVQNNDSTE	40-49	None
RELKDLKGY	10-18	None
LAHKALCSEKLDQ	105-117	None
EQLTKCEVF	1-9	None
WLCEKL	118-123	None
IMCVKKILD	89-97	None
HTSGYDTQ	32-39	None
DKVGINY	97-103	ACE inhibition[21]
WVCTTFHTSG	26-35	None
WVCTTF	26-31	None
IMCVKKILDKVGINY	89-103	None
WLAHKALCSEKLDQ	104-117	DPP IV inhibition[22]
EQLTKCEVFRE	1-11	None
IVQNNDSTE	41-49	None
LKDLKGY	12-18	None
LDDDLTDD	81-88	None
KVGINYW	98-104	None
HTSGYDTQA	32-40	None
PEWVCTTF	24-31	None
SCDKFLDD	76-83	None
LCEKL	119-123	DPPIV inhibitor[22]
CSEKLDQ	111-117	DPPIV inhibitor[22]
CEVFRE	6-11	None
KVGINY	98-103	None
IVQNND	41-46	None
CTTFHTSG	28-35	None
LTKCEVF	3-9	None
YDTQA	36-40	None
ALCSEKLDQ	109-117	None

TKCEVF	4-9	None

Results obtained from the identification of peptides released in the gastric phase could be useful in studies on protein allergenicity. Peptides were found also from regions 1-19, 90-109 and 105-123 of α-lactalbumin sequence that are known to be IgE-binding epitopes[23]. The length of the identified peptides varies from 5 to 15 amino acids in Fraction 3 and from 5 to 14 in Fraction 5. It was previously described that the length of an epitope might be around 12-15 amino acids[24,25], in this light some of the identified peptides could be potential IgE-binding epitopes. In Table 4.3.3 are the identified peptides in the epitope regions reported with a corresponding length.

Table 4.3.3 Identified α-lactalbumin peptides that could be IgE-binding epitopes (signal peptide was subtracted from the sequence).

Peptide sequence	Amino acids residues	Digestive Fraction
EQLTKCEVFRE	1-11	Fraction 3
IMCVKKILDKVGINY	89-103	Fraction 3
LAHKALCSEKLDQ	105-117	Fractions 3 and 5
WLAHKALCSEKLDQ	104-117	Fractions 3 and 5

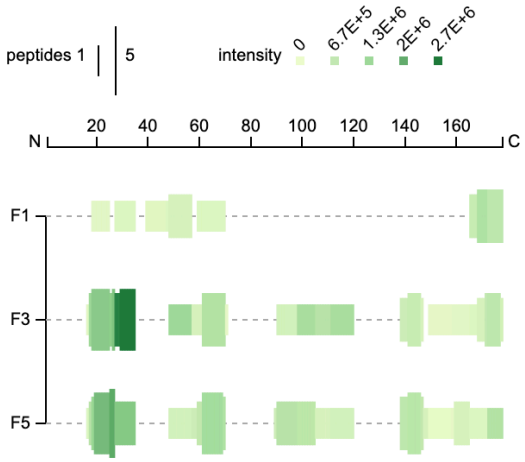


Figure 4.3.8 β-lactoglobulin peptides identified in Fractions 1, 3 and 5 of the gastric phase. In the graph, the amino acids that were identified in peptide sequences are represented with vertical bars. The intensity of the colour is proportional to the sum of identified peptides' intensities. The dimension is

proportional to the number of peptides in which was identified that amino acid in that position. The first 16 amino acids of the graph are the ones relative to the signal peptide.

Figure 4.3.8 shows the peptides released from β-lactoglobulin, confirming partial digestion of this protein in the gastric phase. From the figure above it is possible to follow the advancement of the digestion. Fraction 1 (18 min) contains few peptides in the regions 3-55 and 150-162. The terminal parts of the protein seem to be more susceptible to hydrolysis, maybe due to easier accessibility of the digestive enzymes. In Fraction 3 (54 min) the digestion of these regions advances and the central part of the protein starts to be digested (regions 74-104 and 133-150). In Fraction 5 there is a change in the intensity of peptides plus an increase in the amount of peptides due to the progress of the digestion. Findings are in agreement with what is reported in literature for the *in vitro* static gastric digestion of this protein using the harmonized INFOGEST protocol[17], but a better coverage was observed. Indeed, with respect to the static digestion, were identified peptides from regions 74-104 and 133-150. Interestingly, peptides from this region were observed after the dynamic gastric digestion of skimmed milk powder[18]. The length of the peptides varies from 6 to 13 amino acids in all the fractions. In table 4.3.4 is reported the list of identified peptides, for some of them was found in literature a reported bioactivity[20].

Table 4.3.4 List of all the β-lactoglobulin peptides identified in the samples and their eventual reported bioactivity (signal peptide amino acids residues were substracted).

Peptide sequence	**Amino acids residues**	**Reported Bioactivity**
EEQCHI	157-162	None
DAQSAPLRV	33-41	None
LDAQSAPLRV	32-41	None
PTQLEEQCHI	153-162	None
SFNPTQLEEQCHI	150-162	None
MAASDISL	24-31	None
SFNPTQL	150-156	None

IQKVAGTW	12-19	Ace inhibitor/ DPPIV inhibitor[26]
EELKPTPEGDL	44-54	None
VTQTMKG	3-9	None
IVTQTMKGLD	2-11	None
DTDYKKYLL	96-104	None
LDIQKVAGTW	10-19	None
YVEELKPTPEGDL	42-54	None
VRTPEVDD	123-130	None
DIQKVAGTW	11-19	None
LPMHIRL	143-149	None
VRTPEVDDE	123-131	None
LIVTQTMKGLD	1-11	None
EKFDKALKA	134-142	None
LKPTPEGDLE	46-55	DPPIV inhibitor[22]
LKPTPEGDL	46-54	DPPIV inhibitor[22]
LEEQCH	156-161	None
IVTQTMKG	2-9	None
KIDALNE	83-89	None
KTKIPAVF	75-82	None
NKVLVL	90-95	None
PEVDDE	126-131	None
KVAGTW	14-19	None
TQTMKGLD	4-11	None
LKPTPEGD	46-53	None
KIDALNENKVLVL	83-95	None
PMHIRL	144-149	None
EKTKIPAVF	74-82	None
VTQTMKGLD	3-11	None
VRTPEVDDEAL	123-133	None

Looking at the peptides that were identified, we found sequences in regions 1-16, 76-95, 121-140 and 136-150 that are known to be IgE-binding epitopes.[27,28] Also for this protein, peptides were identified

with a length that might define them as possible epitopes and are reported in table 4.3.5.

Table 4.3.5 Identified β-lactoglobulin peptides that could be IgE-binding epitopes (signal peptide amino acids were subtracted).

Peptide sequence	Amino acids residues	Digestive Fraction
LIVTQTMKGLD	1-11	Fractions 3 and 5
KIDALNENKVLVL	83-95	Fraction 5
VRTPEVDDEAL	123-133	Fraction 5

Concerning peptides bioactivity, it was identified the peptide DKVGINY, deriving from α-lactalbumin, with reported ACE inhibitory activity[21]. Some peptides were identified with dipeptidyl peptidase IV (DPP IV)-inhibitory activity[22]: peptides WLAHKALCSEKLDQ, LCEKL and CSEKLDQ from α-lactalbumin and LKPTPEGDL and LKPTPEGDL from β-lactoglobulin. One peptide was identified for which a double activity was reported, peptide IQKVAGTW that shows both DPP IV inhibitory and ACE inhibitory activity[26]. Peptides with ACE inhibitory activity are important for the treatment of hypertension while DPP IV-inhibitors can potentiate the insulinotropic effects and the glycemic control increasing the half-life of active GLP-1, the hormone involved in the insulin secretion.[29] Since α-lactalbumin and β-lactoglobulin are two important milk allergens, peptides deriving from proteins' epitopes were identified in the collected gastric fractions (Tables 4.3.3 and 4.3.5). For potential allergenicity assessment, the study of allergens modification during the gastro-intestinal digestion is crucial, and in particular the possible influences on their effect. It is known that protein hydrolysis (i.e. digestion) can alter allergenicity. Indeed, it was previously described that after digestion the IgE-binding capacity of allergens may be reduced, increased or it may remain unchanged[30].

4.3.5 Intestinal phase

The intestinal phase was performed in a static way on three of the five gastric fractions collected: Fraction 1, 3 and 5. These three fractions were selected to represent the whole digestion as the main differences were found between them. From the MS/MS spectra, some peptides were identified. Fractions 1, 3 and 5 of the gastric phases

underwent two hours of intestinal digestion each one, so the intestinal digested will be named as the gastric phase from which they were obtained.

For α-lactalbumin very few peptides were identified in the digested samples, probably due to the fact that the digestion of this protein mainly occurs in the gastric phase due to its susceptibility to pepsin at pH below 4[16]. Results are reported in table 4.3.6.

Table 4.3.6 α-lactalbumin peptides identified in the intestinal digested sample (signal peptides amino acids were subtracted).

Peptide sequence	Amino acid residues	Fraction 1	Fraction 3	Fraction5
YDTQA	36-40		x	
DDQNPH	63-68	x	x	x
CKDDQNPH	61-68			x
DDDLTDDI	82-89	x		
LDDDLTDDI	81-89	x		
LDDDLTDD	81-88		x	
LDDDL	81-85		x	x

From the list above it is possible to observe that it seems like that peptide 81-89 is degraded to peptide 81-88 and 81-85 with the advancement of digestion. It was not observed the same for peptide 82-89 identified in Fraction 1. This might be explained with possible instrumental limits in the detection of eventual fragments in the other fractions. The few peptides that were identified belong to two regions of protein sequence, 81-89 and 61-68 amino acids residues, except for the first peptide reported in table 4.3.6. These regions were previously found resistant to gastrointestinal *in vitro* digestion[19]. Moreover, region 80-90 was reported as the unique region present in human intestinal digestion in significant amount[31]. Peptide DDQNPH and LDDDLTDDI were found related to ACE-I inhibitory activity on studies on milk fermentation[32]. In figure 4.3.9 are reported the β-lactoglobulin peptides that were identified.

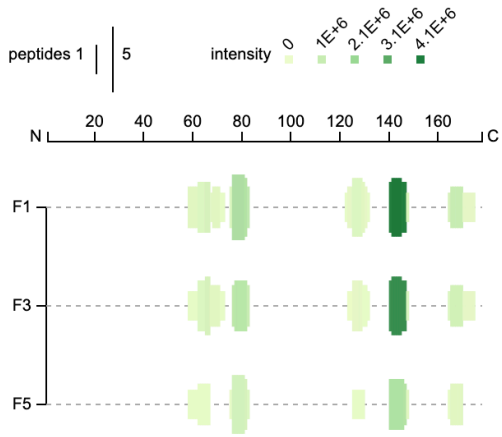


Figure 4.3.9 β-lactoglobulin peptides identified in the three intestinal digested sample. In the graph, the amino acids that were identified in peptide sequences are represented with vertical bars. The intensity of the colour is proportional to the sum of identified peptides' intensities. The dimension is proportional to the number of peptides in which was identified that amino acid in that position. The first 16 amino acids of the graph belong to the signal peptide.

As shown in figure 4.3.9, the differences between the three fractions are very little. This indicates that the differences in protein and peptide content observed in the gastric phase collected, due to the gastric emptying, are not present in the intestinal phase. Indeed, β-lactoglobulin is well and rapidly digested by pancreatic enzymes[16,33], and applying the same incubation time to all the collected fractions might have homogenized the results. However, there are slight differences in the intensities of the bars and fraction 5 presents fewer peptides that the other two fractions. The length of the identified peptides ranges from 5 to 9 amino acid residues in all the digested samples. Peptides were found mainly in four regions of the protein: 43-67, 107-116, 125-132 and 149-160 amino acid residues on protein sequence. These findings are in agreement with previously reported *in vitro* gastrointestinal digestion, in particular for regions approximately 42-60 and 125-135[14,19]. Peptides form regions 9-20 and 92-105 for which bioactivity was reported were not identified[14,34]. In Table 4.3.7 are reported the peptides identified in the samples.

Table 4.3.7 List of all the β-lactoglobulin peptides identified in the samples (signal peptide amino acids were subtracted).

Peptide sequence	**Amino acids residues**	**Peptide sequence**	**Amino acids residues**
LSFNPT	152-157	VEELKPTPE	46-54
SFNPT	153-157	QLEEQ	158-162
TPEVD	128-132	MENSAEPE	110-117
SAEPEQ	113-118	ENSAEP	111-116
DLEIL	56-60	KWENDEC	63-69
WENGE	64-68	TPEVDDEA	128-135
KWENDECA	63-70	PEVDDE	129-134
SAEPEQS	113-119	WENDEC	64-69
TPEVDDE	128-134	ENDECA	65-70
VEELKPT	46-52	KPTPEG	50-55
PEGDLE	53-58	TPEVDD	128-133
KPTPE	50-54	SAEPE	113-117
WENDECA	64-70		

The semi-dynamic model developed was based on *in vivo* digestion parameters obtained for the digestion of dairy products[1]. With respect to the static digestion, the semi-dynamic *in vitro* gastrointestinal digestion performed is more similar to the *in vivo* situation, where in the gastric phase the pH decreases gradually and there is the gastric emptying. A study on the effects of the food structure on the digestion of nutrients applying the semi-dynamic model showed a good comparability with the *in vivo* digestion[10]. Moreover, another study applied the semi-dynamic model for studying the effect of heat treatment on milk digestibility, underlying the importance of the gastric emptying[13]. Adding this parameter, this model allows to follow protein degradation during gastric digestion, determining protein digestion times. It was previously reported that with the harmonized INFOGEST protocol[1] protein coverage was low with respect to other static *in vitro* models, and the difference was explained as a high degree of digestion in the harmonized protocol[17]. With the semi-dynamic model, combining findings from the gastric and intestinal β-lactoglobulin digestion obtained in this study, a higher protein coverage was found. Only regions 35-42, 68-73 and 117-124 were not identified. Concerning α-lactalbumin a good coverage was observed

combining results from both the digestive phases, with only regions 41-60 and 69-75 not identified. Moreover, performing the gastric emptying allows to follow formation and degradation of peptides, for some of which a reported bioactivity or a potential IgE binding was found. This information might be useful for studies on the release of peptides with potential bioactivity from proteins.

4.4 Conclusions

The semi-dynamic digestion experiments performed showed a good repeatability and interesting results on protein degradation and peptide release were obtained. From SDS-PAGE and UPLC-UV analysis of the five fractions collected in the gastric phase it was possible to observe that, in these conditions, α-lactalbumin is digested in the first 36 min of the gastric phase, while β-lactoglobulin was found until the end of the phase. From protein quantification it was possible to see the decrease of β-lactoglobulin amount during gastric phase, due to a partial degradation of this protein. This was confirmed from the HPLC-MS analysis where it was possible to identify peptides deriving from this protein in the gastric fractions. In the first fraction were mainly found peptides deriving from the terminal parts of protein sequence, while in the following fractions it was possible to observe the digestion of more central regions of the sequence. From peptides intensities and their amount, differences were observed in the fractions suggesting the digestion advancement. A better protein coverage for both the whey proteins was observed with this model with respect to what was reported in literature using the static harmonized protocol. Moreover, among the peptides identified it was possible to recognize some peptides with reported bioactivity.
The digestion protocol here described will be used to test other whey protein samples obtained with different processes, as described in Chapter 5.

References

1. Brodkorb, A. *et al.* INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nat. Protoc.* **14**, 991–1014 (2019).
2. Brandon, E. F. A. *et al.* Consumer product in vitro digestion model: Bioaccesibility of contaminants and its application in risk assessment. *Regul. Toxicol. Pharmacol.* **44**, 161–171 (2006).
3. Hur, S. J., Lim, B. O., Decker, E. A. & McClements, D. J. In vitro human digestion models for food applications. *Food Chem.* **125**, 1–12 (2011).
4. S., B. & B.O., E. Critical evaluation of in vitro methods for estimating digestibility in simple-stomach animals. *Nutr. Res. Rev.* **4**, 141–162 (1991).
5. Guerra, A. *et al.* Relevance and challenges in modeling human gastric and small intestinal digestion. *Trends Biotechnol.* **30**, 591–600 (2012).
6. Minekus, M., Marteau, P., Havenaar, R. & Huis in't Veld, J. H. J. A Multicompartmental Dynamic Computer-controlled Model Simulating the Stomach and Small Intestine. *ATLA* **23**, 197–209 (1995).
7. Dupont, D. *et al.* Can dynamic in vitro digestion systems mimic the physiological reality? *Crit. Rev. Food Sci. Nutr.* (2018). doi:10.1080710408398.2017.1421900
8. Molly, K., Vande Woestyne, M. & Verstraete, W. Applied AFwrobiology Biotechnology Development of a S-step multi-chamber reactor as a simulation of the human intestinal microbial ecosystem. *Appl Microbiol Biotechnol* **39**, 254–258 (1993).
9. Minekus, M. *et al.* A standardised static in vitro digestion method suitable for food-an international consensus. *Food Funct.* **5**, 1113–1124 (2014).
10. Mulet-Cabero, A. I., Rigby, N. M., Brodkorb, A. & Mackie, A. R. Dairy food structures influence the rates of nutrient digestion through different in vitro gastric behaviour. *Food Hydrocoll.* **67**, 63–73 (2017).
11. Manguy, J. *et al.* Peptigram: A Web-Based Application for Peptidomics Data Visualization. *J. Proteome Res.* **16**, 712–719 (2017).
12. Bateman, A. UniProt: A worldwide hub of protein knowledge. *Nucleic Acids Res.* **47**, D506–D515 (2019).
13. Mulet-Cabero, A. I., Mackie, A. R., Wilde, P. J., Fenelon, M. A. & Brodkorb, A. Structural mechanism and kinetics of in vitro gastric digestion are affected by process-induced changes in bovine milk. *Food Hydrocoll.* **86**, 172–183 (2019).
14. Picariello, G. *et al.* Peptides surviving the simulated gastrointestinal digestion of milk proteins: Biological and toxicological implications. *J.*

Chromatogr. B Anal. Technol. Biomed. Life Sci. **878**, 295–308 (2010).
15. Dupont, D. *et al.* Comparative resistance of food proteins to adult and infant in vitro digestion models. *Mol. Nutr. Food Res.* **54**, 767–780 (2010).
16. Kitabatake, N. & Kinekawa, Y. I. Digestibility of Bovine Milk Whey Protein and β-Lactoglobulin in Vitro and in Vivo. *J. Agric. Food Chem.* **46**, 4917–4923 (1998).
17. Egger, L. *et al.* The harmonized INFOGEST in vitro digestion method: From knowledge to action. *Food Res. Int.* **88**, 217–225 (2016).
18. Sánchez-Rivera, L., Ménard, O., Recio, I. & Dupont, D. Peptide mapping during dynamic gastric digestion of heated and unheated skimmed milk powder. *Food Res. Int.* **77**, 139–132 (2015).
19. Sanchón, J. *et al.* Protein degradation and peptide release from milk proteins in human jejunum. Comparison with in vitro gastrointestinal simulation. *Food Chem.* **239**, 486–494 (2018).
20. Nielsen, S. D., Beverly, R. L., Qu, Y. & Dallas, D. C. Milk bioactive peptide database: A comprehensive database of milk protein-derived bioactive peptides and novel visualization. *Food Chem.* **232**, 673–682 (2017).
21. Tavares, T. *et al.* Novel whey-derived peptides with inhibitory effect against angiotensin-converting enzyme: In vitro effect and stability to gastrointestinal enzymes. *Peptides* **32**, 1013–1019 (2011).
22. Lacroix, I. M. E. & Li-Chan, E. C. Y. Isolation and characterization of peptides with dipeptidyl peptidase-IV inhibitory activity from pepsin-treated bovine whey proteins. *Peptides* **54**, 39–48 (2014).
23. Hochwallner, H. *et al.* Visualization of clustered IgE epitopes on α-lactalbumin. *J. Allergy Clin. Immunol.* **125**, (2010).
24. Kringelum, J. V., Nielsen, M., Padkjær, S. B. & Lund, O. Structural analysis of B-cell epitopes in antibody: Protein complexes. *Mol. Immunol.* **53**, 24–34 (2013).
25. Buus, S. *et al.* High-resolution mapping of linear antibody epitopes using ultrahigh-density peptide microarrays. *Mol. Cell. Proteomics* **11**, 1790–1800 (2012).
26. Lacroix, I. M. E., Meng, G., Cheung, I. W. Y. & Li-Chan, E. C. Y. Do whey protein-derive peptides have dual dipeptidyl-peptidase IV and angiotensin I-converting enzyme inhibitory activities? *J. Funct. Foods* **21**, 87–96 (2016).
27. Cong, Y. J. & Li, L. F. Identification of the Critical Amino Acid Residues of Immunoglobulin E and Immunoglobulin G Epitopes in α-Lactalbumin by Alanine Scanning Analysis. *J. Dairy Sci.* **95**, 6307–6312 (2012).

