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Study of Protein Aggregation Machinery in Processed Food Products: New Details for a Supramolecular Organization

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La scienza è una cosa meravigliosa
per chi non deve guadagnarsi da vivere con essa.
(Albert Einstein)

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Study of Protein Aggregation Machinery in Processed Food Products: new details for a Supramolecular Organization

Abstract

This PhD thesis deals with the assessment of the protein aggregation machinery in cereal, meat and milk products. The protein aggregation systems in pasta products and baked products, in cooked meat products and in UHT milk were studied through the application of size exclusion liquid chromatography (SE-HPLC), electrophoretic techniques (SDS-PAGE, UREA-PAGE, 2D-electrophoresis) and mass spectrometry analyses. The overall results of this thesis provide for new details in the assessment of the protein organization in these systems, where the formation of spontaneous and/or heat-induced new supramolecular architectures can be accountable for the nutritional and technological food properties.

Prefazione

Questa tesi di dottorato riguarda lo studio dei meccanismi di aggregazione proteica spontanea e/o indotta, anche di natura supramolecolare, in alcune matrici alimentari. L'organizzazione proteica in prodotti trasformati, quali pasta, biscotti, salumi cotti e latte UHT è stata studiata mediante l'applicazione di tecniche cromatografiche (cromatografia ad