28. Cerecedo, I. *et al.* Mapping of the IgE and IgG4 sequential epitopes of milk allergens with a peptide microarray-based immunoassay. *J. Allergy Clin. Immunol.* **122**, 589–594 (2008).
29. Brandelli, A., Daroit, D. J. & Corrêa, A. P. F. Whey as a source of peptides with remarkable biological activities. *Food Res. Int.* **73**, 149–161 (2015).
30. Verhoeckx, K. *et al.* The relevance of a digestibility evaluation in the allergenicity risk assessment of novel proteins. Opinion of a joint initiative of COST action ImpARAS and COST action INFOGEST. *Food Chem. Toxicol.* **129**, 405–423 (2019).
31. Boutrou, R. *et al.* Sequential release of milk protein – derived bioactive peptides in. *Am. J. Clin. Nutr.* **97**, 1314–1323 (2013).
32. Rodríguez-Figueroa, J. C., González-Córdova, A. F., Torres-Llanez, M. J., Garcia, H. S. & Vallejo-Cordoba, B. Novel angiotensin I-converting enzyme inhibitory peptides produced in fermented milk by specific wild Lactococcus lactis strains. *J. Dairy Sci.* **95**, 5536–5543 (2012).
33. Mandalari, G. *et al.* In vitro digestibility of β-casein and β-lactoglobulin under simulated human gastric and duodenal conditions: A multi-laboratory evaluation. *Regul. Toxicol. Pharmacol.* **55**, 372–381 (2009).
34. Pihlanto-Leppälä, A. Bioactive peptides derived from bovine whey proteins: Opioid and ace-inhibitory peptides. *Trends Food Sci. Technol.* **11**, 347–356 (2000).

Chapter 5

Evaluation of the effects of processing on whey proteins digestibility and allergenicity

5.1 Introduction

Whey ingredients are used to increase food nutritional value (i.e. sports supplements, infant formulas, meat, bakery, dairy, confectionaries, etc.). Moreover, they are used also as technological agents for thermal stability, emulsification, gelation and foam formation[1]. Whey proteins are isolated from whey with membrane-based separation techniques, such as the ultrafiltration (UF), that concentrates proteins coupled to diafiltration (DF) that remove lactose, minerals and low molecular components[2]. Whey protein concentrates (WPC) are usually classified by their protein content: WPC35, WPC50, WPC65 and WPC80 are the most common products and the number indicates the percentage of proteins content (w/w). Instead, whey protein isolates (WPI) have a higher amount of proteins, more than 90%[3]. After being concentrated, these proteins are usually spray-dried in order to have a product more stable for storage and transport[2,4]. During the production of WPCs and WPIs, the protein content can be altered affecting its nutritional value. Among the others, denaturation and aggregation phenomena can occur, affecting both protein digestibility and allergenicity[5]. Indeed, denatured proteins may expose more cleavage sites to the action of the digestive enzymes, affecting their digestibility.

In parallel, since whey proteins are known allergens, changes in proteins structure may cause the loss of the conformational epitopes[6]. Due to the technological treatments, proteins can be involved also in the Maillard reaction (reaction between lactose and lysine residues). The presence of lactose bound to them alters their primary and secondary structure and can affect their properties such as the digestibility, or modulate their functionalities, such as allergenicity. Recent studies showed that mostly all the lysine residues can be involved in the lactosylation[7,8]. This means that also lysine residues present in the epitopes' sequences could be modified as well,

resulting in a possible effect of the sugar in the binding with the human IgE, which is not known so far.

Apart from processing, another parameter that may influence protein allergenicity is the gastrointestinal digestion. During digestion, depending on accessibility and solubility, proteins are released from food at different levels of ingestion. Then, enzymatic hydrolysis occurs from the stomach, through the duodenum and finally in the mucus layer, where there are brush border enzymes before the intestinal absorption[9]. Commonly the resistance of the protein to the gastrointestinal digestion was considered fundamental, as the potential allergic response was attributed to intact proteins[10] or large protein fragments, with a lower size limit of nearly 30 amino acids or 3 kDa[11]. However, more recently, it was reported that also smaller peptides may still induce an allergic response[12,13]. The relationship between allergenicity and peptides molecular weight is relevant for the production of hypoallergenic formulas, which are commonly categorized as partially (mixtures with molecular weight between 3 and 10 kDa) or extensively (mixtures with molecular weight lower than 3 kDa) hydrolysed[14]. The effects of the gastrointestinal digestion on the allergenicity are different: it was reported that it may reduce, eliminate or increase the allergic response with respect to the undigested protein, in some cases it may even not induce any changes[15]. Important parameters in the evaluation of the effects of digestibility on allergenicity are the selection of the right digestion model, the effect of the pH and processing, among the others (i.e. the use of Brush Border Membrane (BBM) enzymes, the influence of the microbiota, the effect of the food matrix)[15]. Thus, processing and gastrointestinal digestion are of big relevance in determining proteins allergenicity.

The aim of this study is to investigate the effects of processing on whey proteins digestibility and allergenicity. To evaluate the effects on digestibility, a sample of whey proteins isolates (WPI) and a sample of whey obtained after cheese production (CW) were digested applying the semi-dynamic model described in Chapter 4. Digested fractions were analysed by HPLC-MS/MS to evaluate protein digestibility also through the released peptides. ELISA inhibition test was performed to evaluate the effects on the allergenicity. This study

was performed at the Institute of Food Science Research (CIAL, CSIC-UAM, Madrid), in collaboration with the BIOPEP research group.

5.2 Materials and methods

5.2.1 Reagents

Whey protein isolates (WPI) were purchased on a local store. Raw milk was purchased from a local milk producer. Pepsin from porcine gastric mucosa 3,200-4,500U/mg, Pancreatin from porcine pancreas, KCl, KH_2PO_4, $NaHCO_3$, Bile salts, Dithiothreitol (DTT), Tris-HCl, β-Mercaptoethanol, NaOH, Formic acid 98-100%, Na_2HPO_4, Tween 20 and Urea were all purchased from Sigma Aldrich (St. Luis, MO, USA). $MgCl_2$, Sodium dodecyl sulphate (SDS), Trifluoroacetic Acid (TFA) and Bromophenol Blue were purchased from Merk. NaCl, $(NH_4)_2CO_3$, $CaCl_2$, NH_4HCO_3, HCl, glass wool and glycerol were purchased from Panreac (ITW reagents, Barcelona, Spain). Acetonitrile and H_2SO_4 96% were purchased from VWR (Milford, MA, USA). Criterion™ XT precast gel 12% Bis-Tris, Running Buffer 20x, Precision Plus Protein™ Unstained protein standards were purchased from BIO-RAD (Hercules, CA, USA). Instant blue™ protein stain was purchased from Expedeon (Cambridge, United Kingdom). Sinapinic Acid and Bacterial Test Standard (BTS) were purchased from Bruker Daltonics (Bruker, Billerica, MA USA). Polyclonal Rabbit Anti-Human IgE and Polyclonal Swine Anti-Rabbit IgG were purchased from Dako Denmark A/S (Glostrup, Denmark). TMB 1x Substrate solution was purchased from eBioscience (Thermo Fisher Scientific, Waltham, MA, USA). Serum samples from milk allergic patients were obtained from our human blood serum bank. Human samples were collected with written consent from the next of kin, caretakers, or guardians on the behalf of the minors/children involved in the performed studies. All experiments were conducted according to the principles expressed in the Declaration of Helsinki and the Bioethics Committee from the Consejo Superior de Investigaciones Científicas (CSIC), Spain. The researchers involved in the process handled and used such material sensitively and responsibly. Individual features of human sera are shown in Table 5.2.1.

Table 5.2.1 Individual sera of the pool used for the ELISA IgE inhibition test (na: data not available).

Patient	Age (years)	IgE (kU/L) Cow milk	IgE (kU/L) Casein	IgE (kU/L) β-LG	IgE (kU/L) α-LA
1	2	na	12.1	17.2	33.9
2	1	35.5	37.7	3.7	22.5
3	1	>100	>100	>100	>100
4	2	30.9	14.7	23.7	0.7
5	7	41.0	34.0	12.0	14.0

5.2.2 Whey production from cheese (CW sample)

Nearly 3 L of raw milk was collected from a local milk producer. Sample was previously defatted centrifuging at 4000 rpm at 5 °C for 20 min and then removing the fat manually. The defatted milk was filtered on wool glass and then used to produce cheese. Nearly 2 L of sample was used and warmed to 33°C. Then 50,62 mg of $CaCl_2$ was added, sample was mixed for 2 min and 500 µl of rennet was added. After 3 min mixing it was left at 33°C for 1h. The obtained cheese was cut in small pieces and filtered on a strainer covered with the proper cloth. The obtained whey was then centrifuged at 4000 g and 5°C for 10 min and filtered on glass wool. Aliquots were stored at -20°C.

5.2.3 MALDI-TOF characterization

Samples were characterized with MALDI-TOF using a Bruker Autoflex Speed spectrometer (Bruker Daltonics GmbH, Bremen, Germany). Samples were dissolved in MilliQ water at 1% of protein concentration. Then they were diluted 1:100 in MilliQ water. One µl was loaded on a Bruker MTP 384 Polished Steel target. Then on this dry spot was loaded 1 µl of sinapinic acid, used as matrix. For calibration 0,5 µl of BTS was loaded on the target. Then 0,5 µl of sinapinic acid was loaded on the dry spot and allowed to dry prior to analysis.

Table 5.2.2 Molecular weight of the whey proteins identified in the MALDI-TOF spectra.

	Uniprot	**CW spectrum**	**WPI Spectrum**
α-lactalbumin	14176 Da	14173 Da	14177 Da
β-lactoglobulin B	18269 Da	18265 Da	18274 Da
β-lactoglobulin A	18355 Da	18352 Da	18343 Da

5.2.4 Determination of protein content

In the two samples, whey protein isolates and whey extracted from cheese, the percentage of nitrogen was determined with Kjeldahl analysis as described in Chapter 3 paragraph 3.2.3. The percentage of protein was determined multiplying the obtained value for the conversion factor (6,38).

5.2.5 In vitro semi-dynamic digestion

The *in vitro* semi-dynamic digestion experiments were performed following the INFOGEST protocol[16] as described in Chapter 4 paragraph 4.2.3. The two samples, whey from cheese (CW) and whey protein isolates (WPI) were used at the same protein concentration (0.95%, as determined from Kjeldahl analysis for the CW sample). For the Simulated Gastric Fluid (SGF) the amount of HCl required to reach pH 2 was determined before the application of the protocol in a test experiment. SGF composition for WPI digestion was 79,4% of SGF 1.25x at pH 7 prepared following the mentioned protocol, 0,98 % of 6M HCl, 0,05% of 0.3M $CaCl_2(H_2O)_2$, 0,62% of pepsin, 18,97% of MilliQ water. The SGF composition for the digestion of CW was the same except for 6M HCl that was 1,66%, and MilliQ water was 18,29%. Five aliquots were collected from the digestion at 18 min, 36 min, 72 min, 54 min and 90 min (nearly 25 mL) in order to mimic the gastric emptying. Fractions collected for each sample were analysed with UPLC-UV as described in paragraph 4.2.7. UV detection was performed at 214 nm. Fractions 1, 3 and 5 were also analysed with HPLC-MS/MS analysis following the protocol described in paragraph 4.2.5. For peptides visualization, it was used the web application Peptigram[17]. Uniprot[18] whey proteins sequences were used (code P00711 for α-lactalbumin and P02754 for β-lactoglobulin). For

Peptigram's graphs signal peptide sequence was maintained, while for the discussion and in the tables it was removed from the amino acids numbering.

5.2.6 SDS-PAGE analysis

The SDS-PAGE analysis was performed following the protocol described in Chapter 4 paragraph 4.2.6.

5.2.7 In vitro static intestinal digestion

The *in vitro* static intestinal digestion was performed following the protocol described in Chapter 4 paragraph 4.2.4. Samples were analysed with HPLC-MS/MS analysis as described in paragraph 4.2.5.

5.2.8 ELISA Inhibition test

For the ELISA test, tested samples were: undigested WPI and CW, Fractions 1,3 and 5 from the gastric digestion, Fractions 1,3, and 5 from the intestinal digestion. As a control, a reference sample of standard whey proteins was used. Samples were prepared at a concentration of 9.5 mg/mL in PBS pH 7.4 (stock solutions). From each stock solution, dilutions were made as reported in Table 5.2.3 and Table 5.2.4.

Table 5.2.3 Dilutions prepared for WPI, CW and control sample.

	Concentration
Std 1	100 µg/mL
Std 2	10 µg/mL
Std 3	3.3 µg/mL
Std 4	1 µg/mL
Std 5	0.3 µg/mL
Std 6	0.1 µg/mL
Std 7	0.03 µg/mL
Std 8	0.01 µg/mL

Table 5.2.4 Dilutions prepared for gastric and intestinal digested fractions.

	Concentration
Std 1	1000 µg/mL
Std 2	100 µg/mL
Std 3	10 µg/mL
Std 4	1 µg/mL
Std 5	0.1 µg/mL

The ELISA test was performed in triplicate. Plates (Corning Costar Assay plates, 96 wells) were coated with the reference sample of standard whey proteins diluted in PBS pH 7.4 (50 µg/mL) pouring 50 µl in each well. Plates were left overnight at 4°C. Then were left for 4 hours at room temperature with 250 µl of PBST 2,5%. Meanwhile, for each stock solution and prepared dilution (Tables 5.2.2 and 5.2.3), 80 µl were mixed with 80 µl of patient sera (pool of sera diluted 1:50 in PBST 0.05%) and incubated for 2 hours at room temperature at 300 rpm. Samples were poured in the wells and left overnight at 4°C. In the plates also 2 control samples were introduced: the positive control (80 µl of patients sera diluted 1:50 in PBST 0.05% and 80 µl of PBS) and the negative control (80 µl of whey protein standard 9.5 mg/mL and 80 µl of PBST 0.05). Plates were incubated first with polyclonal rabbit Anti-Human IgE (diluted 1:1000 in PBST 0.05%) for one hour at room temperature, then with polyclonal swine Anti-Rabbit IgG-HRP (diluted 1:2000 in PBST 0.05%) for 30 min at room temperature. Amplification of the signal was performed using the ELAST® ELISA Kit. Plates were incubated with Biotinyl tyramide 5 µl/mL (diluted 1:1 with water) for 15 min at room temperature. Then they were incubated with Streptavidin-HRP (dilute 1: 2000 in PBST 0.05%) for 30 min at room temperature. Then 50 µl of TMB was added in each well and rapidly after 50 µl of H_2SO_4 0.5M was added to stop the reaction. After each step of the procedure and before the addition of TMB, plates were washed 4 times with PBST 0.005% (Biotek ELx50 Microplate Washer). Absorbance was measured at 450 nm with a Multiskan™ FC Microplate Photometer (ThermoFisher Scientific). Statistical analysis (Unpaired T test) was performed with the Prism software (GraphPad, San Diego, CA, USA). UPLC-UV analysis on samples (stock solutions) was performed as described in Chapter 4 paragraph 4.2.8.

5.3 Results and Discussion

5.3.1 Protein Characterization

In order to evaluate the effects of the processing on whey proteins in the production of whey protein isolates, a sample of untreated whey was produced from raw milk. This sample was obtained from the homemade production of cheese, as most of the whey proteins that are commercially available derive from whey collected after cheese production. Whey produced from cheese (CW) and whey protein isolates (WPI) were analysed with MALDI-TOF mass spectrometry.
Figures 5.3.1 and 5.3.2 show the obtained spectra in which is possible to identify the two principal whey proteins, α-lactalbumin (α-LA) and β-lactoglobulin (β-LG).

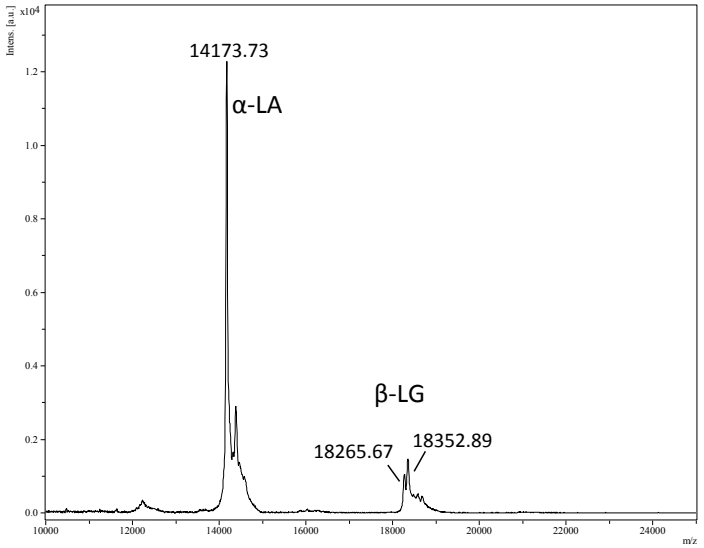


Figure 5.3.1 MALDI-TOF spectrum of CW sample (α-LA= α-lactalbumin; β-LG= β-lactoglobulin). Intensity is on the y axis, mass-to-charge ratio is on the x axis.

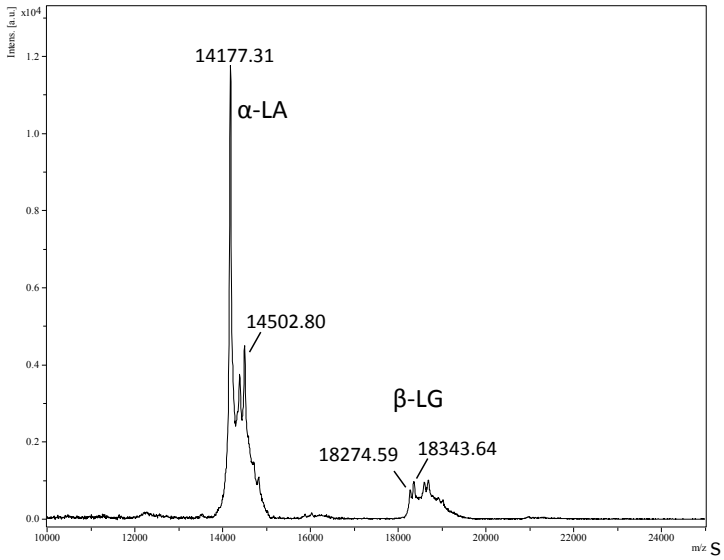


Figure 5.3.2 MALDI-TOF spectrum of WPI sample (α-LA= α-lactalbumin; β-LG= β-lactoglobulin). Intensity is on the y axis, mass-to-charge ratio is on the x axis.

In the WPI sample, apart from the signal relative to α-lactalbumin (14177 Da), a signal corresponding to the addition of 324 Da to the protein mass was also detected (14502 Da). This signal may be attributed to the lactosylated form of the same protein (lactose molecular weight is 324 Da). WPI processing may induce chemical modifications to the proteins, like lysine lactosylation (first step of the Maillard reaction). This finding may be confirmed looking at the CW spectrum where this signal was not detected. These samples will be further analysed with UPLC-ESI-MS analysis applying the protocol described in Chapter 1[19] to better investigate the difference in protein lactosylation among the two samples.

5.3.2 Semi-dynamic gastric digestion

The *in vitro* gastrointestinal digestion was performed following the protocol reported in literature[16,20] and described in Chapter 4. In particular, the gastric phase was performed in a semi-dynamic way, while the intestinal phase in a static way. Total digestion time of the gastric phase was 90 min in which five fractions were collected (Table 5.3.1) simulating the gastric emptying.

Table 5.3.1 List of the fractions collected during the digestion

	Digestion time (min)	Volume (mL)
Fraction 1 (F1)	18,08	25
Fraction 2 (F2)	36,15	25
Fraction 3 (F3)	54,23	25
Fraction 4 (F4)	72,30	25
Fraction 5 (F5)	90,38	25

In order to compare the results, protein percentage of the undigested sample was determined by Kjeldahl (80,5% for WPI and 0,95% for CW) and samples were digested at the same protein concentration (0,95%). In figure 5.3.3 are reported the pH profiles of the two gastric phases performed.

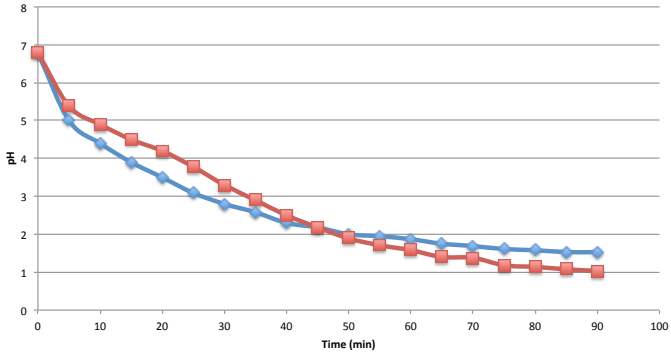


Figure 5.3.3 pH profiles of the gastric digestions of WPI (blue line) and CW (red line).

As it is possible to observe, the trends are quite similar. Both the samples start from the same pH value (6.8) after the initial addition of the gastric phase and reach a final pH of 1,05±0,02 (1,47±0,09 for WPI). The pH profiles were confirmed with the external pH measurements performed measuring the pH of the collected fractions. The obtained pH profiles are in agreement with the findings described and discussed in Chapter 4 paragraph 4.3.2.

5.3.3 Protein degradation

The digested samples collected from the two gastric digestions were analysed with SDS-PAGE and UPLC-UV analysis in order to follow whey proteins degradation during digestion.

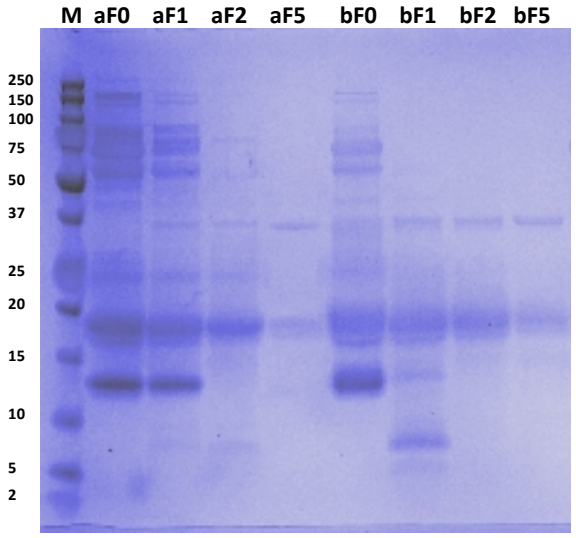


Figure 5.3.4 SDS-PAGE gel image of the two gastric digestions performed on CW (a) and WPI (b) samples (F0= undigested protein; F1= Fraction 1; F2= Fraction 2; F5= Fraction 5). Marker values are expressed as kDa.

In figure 5.3.4 SDS-PAGE results of the two digestions are reported. For each case the following fraction are present: 1 (18 min), 2 (36 min), 5 (90 min) plus the undigested sample (F0). Bands relative to β-lactoglobulin at around 18 kDa are clearly detectable as well as a little decrease in the intensity of the signals from fraction 1 to 5 can be observed. This finding could be due both to a partial digestion of the protein in the gastric phase and to the different dilution of the five samples. The band corresponding to α-lactalbumin, at around 14 kDa is present only in Fraction 1. Being absent in Fraction 2, it can be assumed that this protein is completely digested in the first 36 min. This finding is in agreement with what is reported and discussed in Chapter 4 paragraph 4.3.3. Comparing the two digestions, they show the same profile with a slight difference for Fraction 1 (lines aF1 and bF1) where for the WPI sample the band relative to α-lactalbumin is

very faint. At around 35 kDa it is possible to see the presence of pepsin in all the digested fractions.
Fractions collected were analysed with UPLC-UV and the obtained chromatograms are reported in figures 5.3.5 and 5.3.6.

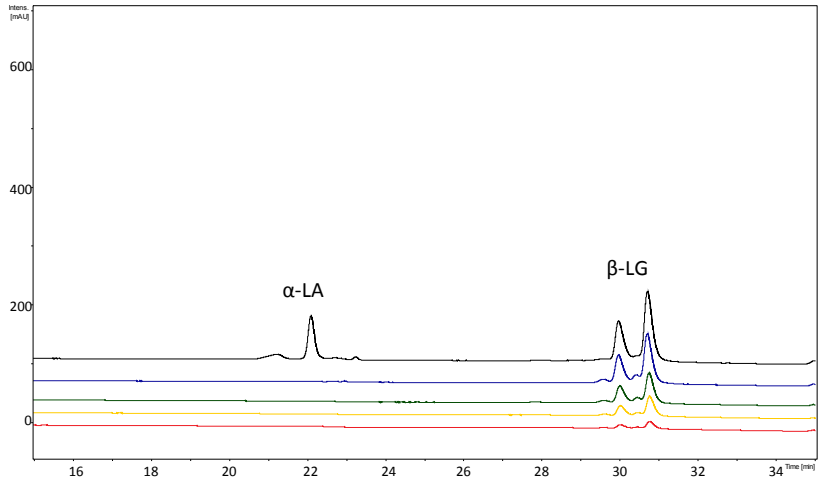


Figure 5.3.5 UPLC-UV chromatograms of the five collected fractions from the digestion of the CW (Fraction 1 in black, Fraction 2 in blue, Fraction 3 in green, Fraction 4 in yellow and Fraction 5 in red). Intensity is on the y axis (mAU) while time on the x axis (min).

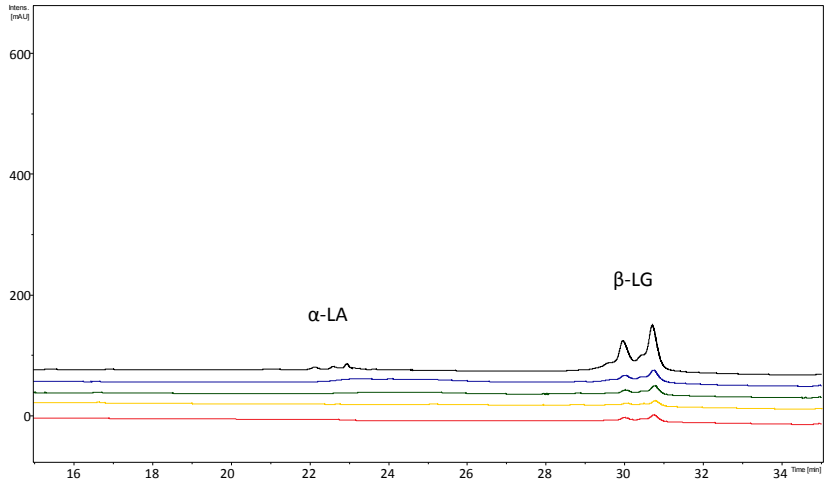


Figure 5.3.6 UPLC-UV chromatograms of the five collected fractions from the digestion of the WPI (Fraction 1 in black, Fraction 2 in blue, Fraction 3 in green, Fraction 4 in yellow and Fraction 5 in red). Intensity is on the y axis (mAU) while time on the x axis (min).

For both the samples is possible to observe the disappearance of α-lactalbumin after Fraction 1, as anticipated from the SDS-PAGE analysis, and the decrease in intensity of β-lactoglobulin signal with the advancement of digestion. Using an external calibration curve, whey proteins in the collected fractions were quantified by integration of the chromatographic peaks areas. In the quantification, dilution factors were calculated considering the gastric emptying and the rate of gastric solution addition. This factor was applied to better evaluate protein degradation. Results are reported in figure 5.3.7 and 5.3.8.

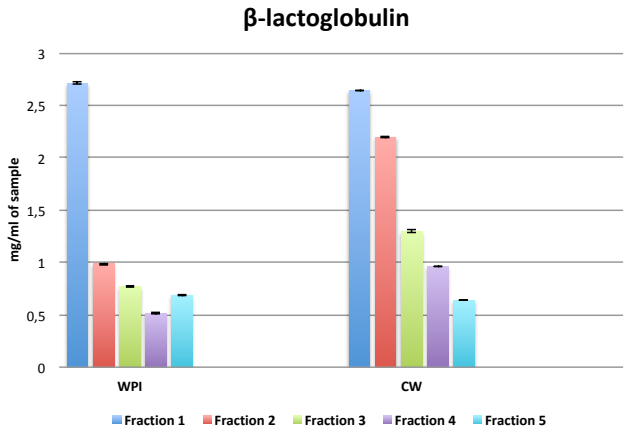


Figure 5.3.7 β-lactoglobulin quantification in the five collected fractions from WPI and CW gastric digestion (Fraction 1=18 min, Fraction 2= 36 min, Fraction 3= 54 min, Fraction 4= 72 min, Fraction 5= 90 min).

Regarding β-lactoglobulin quantification, some consideration may be drawn. As already discussed in Chapter 4, this protein is known to be resistant to gastric digestion. It is possible to observe that the amount of β-lactoglobulin in Fraction 1 is similar for both the samples, but it slowly decreases during digestion. During CW digestion, β-lactoglobulin amount decreases gradually from Fraction 1 to Fraction 5. During WPI digestion there is a significant difference between Fraction 1 and the other four fractions. Moreover, in the last fraction, there is a little increase of protein with respect to Fraction 4. In the last part of the gastric digestion, the volume was reduced and the sample was not completely homogenous creating some difficulties in sample

collection. Thus, the observed differences in the last part of the digestion (Fractions 4 and 5) might be ascribed to technical issues.
Differences in the digestibility of this protein between the two samples lead to the assumption that the WPI sample is more digestible than the CW one. This may be related to the processing involved in the production of WPI that may affect protein structures, denaturing them and making them more digestible, as discussed in Chapter 4. As an example, differences in β-lactoglobulin digestibility between heated and unheated skimmed milk powder were recently reported in a dynamic gastric digestion study, resulting in higher resistance to digestion of the unheated sample[21]. Another example, a digestion study performed with the semi-dynamic model on raw milk and UHT milk, reported a lower degree of hydrolysis of β-lactoglobulin in the gastric phase for the raw milk[22].

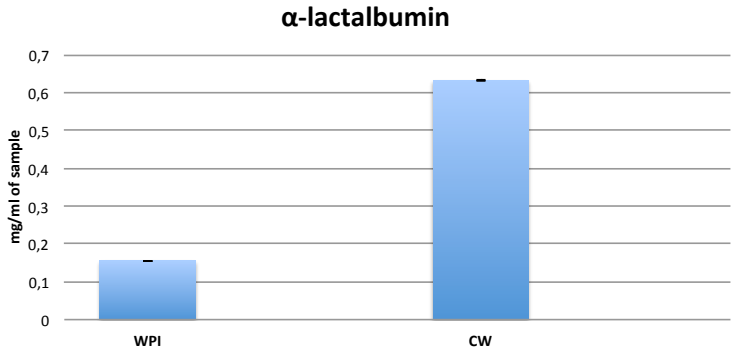


Figure 5.3.8 α-lactalbumin quantification in gastric Fraction 1 (18 min) of WPI and CW samples digestions.

Alpha-lactalbumin was detected only in Fraction 1 showing a different amount in the two samples. This finding might be the result of a higher susceptibility to digestion due to changes in the native structure induced by the processing in the WPI sample, similarly to what discussed for β-lactoglobulin. Moreover, processing might have induced protein denaturation and aggregation, thus decreasing the amount of soluble α-lactalbumin with respect to the untreated sample (CW).

5.3.4 Peptides identification in CW sample after gastric semi-dynamic digestion

The five collected fractions obtained from the gastric digestion were analysed with HPLC-MS/MS analysis to monitor peptide release during digestion. Peptides were identified with MASCOT software using a homemade database for whey proteins. In figures 5.3.9 and 5.3.10 are reported the peptides identified in Fraction 1, 3 and 5 of the gastric phase of the two whey proteins in exam.

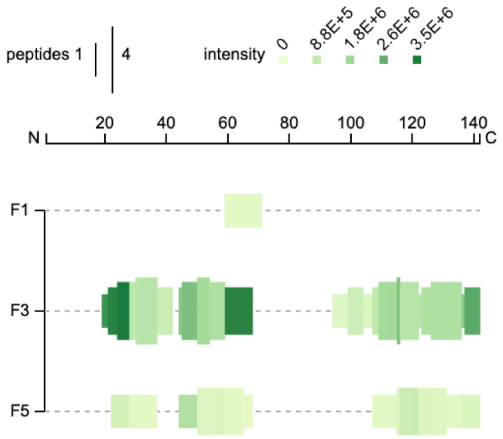


Figure 5.3.9 α-lactalbumin peptides identified in Fraction 1, Fraction 3 and Fraction 5 of the gastric phase (CW sample). In the graph, the amino acids that were identified in peptide sequences are represented with vertical bars. The intensity of the colour is proportional to the sum of identified peptides' intensities. The dimension of the bar is proportional to the number of peptides in which was identified that amino acid in that position. The first 19 amino acids of the graph are the ones relative to the signal peptide.

Figure 5.3.9 shows the peptides identified in α-lactalbumin. In Fraction 1 (18 min) it was possible to identify only a peptide: IVQNNDSTEYGL (41-52 amino acids residues). In Fraction 3 (54 min) were identified peptides deriving from two main regions (1-52 and 76-123 amino acids residues) of protein sequence. The amount of peptides is lower in Fraction 5 suggesting the advancement of digestion. In fact, by the end of the gastric phase α-lactalbumin is completely digested. In Table 5.3.2 the list of identified peptides in the whole gastric digestion is reported. Peptides length varies from 5 to 15 amino acids residues in

Fraction 3 and from 6 to 14 in Fraction 5. For some of them the potential reported[23] bioactivity is indicated.

Table 5.3.2 List of all the α-lactalbumin peptides identified in the samples and their potential reported bioactivity (signal peptide amino acids were subtracted).

Peptide sequence	**Amino acids residues**	**Reported Bioactivity**
IVQNNDSTEYGL	41-52	None
IMCVKKILDKVGINY	89-103	None
RELKDLKGYGGVSL	10-23	None
AIVQNNDSTE	40-49	None
LAHKALCSEKLDQ	105-117	None
WLAHKALCSEKLDQ	104-117	DPP IV-inhibitory activity[24]
CEVFRE	6-11	None
WLCEKL	118-123	None
HTSGYDTQA	32-40	None
WVCTTF	26-31	None
LTKCEVF	3-9	None
LCEKL	119-123	None
DKVGINY	97-103	ACE-inhibitory activity[25]
CVKKILD	91-97	None
LKDLKGY	12-18	None
YDTQA	36-40	None
SCDKFLDDDL	76-85	None
LDDDLTDD	81-88	None
IMCVKKILD	89-97	None
EQLTKCEVF	1-9	None
LKDLKGYGGVSL	12-23	None
WVCTTFHTSG	26-35	None
KVGINY	98-103	None
HTSGYDTQ	32-39	None
KALCSEKLDQ	108-117	None
RELKDLKGY	10-18	None
VCTTFHTSG	27-35	None

IVQNNDSTE	41-49	None
IVQNND	41-46	None
TKCEVF	4-9	None
WLAHKALCS	104-112	None

Regions 1-19, 13-26 and 90-123 of protein sequence are reported in literature to be IgE-binding epitopes[26,27]. It was previously reported that the estimated length of an epitope might be around 12-15 amino acids[28,29], some peptides deriving from these regions could be potentially IgE-binding peptides (Table 5.3.3).

Table 5.3.3 Identified α-lactalbumin peptides that could be IgE-binding epitopes (signal peptide amino acids were subtracted).

Peptide sequence	Amino acids residues	Digestive fraction
IMCVKKILDKVGINY	89-103	Fraction 3
RELKDLKGYGGVSL	10-23	Fraction 3
LKDLKGYGGVSL	12-23	Fraction 3
LAHKALCSEKLDQ	105-117	Fraction 3
WLAHKALCSEKLDQ	104-117	Fractions 3 and 5

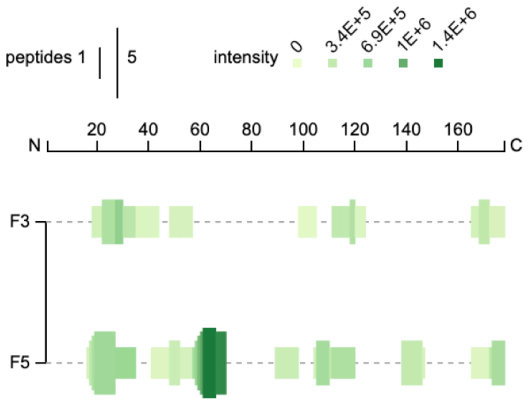


Figure 5.3.10 β-lactoglobulin peptides identified in Fractions 3 and 5 of the gastric phase (CW sample). In the graph, the amino acids that were identified in peptide sequences are represented with vertical bars. The intensity of the colour is proportional to the sum of identified peptides' intensities. The dimension of the bar is proportional to the number of peptides in which was identified that amino acid in that position. The first 16 amino acids of the graph are the ones relative to the signal peptide.

As emerges from Figure 5.3.10, in Fraction 1 it was not possible to identify peptides. A possible explanation could be an instrumental limitation in the detection due to the very low degree of hydrolysis in this fraction. A little amount of peptides appears in Fraction 3 and increase in Fraction 5. Peptide identification confirmed a partial digestion of β-lactoglobulin in the gastric phase. Peptides were identified in regions 1-54, 74-108, 123-131 (only in Fraction 5), 150-162 amino acids residues. Below the list of identified peptides reported in Table 5.3.4.

Table 5.3.4 List of all the β-lactoglobulin peptides identified in the samples and their eventual reported bioactivity (signal peptide amino acids were subtracted).

Peptide sequence	**Amino acids residues**	**Reported Bioactivity**
DAQSAPLRV	33-41	None
SFNPTQL	150-156	None
VTQTMKGLD	3-11	None
LLFCME	103-108	None
YSLAMAASD	20-28	None
PTQLEEQCHI	153-162	None
DTDYKKYLL	96-104	None
IQKVAGTW	12-19	Ace inhibitor/ DPPIV inhibitor[30]
KIDALNE	83-89	None
MKGLDIQK	7-14	None
NKVLVL	90-95	None
TQTMKGLD	4-11	None
EEQCHI	157-162	None
EQCHI	158-162	None
VEELKPTP	43-50	None
ENKVLV	89-94	None
YVEELKPTPEGDL	42-54	None
VRTPEVDDE	123-131	None
EKTKIPAVF	74-82	None
VRTPEVDD	123-130	None
LIVTQTMKGLD	1-11	None

LKPTPEGDL	46-54	DPP IV- inhibitory activity[24]
ELKPTPEGDL	45-54	None
EELKPTPEGDL	44-54	None
ASDISLLDAQS	26-36	None
IVTQTMKGLD	2-11	None

Peptides length ranges from 6 to 10 in Fraction 3 and 5 to 13 amino acid residues in Fraction 5. Regions 1-16, 31-60, 76-95, 121-140 are known to be IgE-binding epitopes,[27,31,32] so peptides identified in these regions could probably be epitopes (Table 5.3.5).

Table 5.3.5 Identified β-lactoglobulin peptides that could be IgE-binding epitopes (signal peptide amino acids were subtracted).

Peptide sequence	Amino acids residues	Digestive fraction
LIVTQTMKGLD	1-11	Fraction 5
EELKPTPEGDL	44-54	Fraction 5
YVEELKPTPEGDL	42-54	Fraction 5

As described in Chapter 4, the intestinal phase was performed in a static way on fractions 1, 3 and 5. The intestinal digested samples will be named as the corresponding gastric fractions. The intestinal samples were analysed with HPLC-MS/MS for peptides identification. In table 5.3.6 are reported the peptides identified deriving from α-lactalbumin.

Table 5.3.6 List of the α-lactalbumin peptides identified in the samples (signal peptide amino acids were subtracted).

Peptide sequence	Amino acid residues	Fraction 1	Fraction 3	Fraction 5
NNDSTE	44-49	x		
NDSTE	45-49		x	
CKDDQNPH	61-68	x	x	x
DDQNPH	63-68	x	x	x
LDDDL	81-85		x	
LDDDLTDD	81-88		x	
DDDLTDDI	82-89	x		
KVGIN	98-102		x	

The peptides identified are very few as expected as this protein is completely digested in the gastric phase. Interestingly it was observed that peptide LDDDLTDD was identified both in the gastric and intestinal phase. In figure 5.3.11 are reported the β-lactoglobulin peptides that were identified.

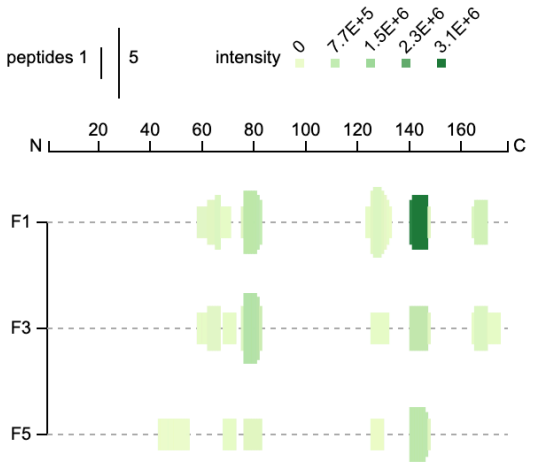


Figure 5.3.11 β-lactoglobulin peptides identified in the three intestinal digested sample (CW sample). In the graph, the amino acids that were identified in peptide sequences are represented with vertical bars. The intensity of the colour is proportional to the sum of identified peptides' intensities. The dimension of the bar is proportional to the number of peptides in which was identified that amino acid in that position. The first 16 amino acids of the graph are the ones relative to the signal peptide.

Peptides were identified mainly in regions 28-86, 108-132 and 149-159 (Fractions 1 and 3) amino acid residues. Peptide length varies from 5 to 9 amino acid residues in Fraction 1 and 3, in Fraction 5 it varies from 5 to 8 amino acid residues with the exception of one peptide of 12. As it is possible to see in the figure the intensity and the length of the peptides decrease from Fraction 1 to 5 suggesting the advancement of the digestion. In table 5.3.7 is reported the list of identified peptides.

Table 5.3.7 List of all the β-lactoglobulin peptides identified in the samples (signal peptide amino acids were subtracted).

Peptide sequence	Amino acids residues	Peptide sequence	Amino acids residues
VEELKPTPE	43-51	ENSAEP	108-113
TPEVDDEA	125-132	TPEVDDE	125-131
PEVDDE	126-131	KPTPE	47-61
KWENDECA	60-67	WENDECA	61-67
WENDEC	61-66	SAEPEQS	110-116
LSFNPT	149-154	ENSAEPE	108-114
PEGDLE	50-55	KWENDEC	60-66
SFNPT	150-154	DLEIL	53-57
SAEPE	110-114	QLEEQ	155-159
SAEPEQ	110-115	DISLLDAQSAPL	28-39
WENGE	61-65	TPEVDD	125-130

5.3.5 Peptides identification in Whey Protein Isolates (WPI) digestion

After semi-dynamic gastric digestion, the five collected fractions were analysed with HPLC-MS/MS analysis. Results are showed below.

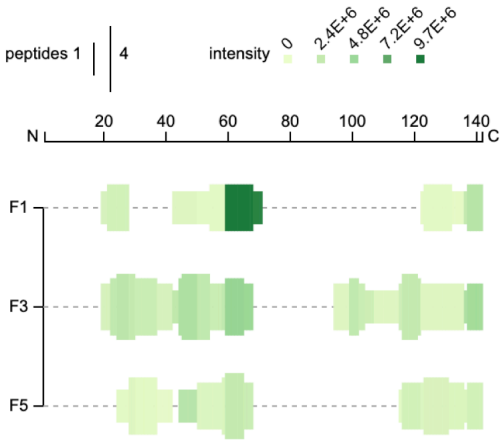


Figure 5.3.12 α-lactalbumin peptides identified in the three fractions of the gastric phase (WPI sample). In the graph, the amino acids that were identified in peptide sequences are represented with vertical bars. The intensity of the colour is proportional to the sum of identified peptides' intensities. The dimension of the bar is proportional to the number of peptides in which was

identified that amino acid in that position. The first 19 amino acids of the graph are the ones relative to the signal peptide.

With respect to the CW digestion mixtures, here in Fraction 1 were identified more peptides. The different distribution among the fractions (higher in Fraction 3 and lower in the others) suggests the digestion advancement. Two main regions were identified in protein sequence from which peptide derives: 1-52 and 76-123. Peptides length varies from 5 to 14 amino acids residues in all the fractions. In Table 5.3.8 is reported the list of identified peptides.

Table 5.3.8 List of all the α-lactalbumin peptides identified in the samples and their eventual reported bioactivity (signal peptide amino acids were subtracted).

Peptide sequence	**Amino acids residues**	**Reported Bioactivity**
HTSGYDTQA	32-40	None
WLCEKL	118-123	None
YDTQA	36-40	None
LCEKL	119-123	None
LTKCEVF	3-9	None
WLAHKALCSEKLDQ	104-117	DPP IV-inhibitory activity[24]
IVQNNDSTEYGL	41-52	None
EQLTKCEVF	1-9	None
LAHKALCSE	105-113	None
IVQNNDSTE	41-49	None
PEWVCTTF	24-31	None
LKDLKGYGGVSL	12-23	None
SCDKFLDDDL	76-85	None
AIVQNNDSTE	40-49	None
KVGINY	98-103	None
WVCTTFHTSG	26-35	None
HTSGYDTQ	32-39	None
WVCTTF	26-31	None
EQLTKCEVFRE	1-11	None
VCTTFHTSG	27-35	None
IMCVKKIL	89-96	None

RELKDLKGYGGVSL	10-23	None
LAHKALCSEKLDQ	105-117	None
LDDDLTDD	81-88	None
DKVGINY	97-103	ACE-inhibitory activity[25]
IVQNND	41-46	None
CEVFRE	6-11	None
KVGINYW	98-104	None
TKCEVF	4-9	None
RELKDLKGY	10-18	None
DKVGIN	97-102	None
SCDKFLDD	76-83	None
AIVQNND	40-46	None
LKDLKGY	12-18	None
WLAHKALCS	104-112	None

Also in this case some peptides were found in the IgE-binding regions of the protein (1-19, 13-26 and 90-123 amino acids residues)[26,27]. In table 5.3.9 are reported the once that were found that could be IgE-binding epitopes.

Table 5.3.9 Identified α-lactalbumin peptides that could be IgE-binding epitopes (signal peptide amino acids were subtracted).

Peptide sequence	**Amino acids residues**	**Digestive fraction**
EQLTKCEVFRE	1-11	Fraction 3
LKDLKGYGGVSL	12-23	Fraction 3
RELKDLKGYGGVSL	10-23	Fractions 3 and 5
LAHKALCSEKLDQ	105-117	Fractions 3 and 5
WLAHKALCSEKLDQ	104-117	Fractions 1, 3 and 5

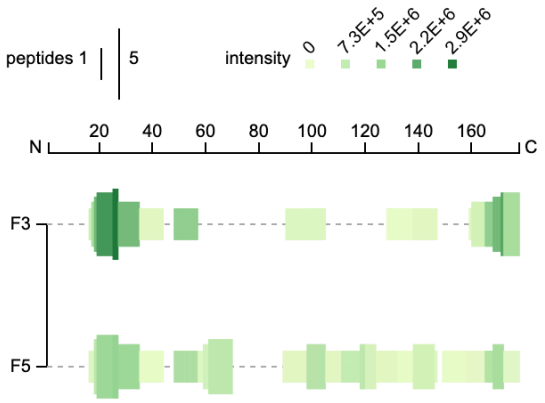


Figure 5.3.13 β-lactoglobulin peptides identified in the gastric digested sample (WPI sample). In the graph, the amino acids that were identified in peptide sequences are represented with vertical bars. The intensity of the colour is proportional to the sum of identified peptides' intensities. The dimension of the bar is proportional to the number of peptides in which was identified that amino acid in that position. The first 16 amino acids of the graph are the ones relative to the signal peptide.

Even in this case in Fraction 1 were not found peptides. Again, a possible explanation could be an instrumental limitation in the detection due to the very low degree of hydrolysis in this fraction. Peptide identification confirmed the partial degradation of this protein in the gastric phase. With respect to the CW digestion in Fraction 3 peptides have a higher intensity than in Fraction 5, this could be explained with a higher digestibility of this protein in this sample. Peptides from the region 55-73 amino acid residues were not found. In Table 5.3.10 are listed the identified peptides and the reported bioactivity.

Table 5.3.10 List of all the β-lactoglobulin peptides identified in the gastric digested samples and their eventual reported bioactivity (signal peptide amino acids were subtracted).

Peptide sequence	**Amino acids residues**	**Reported Bioactivity**
LIVTQTMKGLD	1-11	None
SFNPTQLEEQCHI	150-162	None
DAQSAPLRV	33-41	None

VTQTMKGLD	3-11	None
LDIQKVAGTW	10-19	None
PEQSLVCQCL	113-122	None
KIDALNE	83-89	None
VRTPEVDDE	123-131	None
IVTQTMKGLD	2-11	None
PTQLEEQCHI	153-162	None
YSLAMAASD	20-28	None
IQKVAGTW	12-19	Ace inhibitor/ DPPIV inhibitor[30]
PMHIRL	144-149	None
MHIRL	145-149	None
SFNPTQL	150-156	None
KTKIPAVF	75-82	None
EEQCHI	157-162	None
TQTMKGLD	4-11	None
LEEQCHI	156-162	None
KIDALNENKVLVL	83-95	None
LVCQCL	117-122	None
EELKPTPEGDL	44-54	None
EKTKIPAVF	74-82	None
VTQTMKG	3-9	None
LLFCME	103-108	None
YVEELKPTPEGDL	42-54	None
EKFDKALKA	134-142	None
DTDYKKYLL	96-104	None
VRTPEVDD	123-130	None
LPMHIRL	143-149	None
LKPTPEGDL	46-54	DPP IV-inhibitory activity[24]
FCMENSAEPEQS	105-116	None

Peptides length varies between 5 and 13 amino acids residues in all the fractions. Peptides were found also from regions 1-16, 31-60, 76-95, 121-140 and 136-150 that are known to be IgE-binding epitopes (Table 5.3.11).[27,31,32]

Table 5.3.11 Identified β-lactoglobulin peptides that could be IgE-binding epitopes (signal peptide amino acids were subtracted).

Peptide sequence	Amino acids residues	Digestive fraction
LIVTQTMKGLD	1-11	Fractions 3 and 5
EELKPTPEGDL	44-54	Fraction 5
YVEELKPTPEGDL	42-54	Fraction 5
KIDALNENKVLVL	83-95	Fraction 5

From the intestinal digestion, peptides deriving from the digestion of the two whey proteins in Fraction 1, 3 and 5 were found. In Table 5.3.12 are listed the peptides identified after the intestinal digestion of α-lactalbumin.

Table 5.3.12 List of the α-lactalbumin peptides identified in the samples (signal peptide amino acids were subtracted).

Peptide sequence	Amino acid residues	Fraction 1	Fraction 3	Fraction 5
GGVSLPE	19-25	x		
YDTQA	36-40		x	
NDSTE	45-49	x		
CKDDQNPH	61-68	x	x	
DDQNPH	63-68	x	x	x
DDDLTDDI	82-89	x		
LDDDLTDDI	81-89	x		
DDDLTDDIM	82-90	x		
LDDDLTDD	81-88		x	
LDDDL	81-85		x	x
TDDIM	86-90	x		

Few peptides were identified in this digestion phase as this protein is completely digested in the gastric phase. Interestingly in the intestinal phase were identified two peptides that were found also in the gastric phase, peptides LDDDLTDD and YDTQA.
In figure 5.3.14 are shown peptides deriving from the intestinal digestion of β-lactoglobulin.

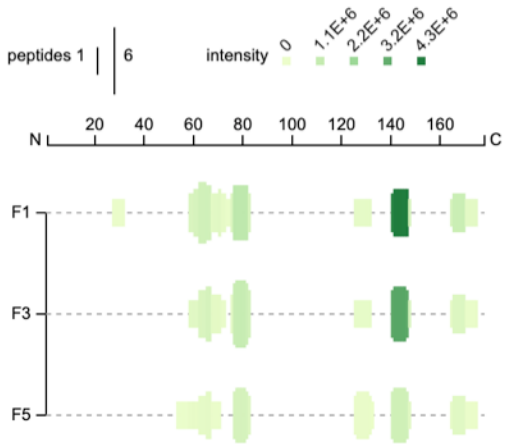


Figure 5.3.14 β-lactoglobulin peptides identified in the intestinal digested fractions (WPI sample). In the graph, the amino acids that were identified in peptide sequences are represented with vertical bars. The intensity of the colour is proportional to the sum of identified peptides' intensities. The dimension of the bar is proportional to the number of peptides in which was identified that amino acid in that position. The first 16 amino acids of the graph are the ones relative to the signal peptide.

Peptides were found mainly in the following regions of the protein sequence: 42-67, 108-116, 125-132 and 149-159. The intensity of the peptides in the figure decreases suggesting the advancement of the digestion. Peptides length ranges between 5 and 9 amino acids residues in all the fractions. In table 5.3.13 are reported the identified peptides.

Table 5.3.13 List of all the β-lactoglobulin peptides identified in the intestinal digested samples (signal peptide amino acids were subtracted).

Peptide sequence	**Amino acids residues**	**Peptide sequence**	**Amino acids residues**
LSFNPT	149-154	WENDEC	61-66
SFNPT	150-154	VEELKPTPE	43-51
QLEEQ	155-159	KWENDEC	60-66
GLDIQ	9-13	KWENDECA	60-67
DLEIL	53-57	PEVDDE	126-131
GDLEI	52-56	WENDECA	61-67
VEELKPT	43-49	WENGE	61-65

TPEVDDE	125-131	TPEVDD	125-130
TPEVDDEA	125-132	ENDECA	62-67
KPTPE	47-51	KPTPEG	47-52
SAEPEQS	110-116	QSAPL	35-39
PEGDLE	50-55	SAEPEQ	110-115
YVEEL	42-46	ENSAEPE	108-114
EELKPT	44-49		

As expected from the findings described in Chapter 4, from the peptides identified in both the digestive phase it was possible to observe that a high protein coverage was obtained. This finding was observed in all samples (CW and WPI) for both the whey proteins. In the CW and WPI samples some peptides with reported bioactivity were identified. In particular, two peptides were identified with DPP IV-inhibitory activity[24], WLAHKALCSEKLDQ (104-117 amino acids residues, from α-lactalbumin) and LKPTPEGDL (46-54 amino acids residues, from β-lactoglobulin), and one peptide with reported ACE-inhibitory activity[25], DKVGINY (97-103 amino acids residues, from α-lactalbumin). Another peptide, for which both DPP IV-inhibitory activity and ACE- inhibitory activity are reported[30], was identified, IQKVAGTW (12-19 amino acids residues, from β-lactoglobulin). Since these peptides were identified in both samples, the process seems not to influence the bioactivity of the peptides.

5.3.6 ELISA Inhibition test

After the gastrointestinal digestion of the two samples (CW and WPI), the allergenicity of the obtained fractions was evaluated. The ELISA inhibition test was performed with a pool of sera from patients allergic to both the whey proteins (sera information are reported in paragraph 5.2.8). An Inhibition ELISA (also known as Competitive ELISA) is performed measuring the concentration of an antigen trough detection of signal interference (Figure 5.3.15).

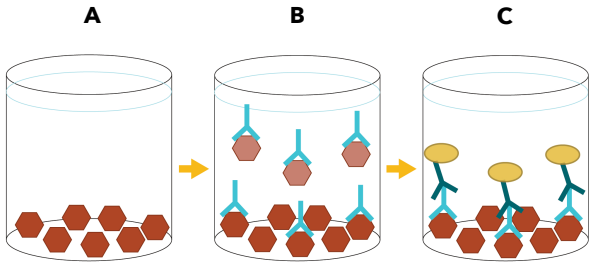


Figure 5.3.15 Schematic representation of an Inhibition ELISA test. Firstly, wells are coated with the reference antigen (A). Then the incubation (B) with a solution of patient sera pre-incubated with the antigen is performed to allow the unbounded IgE to bind the reference antigen. Finally, the incubation with the secondary antibody is performed for the detection (C).

Wells are pre-coated with a reference antigen that competes with the antigen in exam for the binding with the antibody. Sample containing the antigen is incubated with the antibody and when is introduced in the wells the unbound antibodies are free to bind the reference antigen. In this way, the absorbance is inversely proportional to the concentration of the antigen in the sample[33]. Antibodies usually are labelled, in this study was used as a primary antibody a Rabbit Anti-Human IgE for binding the antigen and a secondary antibody Swine Anti-Rabbit IgG labelled with Horseradish Peroxidase (HRP). To obtain a signal to be measured TMB is used as a substrate for the HRP. TMB (3,3,5,5-Tetramethylbenzidine) is colourless before the action of the enzyme that is visible due to the change of colour in blue. To stop this reaction H_2SO_4 0.5M is added and colour change to yellow (Figure 5.3.16).

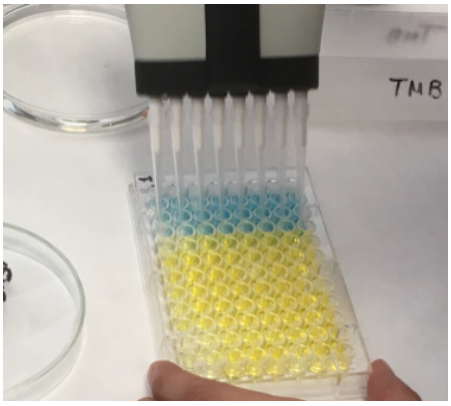


Figure 5.3.16 Elisa plate example, in yellow are the wells where the HRP activity was stopped with sulphuric acid, in blue the one were HRP is still acting on TMB.

Among the sample also three controls were tested: the undigested WPI, the undigested CW and the reference antigen (whey proteins standard solutions).
Here follows the inhibitions curves obtained for the two samples (Figures 5.3.17 and 5.3.18).

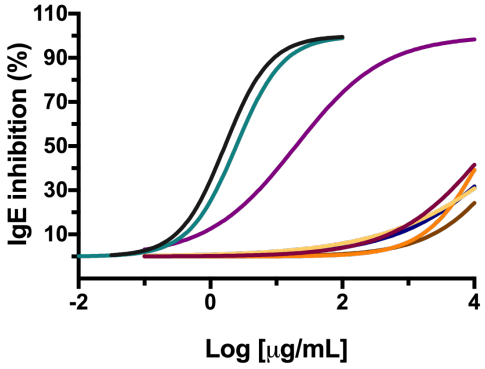


Figure 5.3.17 Inhibition curves obtained for all the samples related to CW. In the graph are presented the curves of the reference antigen (black), the undigested protein (light green), gastric Fraction 1 (purple), gastric Fraction 3 (red), gastric Fraction 5 (yellow), intestinal Fraction 1 (orange), intestinal Fraction 3 (blue), intestinal Fraction 5 (brown).

Inhibition curves were obtained plotting the percentage of IgE inhibition with the logarithm of the concentration. For the reference

antigen and the undigested protein (CW) a sigmoidal curve was obtained while for the digested fraction a low curve was obtained. The only exception is represented by the gastric fraction 1 that keeps a sigmoidal profile.

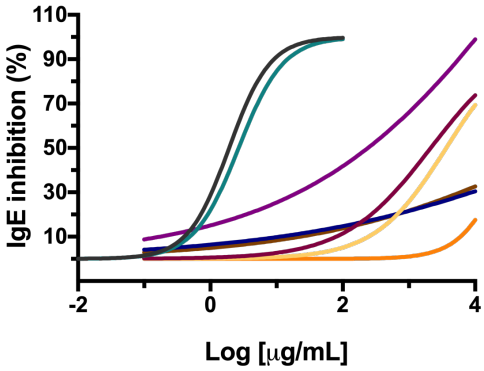


Figure 5.3.18 Inhibition curves obtained for all the samples related to WPI. In the graph are presented the curves of the reference antigen (black), the undigested protein (green), gastric Fraction1 (purple), gastric fraction 3 (red), gastric Fraction 5 (yellow), intestinal fraction 1 (orange), intestinal Fraction 3 (blue), intestinal fraction 5 (brown).

For the WPI samples the obtained profile is different from the CW case. Both the reference antigen and the undigested protein (WPI) present a sigmoidal curve. The gastric fractions here present higher values of percentages of IgE inhibition. For the intestinal fractions here they present lower values as for the CW samples. In Table 5.3.14 are reported the EC50 values calculated for the samples.

Table 5.3.14 EC50 Values (μg/mL) calculated for the tested samples. GF= Gastric Fraction; IF= Intestinal Fraction; Control= Undigested protein; WP= reference antigens.

	Control	GF1	GF3	GF5	IF1	IF3	IF5
WP	1,7	-	-	-	-	-	-
CW	2,2	17,8	1313 6,7	5524 0,7	1325 3,0	41126, 3	48299,7
WPI	2,4	146, 5	2145 ,0	3106 ,7	4827 6,3	830919 ,3	240475, 7

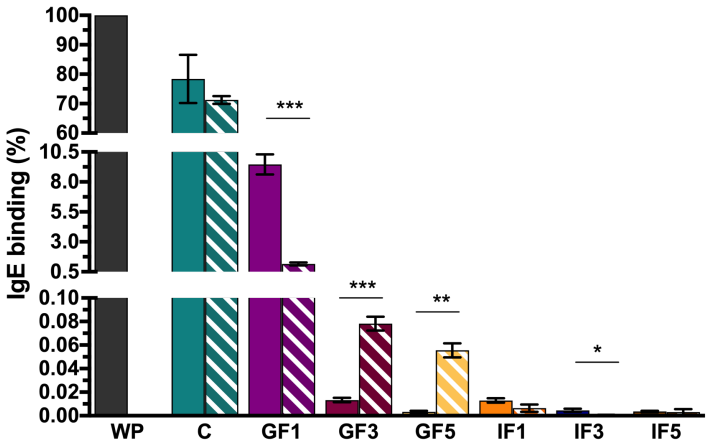


Figure 5.3.19 IgE binding values obtained for the tested samples. Unpaired t test was performed: *=p<0.05; **=p<0.01; ***=p<0.001. Error bars represent mean ± standard error of the mean (SEM). WP= reference sample; C=control sample; GF1=Gastric Fraction1; GF3=Gastric Fraction 3; GF5=Gastric Fraction 5; IF1=Intestinal Fraction1; IF3=Intestinal Fraction 3; IF5=Intestinal Fraction 5. In stripes are the samples relative to WPI digestion.

As it is possible to see in the reported graph, significant differences were found among the tested samples. Globally the allergenicity decreases with the digestion with respect to the undigested sample, but differences were found among the samples during the digestion. From figure 5.3.19 it is possible to deduce that the two undigested samples show the same IgE-binding. While this value decreased after the gastric phase. In Fraction 1 seems to be more allergenic the CW sample: in this fraction were identified both the whey protein and very few peptides, while for the WPI a higher degree of hydrolysis was found. In Fractions 3 and 5 the ratio is reverted, the WPI samples are more allergenic than the CW one. This could be related to the higher degree of hydrolysis of this sample, intact α-lactalbumin was not observed, and more peptides are generated in these fractions. Major differences were found between the gastric fractions. While no significant differences were observed between the intestinal fractions. Indeed, as resulted from the study of the peptides release, similar results were obtained from CW and WPI samples.

To verify the influence of the intact protein on the obtained results, stock solutions were analysed with UPLC-UV analysis. In figure 5.3.20 and 5.3.21 are reported the quantifications of the two whey proteins.

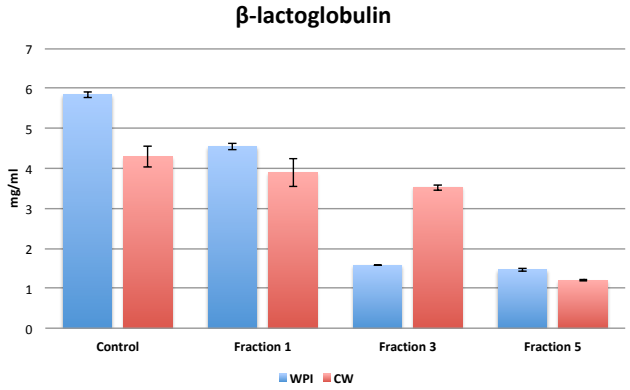


Figure 5.3.20 β-lactoglobulin quantification in the stock solution of the undigested samples (control= CW, WPI) and of the three gastric fractions collected from CW and WPI digestions (Fraction 1=18 min; Fraction 3= 54 min; Fraction 5= 90 min).

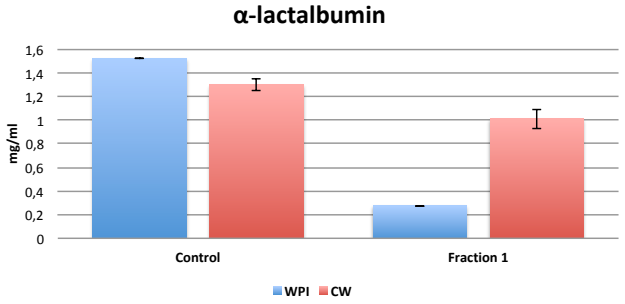


Figure 5.3.21 α-lactalbumin quantification in the stock solutions of the undigested samples (control= WPI, CW) and of gastric Fraction 1 (18 min) from the two digestions on CW and WPI.

WPIs are commonly extracted from whey due to membrane based techniques and then spray-dried to obtain a powder. Thus, some steps involving the use of high temperature may affect protein content inducing protein aggregation/denaturation and chemical modification (i.e. glycation). Processing may alter, hide, expose or destroy allergenic epitopes[5]. Several studies reported different findings on the

effects of heat treatment on whey protein allergenicity. For example, it was reported that heating β-lactoglobulin up to 75°C induced a decrease in the IgE-binding[34,35], while another study reported that up to 90°C there is an increase in IgE-binding for both whey proteins[36]. Above 90°C, the binding capacity decreases below the one of the untreated protein for α-lactalbumin while for β-lactoglobulin still remain higher than for the untreated protein[36]. However, in the present study, it was found that the processing did not affect too much the allergenicity of the product itself. A similar IgE-binding was obtained for CW and WPI undigested sample, meaning that the possible structural modifications (i.e. denaturation) induced by the processing might have not affected the allergenicity. Moreover, it was observed that the gastrointestinal digestion had reduced the IgE-binding of both the samples, with some differences among them. In Fraction 1 the main difference among the sample is that the WPI present fewer amount of α-lactalbumin. This could be the explanation of the higher IgE-binding of CW observed in this fraction. The binding might be related to the intact protein and the more digestibility of α-lactalbumin in WPI might have affected the IgE-binding. Indeed, for α-lactalbumin the allergenicity is mainly related to the conformational epitopes that the process induced denaturation may destroy[27,36]. In Fraction 3 intact α-lactalbumin was not detected in both the samples while β-lactoglobulin was present in higher amount in the CW sample. Unexpectedly, in this fraction was observed a higher IgE-binding for the WPI sample. This finding might be ascribed to the higher degree of hydrolysis that in this case might have caused the exposure of more linear IgE-binding epitopes. Indeed, for β-lactoglobulin it was reported that the allergenicity was mainly related to the presence of linear epitopes[27,36]. In Fraction 5 the amount of this last protein is quite the same in the two samples. Even in this fraction a higher amount of peptides was observed for the WPI for which a higher IgE-binding was registered. Thus, this may confirm the assumption that the binding is related to the released peptides. It might be concluded that the processing used for the production of WPI did not influence much protein allergenicity in the product but it affected the residual allergenicity observed after the gastric digestion.

5.4 Conclusions

The semi-dynamic *in vitro* gastrointestinal digestion model described in Chapter 4 was applied to study the effects of processing on whey protein digestibility. In this study, a sample of commercially available whey protein isolates (WPI) was compared with a sample of whey collected from cheese (CW) that wasn't processed. The digestion model applied allowed to follow protein degradation and peptides release simulating the gastric emptying. Comparing the results obtained for the untreated CW sample with the WPI one, some differences were found that could be related to the processing. Indeed, it was found that in the WPI sample the two whey proteins are present in higher amount in a denatured form, resulting in higher protein digestibility. Indeed, more peptides were identified from the WPI sample. This difference in digestibility may affect also the allergenicity of the proteins. An ELISA inhibition test was performed to evaluate the differences in IgE-binding. The CW sample showed a higher IgE-binding in Fraction 1 where intact α-lactalbumin was present in a higher amount, while in the following analysed fractions the WPI resulted more allergenic probably due to the higher degree of hydrolysis. Thus, it results that processing affected protein residual allergenicity after ingestion. This information is quite important for the determination of the effects of processing on protein allergenicity, underling the importance of performing gastrointestinal digestion studies. To complete the evaluation of the effects of processing on protein digestibility, the intestinal digested fractions 5 of both the CW and WPI sample will be used in transepithelial transport study (described in Chapter 6) to test their possible intestinal absorption.

References

1. Ramos, O. L. *et al. Whey and Whey Powders: Production and Uses*. *Encyclopedia of Food and Health* (Elsevier Ltd., 2015). doi:10.1016/B978-0-12-384947-2.00747-9
2. Kelly, P. *Manufacture of Whey Protein Products*. *Whey Proteins* (Elsevier Inc., 2019). doi:10.1016/b978-0-12-812124-5.00003-5
3. Madureira, A. R., Pereira, C. I., Gomes, A. M. P., Pintado, M. E. & Xavier Malcata, F. Bovine whey proteins - Overview on their main biological properties. *Food Res. Int.* **40**, 1197–1211 (2007).
4. Chegini, G. & Taheri, M. Whey powder: Process technology and physical properties: A review. *Middle East J. Sci. Res.* **13**, 1377–1387 (2013).
5. van Lieshout, G. A. A., Lambers, T. T., Bragt, M. C. E. & Hettinga, K. A. How processing may affect milk protein digestion and overall physiological outcomes: A systematic review. *Critical Reviews in Food Science and Nutrition* 1–24 (2019). doi:10.1080/10408398.2019.1646703
6. Rahaman, T., Vasiljevic, T. & Ramchandran, L. Effect of processing on conformational changes of food proteins related to allergenicity. *Trends Food Sci. Technol.* **49**, 24–34 (2016).
7. Meltretter, J., Wüst, J. & Pischetsrieder, M. Modified peptides as indicators for thermal and nonthermal reactions in processed milk. *J. Agric. Food Chem.* **62**, 10903–10915 (2014).
8. Milkovska-Stamenova, S. & Hoffmann, R. Identification and quantification of bovine protein lactosylation sites in different milk products. *J. Proteomics* **134**, 112–126 (2016).
9. Wickham, M., Faulks, R. & Mills, C. In vitro digestion methods for assessing the effect of food structure on allergen breakdown. *Mol. Nutr. Food Res.* **53**, 952–958 (2009).
10. Astwood, J. D., Leach, J. N. & Fuchs, R. L. Stability of Food Allergens to Digestion in Vitro. *Nat. Biotechnol.* **14**, 1269–1273 (1996).
11. Huby, R. D. J., Dearman, R. J. & Kimber, I. Why Are Some Proteins Allergens? *Toxicol. Sci.* **55**, 235–246 (2000).
12. Bøgh, K. L. & Madsen, C. B. Food Allergens: Is There a Correlation between Stability to Digestion and Allergenicity? *Crit. Rev. Food Sci. Nutr.* **56**, 1545–1567 (2016).
13. Eiwegger, T. *et al.* Gastro-duodenal digestion products of the major peanut allergen Ara h 1 retain an allergenic potential. *Clin. Exp. Allergy* **36**, 1281–1288 (2006).
14. Bøgh, K. L., Barkholt, V. & Madsen, C. B. Characterization of the

Immunogenicity and Allergenicity of Two Cow's Milk Hydrolysates - A Study in Brown Norway Rats. *Scand. J. Immunol.* **81**, 274–283 (2015).

15. Verhoeckx, K. *et al.* The relevance of a digestibility evaluation in the allergenicity risk assessment of novel proteins. Opinion of a joint initiative of COST action ImpARAS and COST action INFOGEST. *Food Chem. Toxicol.* **129**, 405–423 (2019).
16. Minekus, M. *et al.* A standardised static in vitro digestion method suitable for food-an international consensus. *Food Funct.* **5**, 1113–1124 (2014).
17. Manguy, J. *et al.* Peptigram: A Web-Based Application for Peptidomics Data Visualization. *J. Proteome Res.* **16**, 712–719 (2017).
18. Bateman, A. UniProt: A worldwide hub of protein knowledge. *Nucleic Acids Res.* **47**, D506–D515 (2019).
19. Buhler, S. *et al.* UV irradiation as a comparable method to thermal treatment for producing high quality stabilized milk whey. *Lwt* **105**, 127–134 (2019).
20. Mulet-Cabero, A. I., Rigby, N. M., Brodkorb, A. & Mackie, A. R. Dairy food structures influence the rates of nutrient digestion through different in vitro gastric behaviour. *Food Hydrocoll.* **67**, 63–73 (2017).
21. Sánchez-Rivera, L., Ménard, O., Recio, I. & Dupont, D. Peptide mapping during dynamic gastric digestion of heated and unheated skimmed milk powder. *Food Res. Int.* **77**, 139–132 (2015).
22. Mulet-Cabero, A. I., Mackie, A. R., Wilde, P. J., Fenelon, M. A. & Brodkorb, A. Structural mechanism and kinetics of in vitro gastric digestion are affected by process-induced changes in bovine milk. *Food Hydrocoll.* **86**, 172–183 (2019).
23. Nielsen, S. D., Beverly, R. L., Qu, Y. & Dallas, D. C. Milk bioactive peptide database: A comprehensive database of milk protein-derived bioactive peptides and novel visualization. *Food Chem.* **232**, 673–682 (2017).
24. Lacroix, I. M. E. & Li-Chan, E. C. Y. Isolation and characterization of peptides with dipeptidyl peptidase-IV inhibitory activity from pepsin-treated bovine whey proteins. *Peptides* **54**, 39–48 (2014).
25. Tavares, T. *et al.* Novel whey-derived peptides with inhibitory effect against angiotensin-converting enzyme: In vitro effect and stability to gastrointestinal enzymes. *Peptides* **32**, 1013–1019 (2011).
26. Hochwallner, H. *et al.* Visualization of clustered IgE epitopes on α-lactalbumin. *J. Allergy Clin. Immunol.* **125**, (2010).
27. Järvinen, K. M., Chatchatee, P., Bardina, L., Beyer, K. & Sampson, H. A. IgE and IgG Binding Epitopes on α-Lactalbumin and β-Lactoglobulin in Cow's Milk Allergy. *Int. Arch. Allergy Immunol.* **126**, 111–118

(2001).
28. Kringelum, J. V., Nielsen, M., Padkjær, S. B. & Lund, O. Structural analysis of B-cell epitopes in antibody: Protein complexes. *Mol. Immunol.* **53**, 24–34 (2013).
29. Buus, S. *et al.* High-resolution mapping of linear antibody epitopes using ultrahigh-density peptide microarrays. *Mol. Cell. Proteomics* **11**, 1790–1800 (2012).
30. Lacroix, I. M. E., Meng, G., Cheung, I. W. Y. & Li-Chan, E. C. Y. Do whey protein-derive peptides have dual dipeptidyl-peptidase IV and angiotensin I-converting enzyme inhibitory activities? *J. Funct. Foods* **21**, 87–96 (2016).
31. Cong, Y. J. & Li, L. F. Identification of the Critical Amino Acid Residues of Immunoglobulin E and Immunoglobulin G Epitopes in α-Lactalbumin by Alanine Scanning Analysis. *J. Dairy Sci.* **95**, 6307–6312 (2012).
32. Cerecedo, I. *et al.* Mapping of the IgE and IgG4 sequential epitopes of milk allergens with a peptide microarray-based immunoassay. *J. Allergy Clin. Immunol.* **122**, 589–594 (2008).
33. Sharma, N., Hanif, S., Upadhyay, D. & Chhikara, M. K. Inhibition ELISA as a putative tool for the identification and quantification of meningococcal A and X polysaccharides at various stages of vaccine development. *J. Immunol. Methods* **473**, 112634 (2019).
34. Taheri-Kafrani, A. *et al.* Effects of heating and glycation of β-lactoglobulin on its recognition by ige of sera from cow milk allergy patients. *J. Agric. Food Chem.* **57**, 4974–4982 (2009).
35. Ehn, B. M., Ekstrand, B., Bengtsson, U. & Ahlstedt, S. Modification of IgE Binding during Heat Processing of the Cow's Milk Allergen β-Lactoglobulin. *J. Agric. Food Chem.* **52**, 1398–1403 (2004).
36. Bu, G., Luo, Y., Zheng, Z. & Zheng, H. Effect of heat treatment on the antigenicity of bovine α-lactalbumin and β-lactoglobulin in whey protein isolate. *Food Agric. Immunol.* **20**, 195–206 (2009).

Chapter 6

Evaluation of the intestinal absorption of peptides deriving from whey proteins' simulated gastrointestinal digestion

6.1 Introduction

Milk proteins are an important source of bioactive or immunomodulatory peptides[1]. During the gastrointestinal digestion, these peptides are released from the food matrix and they cover a broad range of immunogenic or bioactive sequences[2]. When they are released during the digestion, they might exert their function interacting directly with the intestinal epithelium or after the absorption in the bloodstream from which they are distributed to peripheral organs[3]. The intestinal epithelium presents a large number of enzymes (the brush border membrane enzymes, BBM), including peptidases, that may degrade the peptides before their possible transport[4]. Indeed, after the gastrointestinal digestion, it could be useful to study the effects of BBM enzymes and transport through the intestinal epithelium. In this light, *in vitro* gastrointestinal digestion experiments may be completed with *in vitro* transepithelial transport studies for the assessment of peptides bioactivity[5].

Peptides could be transported through the intestinal epithelium in different ways. Peptides composed of two or three amino acids can be transported through the epithelial barrier or can be hydrolysed into amino acids and be absorbed[3]. Among the main routes in the gut there is the PepT1 transport di/tri- peptides[5], or the SOPT1 and SOPT2 Na^+ coupled for oligopeptides[6,7], transcellular and paracellular (through tight junctions) passive transport or transcytosis[8]. Moreover, peptides permeability is influenced by hydrophobicity, charge and molecular size[9]. Indeed, small peptides (i.e. composed by two or three amino acids residues) are more easily absorbed as compared to long-chain ones[5].

The study described in this chapter is a continuation of the work presented in the former chapter. Due to the identification of peptides

with potential bioactivity or potential IgE-binding functions after the *in vitro* simulated gastrointestinal digestion, it was decided to complete the digestion route testing the possible absorption of the released peptides. For the transepithelial transport study, Caco-2 cells were selected to simulate the intestinal epithelium. This study had three objectives: 1) test the possible transport of digested sample completing the whole gastrointestinal digestion; 2) test the transport of a synthetic peptide found during the digestion of whey proteins isolates and that could be a potential IgE-binding peptide; 3) test the possible presence of lactose bound to a peptide during its intestinal absorbance. Results here described were obtained thanks to a study carried out at the Institute of Food Science and research (CIAL, CSIC-UAM, Madrid) in collaboration with the BIOPEP group.

6.2 Materials and Methods

6.2.1 Reagents

Lucifer Yellow CH dilithium salt, Trypan blue solution 0.4% and Hank's balanced salt solution (HBSS) modified, with sodium bicarbonate, without phenol red, w/o calcium chloride and magnesium sulphate, Dichloromethane (DCM), N,N-diisopropylethylamine (DIPEA), Piperidine, Thioanisole, Triisopropylsilane (Tis) were purchased from Sigma Aldrich (St. Luis, MO, USA). DMEM, Dulbecco's Modified Eagle's medium with 4.5g/L glucose and with L-glutamine, Trypsin-Versene® Mixture and Phosphate Buffered Saline (PBS) without calcium and magnesium were purchased from BioWhittaker® Reagents, Lonza (Basel, Switzerland). Fetal Bovine Serum (FBS), Antibiotic-Antimicotic 100x solution and MEM non essential amino acids 100X without L-glutamine were purchased by Biowest (Nuaillé, France). Lactose monohydrate was purchased from Labkem (Barcelona, Spain). Diethyl ether and Dimethylformamide (DMF) for peptide synthesis were purchased from Carlo Erba Reagents (Milan, Italy). All the amino acids (Fmoc-Ala-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Ile-OH, Fmoc-Lys(Boc)-OH, Fmoc-Leu-OH, Fmoc-Asn(Trt)-OH, Fmoc-Val-OH), N,N,N',N'-Tetramethyl-O-(1H-benzotriazol-1-yl)uranium hexafluorophosphate (HBTU) and the resin (Fmoc-Leu-Wang LL) used

for the synthesis were purchased from Novabiochem (Merk KGaA, Darmstadt, Germany). Trifluoroacetic acid (TFA) was purchased from Acros Organics (Thermo Fisher Scientific, Waltham, MA, USA). Formic acid was purchased from Fisher Scientific (Thermo Fisher Scientific, Waltham, MA, USA). Doubly deionized water was obtained using a MilliQ system (Millipore, Bedford, MA, USA). HPLC grade Acetonitrile (ACN) was purchased from VWR International (Milan, Italy).

6.2.2 Cell culture

Caco-2 cells were cultured in the Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS heat-inactivated, 1% nonessential amino acids and penicillin/streptomycin. Cells were incubated at 37°C in a humidified atmosphere and culture medium was changed every 2-3 days. Cells in log-phase growth were weekly sub-cultured by trypsinisation and seeded in a new flask.

6.2.3 Transepithelial transport studies

For these studies the Hubatsch et al. protocol[10] reported in literature was followed. For transports experiments, cells were seeded at a density of $2{,}6 \times 10^5$ cells cm^{-2} in 12-well filter support inserts (Costar® Transwell® Permeable Supports 12 mm Diameter insert 12 well 0.4 um polycarbonated Membrane, Corning, NY, USA). The integrity of the cell monolayers was tested measuring the transepithelial electrical resistance (TEER) with Evom^2 Epithelial Voltammeter (World Precision Instruments). TEER values were determined subtracting from the measured value the TEER value of the blank (insert without cell monolayer) and multiplied then for the surface area of the insert. Only Caco-2 monolayer with TEER values more than 500 Ω/cm^2 were used in the experiments. Experiments were performed 21-29 days after seeding. Medium was changed 24h before the assay. Inserts were washed with DMEM and with transport medium (HBSS) and then moved to new 12-well clusters filled with 1,5 mL of HBSS. Then 500 µl were added to the apical side and the filters were incubated at 37°C for 20 min. Samples were dissolved in HBSS, at 4 mg/mL final concentration for the digested samples and 1.7mM for peptide[11], and filtered on 0.2 µm filters. Then, inserts were moved

to new 12-well clusters with 1,5 mL of HBSS, on the apical side were added 500 µl of the sample or of HBSS for the blanks. TEER was measured immediately and for each well 100 µl were collected and frozen. Plate was incubated for one hour at 37°C. TEER was measured immediately and samples from the apical and basolateral sides were collected and stored at -80°C. Collected samples were freeze-dried. The assay was performed in triplicate.

6.2.4 Lucifer yellow assay

Lucifer Yellow was used to test the integrity of the Caco-2 monolayers. A solution of 50 µM Lucifer yellow in HBSS was prepared and filtered on 0.2 µm filters. After the assay with samples, inserts were washed 3 times with HBSS and moved to another plate with 1,5 mL of HBSS in the basolateral side. On the apical side, 500 µl of the Lucifer yellow solution was added and TEER was measured. After one hour incubation at 37°C, TEER was measured again and 300 µl of the apical and basolateral side were collected and stored at -80°C. For each collected sample (both apical and basolateral side after one hour incubation and apical site at 0 min of incubation), 100 µl were used for the fluorescence measurement (Costar® Assay plate 96 well, Corning, NY, USA). A calibration curve was prepared with solutions of Lucifer Yellow at different concentrations: 200 µM, 100 µM, 50 µM, 10 µM, 5 µM, 1 µM, 0,5 µM, 0,25 µM, 0,1 µM. Fluorescence was measured using a Fluostar Optima microplater reader (BMG Labtech, Ortenberg, Germany) setting 480 nm of excitation and 520 nm of emission. The analysis was performed in duplicate.

6.2.5 Peptide Synthesis

Synthesis of the peptide KIDALNENKVLVL was performed as described in paragraph 2.2.6. With respect to the procedure described, in this case the acetylation step before the cleavage from the resin was not performed. The resin used (Fmoc-Leu-Wang LL) was already loaded with the C-terminal amino acid, Leucine, so that the loading and capping step described in paragraph 2.2.6 before the synthesis were not performed. The obtained peptide was then lactosylated dissolving peptide and lactose (1:50 ratio) in DMF under

nitrogen atmosphere. The solution was incubated at 70°C for 40h and then solvent was removed under vacuum. Peptides mixture was then freeze-dried and analysed with HPLC-MS analysis as described in paragraph 4.2.5. Proteomics Toolkit (developed by the Institute for Systems Biology, Seattle, WA, USA) was used for the identification of the MS ions.

Table 6.2.1 MS ion of the synthesised peptides identified from the HPLC-MS spectra.

	MS calculated ions	**MS identified ions**
Unreacted peptide *(r. t. 61.2 min)*	1468.8 $[M+H]^{+}$; 734.9 $[M+2H]^{2+}$; 490.29 $[M+3H]^{3+}$; 367.97 $[M+4H]^{4+}$	1470.6 $[M+H]^{+}$; 735.15 $[M+2H]^{2+}$;
Mono-Lactosylated peptide *(r. t. 59.6 min)*	1792.87 $[M+H]^{+}$; 896.94 $[M+2H]^{2+}$; 598.27 $[M+3H]^{3+}$; 448.97 $[M+4H]^{4+}$	1794.57 $[M+H]^{+}$; 897.25 $[M+2H]^{2+}$
Di-Lactosylated peptide *(r. t. 58.9 min)*	2116.87$[M+H]^{+}$; 1058.94 $[M+2H]^{2+}$; 706.27 $[M+3H]^{3+}$; 529.97 $[M+4H]^{4+}$	1059.74 $[M+2H]^{2+}$; 706.7 $[M+3H]^{3+}$

Before the transepithelial transport assay, the mixture was dissolved in water and ultrafiltration was performed with a 1kDa cut off (Microsep advanced centrifugal device, Pall Corporation, Port Washington, NY, USA) to remove the unreacted lactose.

6.2.6 HPLC-MS/MS analysis

For the HPLC-MS/MS analysis samples were reconstituted in eluent A at different concentrations: the apical side collected before the incubation (t0) was reconstituted in 100 µl and then diluted 1:4 for digested samples and 1:12 for peptides samples with eluent A, the apical side after 60 min was reconstituted in 300 µl and then diluted as performed for the samples from the apical side at 0 min, the basolateral samples were reconstituted in 100 µl of eluent A. Then HPLC-MS/MS analysis was performed as described in paragraph 4.2.5.

6.3 Results and Discussion

For this study, it was followed the protocol developed by Hubatsch et al[10]. A transepithelial transport experiment consists mainly of culturing cells on membrane filters, then, when the monolayer is completely formed, cells are incubated with the samples. The side above the cell layer, where the sample is introduced, is the apical side and represent the intestinal lumen, while the side below is called basolateral side (Figure 6.3.1).

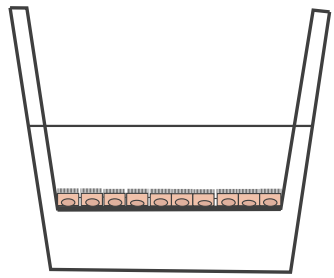


Figure 6.3.1 Schematic representation of a Caco-2 monolayer grown on permeable filter support.

Transepithelial electric resistance (TEER) is measured during culturing and before and after the assay to determine when the monolayer is ready for the assay and to verify then the integrity of the monolayer after the incubation. Samples are collected soon after the TEER measurement, when samples are poured in the apical side, and after one hour of incubation to see the effects of the possible transport and the activity of the peptidases. Apart from the TEER measurement, the integrity of the monolayer after the assay was determined also with the Lucifer Yellow assay. Collected samples were then lyophilized and analysed with HPLC-MS/MS analysis for peptides identification.

The Lucifer Yellow (LY) assay is commonly used to evaluate Caco-2's apical to basolateral paracellular permeability. Lucifer Yellow is a fluorescent dye that could be transported only through the paracellular via, thus if the monolayer integrity is preserved the tight junction shouldn't let the dye cross the monolayer[12]. After the assay with the samples, cells were incubated for one hour with a 50 μM solution of Lucifer Yellow in HBSS, samples from the apical and

basolateral side were collected. The fluorescence signal of the sample collected was then measured and used for the determination of paracellular permeability. As reported in literature[13], this parameter is expressed as $\%LY_{basal}$ and was calculated using the equation

$$\%LY_{basal} \text{ recovery}=[LY_t \text{ (basolateral side)}/LY_0 \text{ (apical side)}]]*100$$

where LY_t is the concentration in the basolateral side after the incubations and LY_0 is the concentration at the beginning of the experiment in the apical side.

6.3.1 Transepithelial transport study on gastrointestinal digested samples

From the digested samples collected in the semi-dynamic gastrointestinal digestion described in the former chapter (Chapter 5), samples that were collected at the end of the gastric phase and then digested in the intestinal phase (fraction 5) were selected for this study. In the intestinal digestion performed the bile salts concentration was lowered with respect to the protocol[14] value to preserve cells in the transepithelial test and to avoid possible negative effects on cells. Both the samples obtained from the digestion of the whey proteins isolated and the whey from cheese were analysed. These samples were diluted in HBSS at 4 mg/mL concentration, then incubated for one hour following the protocol. Sample concentration was determined after some trials and based on concentration values reported in literature[15]. During the experiments, samples were collected from the apical side at the beginning and the end of the one hour incubation, from the basolateral side only at the end of the incubation.

From the Lucifer Yellow assay test, after the incubation of the CW digested sample (fraction 5) a $\%LY_{basal}$ recovery of 1,77±0,72 was determined. A value of 1,81±0,25 was determined after the incubation of the WPI digested sample (fraction 5). The values calculated showed a low value suggesting that the monolayer was not affected by the incubation with the sample.

In figure 6.3.2 and 6.3.3 are shown the HPLC-MS/MS chromatograms of the apical side collected at 0 min and 60 min for the

CW (whey collected from cheese) and WPI (whey protein isolates) samples.

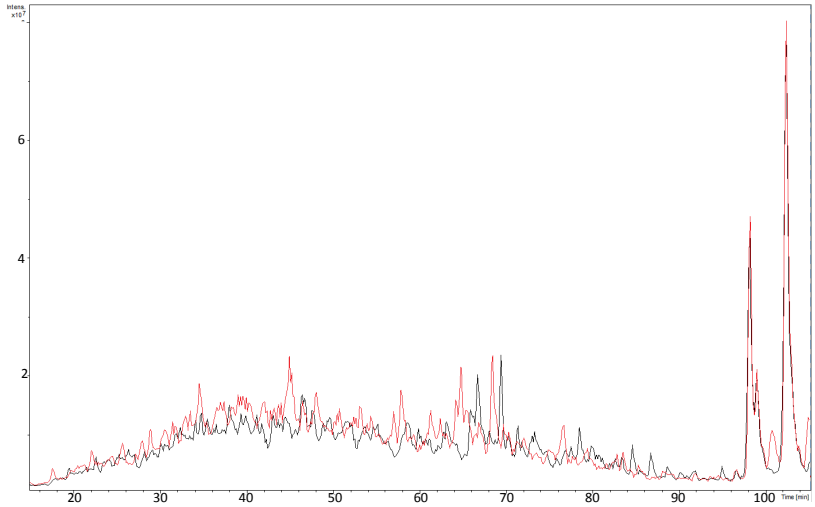


Figure 6.3.2 Apical side chromatogram of the CW sample at the beginning (0 min, red line) and at the end (60 min, black line) of the incubation. On the y axis is reported the intensity ($x10^7$), on the x axis the time (min).

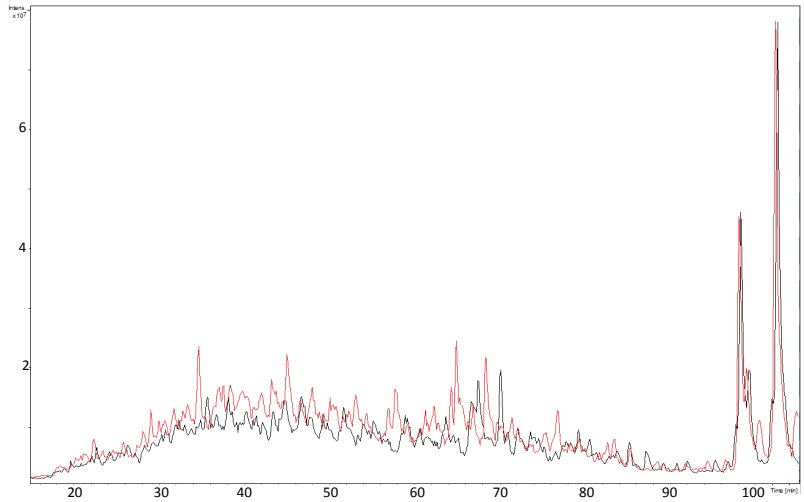


Figure 6.3.3 Apical side chromatograms of the WPI sample at the beginning (0 min, red line) and the end (60 min, black line) of the incubation. On the y axis is reported the intensity ($x10^7$), on the x axis the time (min).

From the chromatographic profile is possible to see little differences between sampling at 0 min and 60 min of incubation,

some peaks are the same in the two incubation times but are slightly shifted in the retention time due to instrumental effects. The MS/MS spectra gave some indications on peptides in the samples.

From the identified peptides in the CW sample, some were found interesting comparing the samples at 0 and 60 min with the digested fraction before the assay. In particular for β-lactoglobulin (Table 6.3.1), peptides TPEVDDE (125-131 amino acids residues) and TPEVDDEA (125-132 amino acids residues) were found in all the samples suggesting their resistance to the peptidases that are present on the surface of the Caco-2. For the same protein, peptides SAEPE (110-114 amino acids residues) and TPEVDD (125-130 amino acids residues) were found in the sample before the test and at 0 min of incubation, suggesting that they were susceptible to the activity of the peptidases.

Concerning α-lactalbumin, peptide DDQNPH (63-68 amino acids residues) was found resistant to the activity of the peptidases.

Table 6.3.1 Peptides from β-lactoglobulin identified in the apical side before and after the incubation, CW sample.

Peptide sequence	Amino acids residues	Digested protein (Fraction 5)	Apical side (0 min incubation)	Apical side (60 min incubation)
TPEVDDE	125-131	x	x	x
TPEVDDEA	125-132	x	x	x
SAEPE	110-114	x	x	-
TPEVDD	125-130	x	x	-

Concerning the WPI sample, some interesting peptides were found comparing the samples at 0 and 60 min of incubation with the digested sample before the assay. For α-lactalbumin, peptide DDQNPH (63-68 amino acids residues) was found resistant to the activity of the peptidases, as was found for the CW sample.

About β-lactoglobulin (Table 6.3.2), peptides VEELKPTPE (43-51 amino acids residues), PEGDLE (50-55 amino acids residues), LSFNT (149-154 amino acids residues), SFNT (150-154 amino acids residues), TPEVDDEA (125-132 amino acids residues), TPEVDDE (125-131 amino acids residues), SAEPEQS (110-116 amino acids residues) and QLEELQ (155-159 amino acids residues) were found resistant to

the peptidases activity on the surface of the Caco-2 cells. While peptides WENGE (61-65 amino acids residues), KTPE (47-51 amino acids residues), ENSAEPE (108-144 amino acids residues) and TPEVDD (125-130 amino acids residues) were found only in the digested samples and at 0 time of incubation suggesting their susceptibility to the activity of the peptidases.

Table 6.3.2 Peptides from β-lactoglobulin identified in the apical side before and after the incubation, WPI sample.

Peptide sequence	Amino acids residues	Digested protein (Fraction 5)	Apical side (0 min incubation)	Apical side (60 min incubation)
TPEVDDE	125-131	x	x	x
TPEVDDEA	125-132	x	x	x
SAEPEQS	110-116	x	x	x
QLEELQ	159-159	x	x	x
VEELKPTPE	43-51	x	x	x
PEGDLE	50-55	x	x	x
LSFNT	149-154	x	x	x
SFNT	150-154	x	x	x
WENGE	61-65	x	x	-
KTPE	47-51	x	x	-
ENSAEPE	108-144	x	x	-
TPEVDD	125-130	x	x	-

Some peptides that were found at the end of the intestinal phase were not found in the sample at 0 min of incubation. This might be due to a quick activity of the Caco-2 peptidases on these peptides. Regarding the CW sample: peptides WENDECA (61-67 amino acids residues), DLEIL, DISLLDAQSAPL from β-lactoglobulin sequence, and peptide CKDDQNPH for α-lactalbumin. Peptide CKDDQNPH might have been hydrolysed to DDQNPH that was found until the end of the incubation time. Peptide DDQNPH was found related to ACE-I inhibitory activity on studies on milk fermentation[16], thus with the present work it was confirmed that this potential bioactive peptide is resistant to the peptidases activity.

Concerning the WPI sample: peptides WENDEC (61-66 amino acids residues), PEVDDE (126-131 amino acids residues), WENDECA

(61-67 amino acids residues), ENDECA (62-67 amino acids residues), QSAPL (35-39 amino acids residues), SAEPEQ (110-115 amino acids residues) from β-lactoglobulin sequence, and peptide LDDDL (81-85 amino acids residues) from α-lactalbumin.

In both the samples where identified peptides deriving from the region 125-135 of β-lactoglobulin. This region was previously reported as highly resistant to digestion[4]. The identification of peptides from this region after the incubation at 60 min in the apical sides confirmed the resistance of the region also to the activity of peptidases as previously reported[4].

Differences among the samples were found in the identified peptides: with the exception of β-lactoglobulin peptide WENDECA (61-67 amino acids residues) that was found in both the sample, different peptides were found susceptible to the rapid activity of the peptidases. Same results were found during the incubation, where only peptide TPEVDD was found susceptible to peptidases activity in both the samples. Concerning the peptides resistant during the incubation only two peptides deriving from β-lactoglobulin and one deriving from α-lactalbumin were found in the CW samples while more peptides were found for the WPI ones. During the whole experiment, more peptides were identified for the WPI sample. This may be related to the higher digestibility of the proteins in this sample due to processing, as described in Chapter 5.

Unexpectedly, in the basolateral side it was not possible to identify any peptides for both WPI and CW samples. It was previously reported that peptides deriving from the two whey proteins may be translocated, in particular the already mentioned peptides deriving from region 125-135 of β-lactoglobulin[4,17]. This finding might be ascribed to the adopted procedure, as an example the incubation time might be prolonged, since in the mentioned study, where peptides were found in the basolateral side, the incubation time was longer[4]. Further analyses are in progress to determine the free amino acids translocation in the basolateral side.

6.3.2 Transepithelial transport study on β-lactoglobulin peptide

The effects of the post-translational modifications on the activity of the brush border peptidase are still under investigation. As an example, it was found that the activity of these enzymes is limited in the digestion of caseinophosphopeptides[18]. It was also reported that the glycation of caseinomacropeptide limited the activity of brush border endopeptidases[19]. However information on the effects of lactosylation on the enzymes activity and intestinal absorption are still lacking.

From the semi-dynamic gastrointestinal simulated digestion performed on the WPI and CW sample, some interesting peptides with possible IgE-binding activity were found. Among the others, peptide KIDALNENKVLVL (83-95 amino acids residues) was found in the WPI gastric digested. This peptide is interesting because is part of a β-lactoglobulin epitope[20] (TKIPAVFKIDALNENKVLVL) for which it was found that the lysine residues in the sequence (lysine 83 and 91) could be lactosylated due to thermal treatments (Chapter 2). Moreover, in the study of the effects of storage conditions on protein content described in Chapter 3, it was found lysine 91 conjugated with a hexose. In this light, it was found interesting to test the possible transport of this peptide through the intestinal barrier and test also if the presence of lactose could affect the transport. The peptide was synthesised following the standard Fmoc protocol for the solid phase peptide synthesis as described in paragraph 2.2.6. Then the peptide was lactosylated in solution using a procedure that was recently developed and described in Chapter 2. Since this peptide contains two lysine residues in the sequence, when the reaction was performed three different products were found, the mono-lactosylated peptide (36%), the unreacted peptide (53%) and the di-lactosylated peptide (10%).

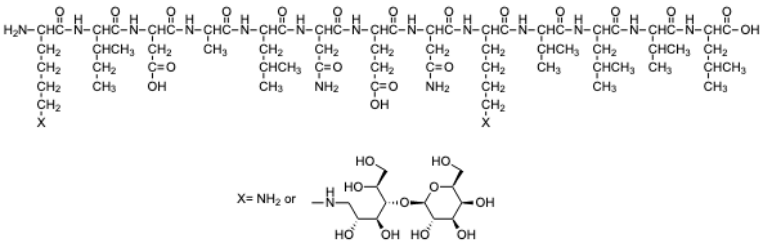


Figure 6.3.4 Peptide sequence KIDALNENKVLVL. Lactose can bind one or both the lysine residues present.

This mixture was then used for the transepithelial transport study. Before the assay, ultrafiltration (1kDa cut-off) was performed to remove the large excess of lactose that could affect cells. Peptide mixture was diluted to a concentration determined from the literature, where the transport of a large peptide (17 amino acids residues) deriving from β-casein was tested on Caco-2 cells[11].

Following the same protocol for the gastrointestinal digested, samples were collected at 0 and 60 min of incubation from the apical side and only at 60 min of incubation for the basolateral side during the assay.

As determined for the digested samples, the $\%LY_{basal}$ recovery values calculated from the Lucifer Yellow assay showed a low value suggesting that the monolayer was not affected by the incubation with the sample.

In Figures 6.3.5, 6.3.6 and 6.3.7 were represented the extracted ion chromatograms (XICs) of the collected samples. These XICs were obtained extracting the characteristic MS ions of three peptides in the mixture: native form, mono-lactosylated form and di-lactosylated form (Table 6.3.3). All the percentages among the peptides are determined by integration of the peaks area.

Table 6.3.3 MS ions of the peptide in the native and modified forms

Peptide	MS ions
Native form	1469,70 $[M+H]^+$; 735,30 $[M+2H]^{2+}$; 490,60 $[M+3H]^{3+}$; 368,19 $[M+4H]^{4+}$
Mono-lactosylated form	1793,70 $[M+H]^+$; 897,30 $[M+2H]^{2+}$; 598,50 $[M+3H]^{3+}$; 449,19 $[M+4H]^{4+}$
Di-lactosylated form	2117,70 $[M+H]^+$; 1059,30 $[M+2H]^{2+}$; 706,50 $[M+3H]^{3+}$; 530,19 $[M+4H]^{4+}$

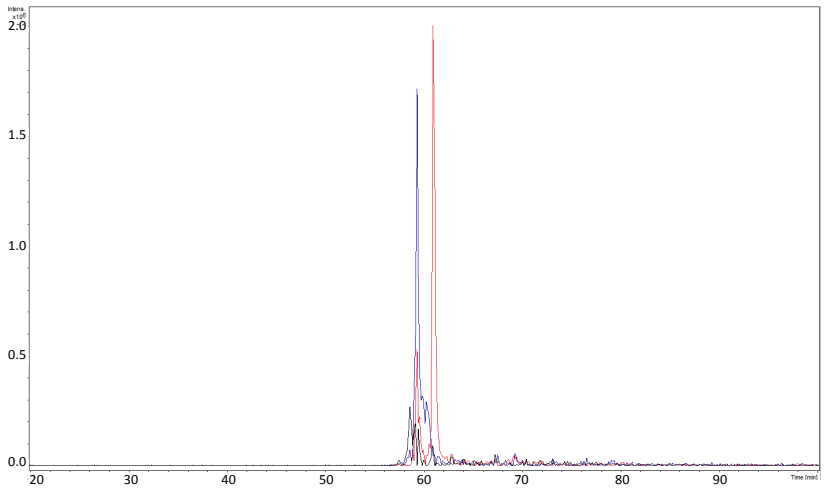


Figure 6.3.5 XICs chromatograms of the peptides in the native form (red), mono-lactosylated form (blue) and di-lactosylated form (black), in the sample collected from the apical side at 0 min. On the y axis is reported the intensity ($x10^6$), on the x axis the time (min).

In the sample collected at 0 min of incubation, all the three forms were identified with the following proportions: 50% of unmodified peptide, 42% of mono-lactosylated peptide, 8% of di-lactosylated peptide. Proportions among the different forms of the peptide are preserved with respect to the starting sample. Nevertheless, in this sample it was possible to see the activity of the peptidases on the peptides. Indeed, some fragments were identified: KIDALNE, NENKVLVL and ALNENKVLVL.

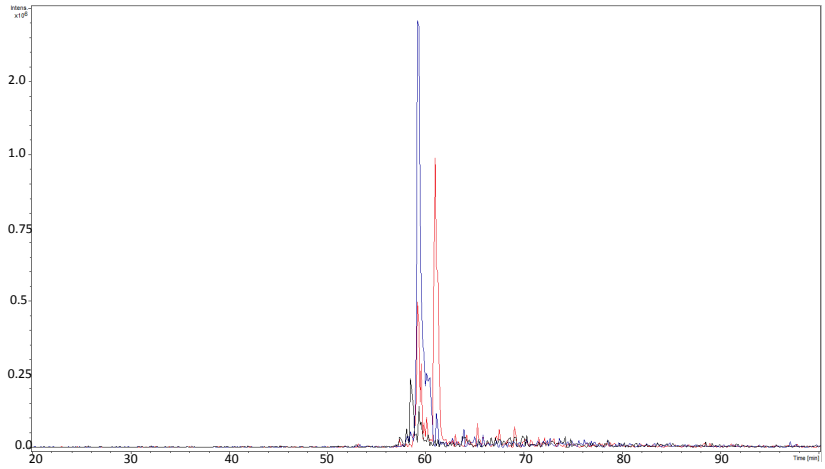


Figure 6.3.6 XICs chromatograms of the peptides in the native form (red), mono-lactosylated form (blue) and di-lactosylated form (black), in the sample collected from the apical side at 60 min. On the y axis is reported the intensity ($x10^6$), on the x axis the time (min).

After 60 min of incubation the proportions among the peptides in the apical side are different: 35% unmodified peptide, 57% lactosylated peptide, 8% di-lactosylated peptide. This difference could be related to an easier transport of the native peptide with respect to the modified forms, or to a more susceptibility of the same to the activity of the peptidases that are present on the surface of the Caco-2 cells. Even in this sample, some peptide's fragments were identified in the MS/MS spectrum: LNENKVLVL that may derive from the peptide ALNENKVLVL found in the apical side at 0 min, and NENKVLVL that was previously found in the apical sample collected at 0 min.

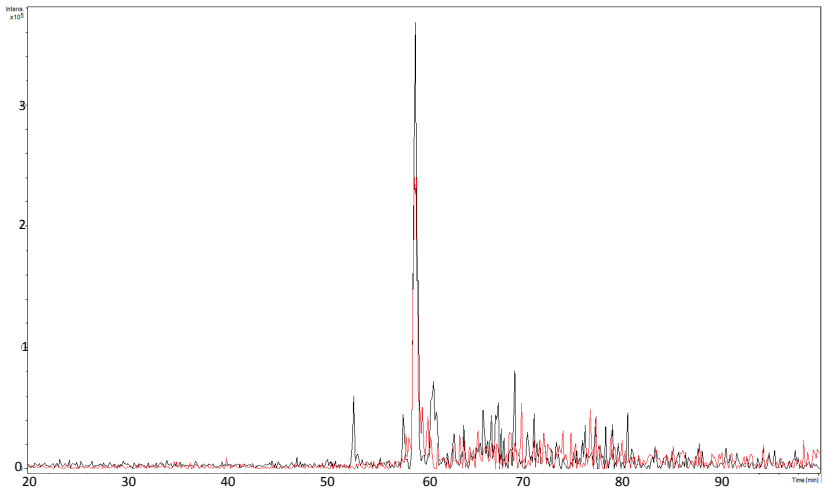


Figure 6.3.7 XICs chromatograms of the peptides in the native form (black) and mono-lactosylated form (red) in the sample collected from the basolateral side at 60 min. On the y axis is reported the intensity ($x10^5$), on the x axis the time (min).

Interestingly at the end, in the basolateral side were found both the peptide in the unmodified (54%) and mono-lactosylated (42%) form, while the di-lactosylated form was not identified. The proportions between the two forms suggest that the native peptide may be more easily transported then the other form. However, the presence of the lactosylated form in this sample suggests that the presence of lactose may influence the transport but it does not prevent it. Even in this sample, some peptide's fragments were identified, NENKVLVL and NENKVLV. The first one was identified in all the collected samples, suggesting that it is formed rapidly at the beginning and it remains stable after one hour of incubation and partially it is transported through the intestinal barrier. The second peptide might derive from the former due to the activity of the BBM enzymes and then is transported too.

6.4 Conclusions

Coupling the *in vitro* gastrointestinal digestion with *in vitro* transport studies is getting more and more relevance in these years, completing the information about nutrients' fate in the intestine. Thus, it gives also information on the activity of the brush border membrane enzymes that are present on the surface and act as a last digestive step.

From this study on peptides transport, promising results were obtained. The transport study on the digested samples showed the activity of the peptidases on the samples from the very beginning of the assay. The concentration of the digested sample and the bile salts used in the intestinal digestion did not affect cells monolayer but more investigation is required to understand if the absent transport was due to the samples or to the protocol conditions. Concerning the synthetic peptides, interestingly it was found that peptide KIDALNENKVLVL, a potential IgE-binding peptide, can be transported by the cell monolayer, both in the native and lactosylated form, and the activity of the peptidases is limited to a little fragmentation of the peptides. A deeper investigation may be required, as an example peptide quantification with an external calibration curve may better represent the ratio between the two peptides forms and give more information on the different absorption of the peptides. Moreover, other assays, trying to inhibit the different possible transport route, may be performed to understand which is the one involved in the transport of the analysed unmodified and modified peptides.

References

1. Brandelli, A., Daroit, D. J. & Corrêa, A. P. F. Whey as a source of peptides with remarkable biological activities. *Food Res. Int.* **73**, 149–161 (2015).
2. Picariello, G. *et al.* Role of intestinal brush border peptidases in the simulated digestion of milk proteins. *Mol. Nutr. Food Res.* **59**, 948–956 (2015).
3. Shimizu, M. Food-derived peptides and intestinal functions. *BioFactors* **21**, 43–47 (2004).
4. Picariello, G. *et al.* Transport across Caco-2 monolayers of peptides arising from in vitro digestion of bovine milk proteins. *Food Chem.* **139**, 203–212 (2013).
5. Giromini, C., Cheli, F., Rebucci, R. & Baldi, A. Invited review: Dairy proteins and bioactive peptides: Modeling digestion and the intestinal barrier. *J. Dairy Sci.* **102**, 929–942 (2019).
6. Chothe, P., Singh, N. & Ganapathy, V. Evidence for two different broad-specificity oligopeptide transporters in intestinal cell line caco-2 and colonic cell line CCD841. *Am. J. Physiol. - Cell Physiol.* **300**, 1260–1269 (2011).
7. Chothe, P. P. *et al.* Identification of a novel sodium-coupled oligopeptide transporter (SOPT2) in mouse and human retinal pigment epithelial cells. *Investig. Ophthalmol. Vis. Sci.* **51**, 413–420 (2010).
8. Vij, R., Reddi, S., Kapila, S. & Kapila, R. Transepithelial transport of milk derived bioactive peptide VLPVPQK. *Food Chem.* **190**, 681–688 (2016).
9. Wang, B. & Li, B. Charge and hydrophobicity of casein peptides influence transepithelial transport and bioavailability. *Food Chem.* **245**, 646–652 (2018).
10. Hubatsch, I., Ragnarsson, E. G. E. & Artursson, P. Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. **2**, 2111–2119 (2007).
11. Regazzo, D. *et al.* The (193–209) 17-residues peptide of bovine β-casein is transported through caco-2 monolayer. *Mol. Nutr. Food Res.* **54**, 1428–1435 (2010).
12. Rastogi, H., Pinjari, J., Honrao, P., Praband, S. & Somani, R. the Impact of Permeability Enhancers on Assessment for Monolayer of Colon Adenocarcinoma Cell Line (Caco-2) Used in in Vitro Permeability Assay. *J. Drug Deliv. Ther.* **3**, 20–29 (2013).
13. Ferraretto, A. *et al.* Morphofunctional properties of a differentiated

Caco2/HT-29 co-culture as an in vitro model of human intestinal epithelium. *Biosci. Rep.* **38**, 1–16 (2018).

14. Brodkorb, A. *et al.* INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nat. Protoc.* **14**, 991–1014 (2019).
15. Wang, C., Wang, B. & Li, B. Bioavailability of peptides from casein hydrolysate in vitro: Amino acid compositions of peptides affect the antioxidant efficacy and resistance to intestinal peptidases. *Food Res. Int.* **81**, 188–196 (2016).
16. Rodríguez-Figueroa, J. C., González-Córdova, A. F., Torres-Llanez, M. J., Garcia, H. S. & Vallejo-Cordoba, B. Novel angiotensin I-converting enzyme inhibitory peptides produced in fermented milk by specific wild Lactococcus lactis strains. *J. Dairy Sci.* **95**, 5536–5543 (2012).
17. Corrochano, A. R. *et al.* Bovine whey peptides transit the intestinal barrier to reduce oxidative stress in muscle cells. *Food Chem.* **288**, 306–314 (2019).
18. Boutrou, R., Coirre, E., Jardin, J. & Léonil, J. Phosphorylation and coordination bond of mineral inhibit the hydrolysis of the β-casein (1 - 25) peptide by intestinal brush-border membrane enzymes. *J. Agric. Food Chem.* **58**, 7955–7961 (2010).
19. Boutrou, R., Jardin, J., Blais, A., Tomé, D. & Léonil, J. Glycosylations of κ-casein-derived caseinomacropeptide reduce its accessibility to endo- but not exointestinal brush border membrane peptidases. *J. Agric. Food Chem.* **56**, 8166–8173 (2008).
20. Matsuo, H., Yokooji, T. & Taogoshi, T. Common food allergens and their IgE-binding epitopes. *Allergol. Int.* **64**, 332–343 (2015).

Summary

Milk and dairy products require processing for their safe consumption and longer shelf life. The processes involved may affect protein content inducing structural (i.e. aggregation and denaturation) and chemical modifications (i.e. glycation). These modifications may influence other properties such as protein digestibility and allergenicity. Due to the increasing use of whey proteins ingredients as food additives, related to their technofunctional properties and high nutritional value, this PhD thesis was aimed at studying the effects of processing on them. Main aims of this study were: a) the study of the effects of the structural and chemical modifications on protein digestibility: b) the study of the modifications, in particular lactosylation, on the allergenicity and intestinal absorption.

Chapter 1

The use of UV irradiation was investigated as a possible alternative to the pasteurization in terms of microbiological safety. A reactor was designed for the treatment of skimmed whey. The system was tested on samples of skimmed whey obtained from the production of Parmigiano-Reggiano cheese. Results were compared with traditional thermal treated samples. It was found that the microbiological safety obtained with the two treatments was comparable. UV treated samples showed a higher amount of soluble whey protein and a lower degree of lactosylation. Indeed, in this study was developed an analytical method for the detection of lactosylated whey proteins with UPLC-ESI-MS analysis. Moreover, the proposed treatment did not alter the nutritional value of the product.

Chapter 2

The work described in this chapter aimed at identifying the lactosylation sites in bovine whey proteins α-lactalbumin and β-lactoglobulin. A screening on different pasteurized and UHT treated samples was performed to confirm the increase in proteins lactosylation degree due to the harshness of the treatment, as reported in previous studies. In solution tryptic and chymotryptic digestion was performed to identify glycated peptides in the samples.

LTQ-Orbitrap and UPLC analysis allowed to identify some lactosylation sites in the samples and the comparison with the known epitopes reported in literature confirmed the presence of possible glycation sites in their sequences. This information was crucial for the development of the work. Indeed, it was then decided to synthesize some of these epitopes in their unmodified and lactosylated form for further ELISA tests that will investigate the effects of lactose on the molecular interaction of the epitopes with human IgE. A procedure for the site-specific lactosylation of the epitope (Fmoc solid phase peptide synthesis and in solution lactosylation in DMF) was thus developed and optimized, with high reaction yields.

Chapter 3

Apart from the processing, also the storage conditions may affect the protein content inducing structural and chemical modifications. Thus, a study on whey protein concentrates (WPC-35) stored at different conditions was then performed. Samples were provided by FrieslandCampina (Amersfoort, the Netherlands) and stored from 20 to 60°C and 3 to 14 days. The molecular characterization performed confirmed an increase of the lactosylation degree and a decrease in the soluble protein content (due to denaturation/aggregation) on whey proteins due to both temperatures and duration of storage. Some other post-translational modifications (PTMs) were found due to the storage conditions applied (i.e. carboxymethylation, hexose glycation). From the analysis of the total amino acids amount and the determination of the chemical score, it was observed that the nutritional value was still acceptable despite the observed modifications. Moreover, in parallel it was investigated the effect of the drying technique on the protein content. Two samples of WPC-35, one freeze-dried and the other spray-dried were compared. The obtained results showed minimal differences in the induced chemical and structural modification, and the nutritional value was comparable.

Chapter 4

For the evaluation of the effects of processing on whey protein digestibility a recently developed semi-dynamic *in vitro* gastrointestinal digestion model was used on a sample of spray-dried

WPC-35 and following the INFOGEST protocol. This model allowed to decrease the pH gradually in the gastric phase and to simulate the gastric emptying. The intestinal phase was performed in a static way on the fractions collected in the simulation of the gastric emptying. With UPLC-UV and HPLC-MS/MS analysis it was possible to follow protein degradation and peptide release. As a result, α-lactalbumin was digested in the first 36 minutes of the gastric phase, while β-lactoglobulin was present in all the gastric phase but a little decrease in the amount was observed, probably due to a higher susceptibility to digestion induced by the processing. From the comparison with reported *in vitro* static model, it was found that with the semi-dynamic model a higher protein coverage was obtained.

Chapter 5

The semi-dynamic digestion protocol described in Chapter 4 was used to compare the digestibility of whey proteins isolates (WPI) with a sample of untreated whey (obtained after the production of cheese). Due to processing, that causes structural modifications in proteins, whey protein isolates sample showed a higher digestibility with respect to the untreated sample. ELISA inhibition tests were performed on the digestive fractions to evaluate the effects of digestion. A pool of patient sera allergic to whey proteins was used to evaluate the potential IgE-binding. Undigested samples showed little differences in the IgE-binding and a residual value was observed in the digested samples, in particular in the gastric fractions. It was observed that, due to processing induced higher digestibility, the WPI digested samples showed higher residual allergenicity in the late gastric phase.

Chapter 6

Transepithelial transport studies were performed with a dual objective: a) to complete the digestion study described in Chapter 5; b) to investigate if the presence of lactose may affect the intestinal absorption. Intestinal digested samples (Fractions 5) from WPI and untreated whey were tested on Caco-2 cells monolayers. From the assay, it was possible to observe the activity of peptidases, present on the surface of the cells, on peptides. However, further studies are required since it was not observed peptide transport as expected.

Peptide KIDALNENKVLVL, for which it was observed that the lysine residues might be lactosylated, was synthesised and lactosylated following the procedure developed in Chapter 2. Synthetic peptides were tested on Caco-2 monolayers. From the performed assays, it was observed the transport of the peptide in both the unmodified and glycated forms, thus the presence of lactose bound to the sequence did not prevent peptide absorption.

Acknowledgements

I would like to express my sincere gratitude to my supervisor Prof. Tullia Tedeschi for her support and supervision during my PhD thesis, and Prof. Arnaldo Dossena and Prof. Stefano Sforza for their mentoring. My sincere thanks go to my thesis co-supervisor Dr. Martine P. van Gool for her support in reviewing the thesis. I wish to thanks also all the professors and researchers of the Food and Drug Department.

I would like to thank the BIOPEP group of the Institute of Food Science Research (CIAL, CSIC-UAM) in Madrid, in particular Dr. Beatriz Miralles and Dr. Isidra Recio for the opportunity they gave me to spend some months in their group and for their support in my work. I am extremely thankful to Dr. Elena Molina and Dr. Sara Benedé for the possibility and the help in performing the ELISA tests on my samples.

Finally, I would like to thank my family, my boyfriend and my friends for their support during these three years. Thank you to my sister, Camilla, for the cover and the illustrations of this thesis.

About the author

Alessandra Gasparini was born on the 2nd of September 1991 in Verona, Italy. In 2010 summer she took part on a scientific expedition across the Silk Road as the responsible for the collection of hearing phenotypes and samples. She started her studies in Chemistry at the University of Trieste in 2010. The bachelor was concluded, in 2013, with a thesis entitled "Design, synthesis and characterization of gold nanoparticles protected by pegylated ligands". The same year she started her master studies in Chemistry, Organic-Biomolecular curriculum, in the same university. The Master degree was concluded, in 2015, with a thesis entitled "Synthesis of cafestol and 16-*O*-methylcafestol derivatives for the quality control of coffee", obtained with full marks. Part of the master thesis was performed in collaboration with the Illy Company's Aromalab, Trieste. In 2016 she gained the national board certification in Chemistry. The same year she started her PhD at the Food and Drug Department of the University of Parma. During the PhD she spent 5 month at the Institute of Food Science Research (CIAL, CSIC-UAM) of Madrid as a visiting PhD student in the BIOPEP group. The results of her PhD research are presented in this thesis.

List of publications

Buhler S, Solari F, Gasparini A, Montanari R, Sforza S, Tedeschi T. "UV Irradiation as a comparable method to thermal treatment for producing high quality stabilized milk whey", *LWT-Food Science and technology*, **105**, 127-134, (2019).

Finotello C, Forzato C, Gasparini A, Mammi S, Navarini L, Schievano E. "NMR quantification of 16-O-methylcafestol and kahweol in *Coffea canephora* var. robusta beans from different geographical origins" *Food Control*, **75**, 62-69, (2017).

Co-author of the book "Marco Polo, genes and flavours on the silk road", ed. White Star, 2015.

Girotto G, Pirastu P, Gasparini A, d'Adamo P, Gasparini P. "Frequency of hearing loss in a series of rural communities of five developing countries located along the Silk Road" *Audiological Medicine*, **9**, 4: 135–140, (2011).

Scientific Meetings/Workshops Attendance

-Second edition of "La chimica degli alimenti e I giovani ricercatori: nuovi approcci in tema di qualità, sicurezza e aspetti funzionali di ingredient alimentari" organised by the Universities of Milano and Parma, short talk, 24 September 2019, Milano.
-"Giornata scientifica della Società Italiana Peptidi dedicate ai Soci Giovani-Premio scienfico Vittorio Erspamer" organised by the Italian Peptide Society, short talk, 21 September 2019, Firenze.
-XXIII Workshop on the Developments in the Italian PhD research on food science technology and biotechnology, organized by Consorzio UNO, poster presentation, 19-20 September 2018, Oristano.
-7th EuCheMS Chemistry Congress, organized by the European Chemical Sciences and the Royal Society of Chemistry, short talk and poster presentation, 26-30 August 2018, Liverpool.

-"Advanced school: Food Proteins" organized by SIB-Protein Group, oral presentation, 2-4 May 2018, Bergamo.
-8th International Symposium on Recent Advances in Food Analysis (RAFA), organized by University of Chemistry and Technology of Prague and RIKILT (University of Wageningen), poster presentation, 7-10 November 2017, Prague.
-5th MS Food Day, organized by Divisione spettrometria di Massa (Italian Chemical Society), oral presentation, 11-13 October 2017, Bologna.
-22th Workshop on the Developments in the Italian PhD research on food science technology and biotechnology, Free University of Bozen, poster presentation, 20-22 September 2017, Bolzano.

Courses/ Summer schools Attendance

-2nd IMaSS Data Analysis School, organized by Imass, 17-19 October 2018, Firenze.
-2nd Advanced school on protein structure and dynamics: from Spectroscopy to Mass spectroscopy, University of Parma-Chiesi Farmaceutici, 27-29 September 2017, Parma.
-"In Silico Methods for Food Science", European Food Safety Authority (EFSA), 13-15 June 2017, Parma.
-3rd APP-MS School, organized by IMaSS, 23-30 April 2017.
-"Quality Assurance and qualification of the operators: monitoring with Shewhart's control charts" training course, organized by Ultra Scientific Italia, 27 October 2016, Ferrara.
-"In silico/in vitro approaches for Food Science", European Food Safety Authority (EFSA), 9 September 2016, Parma.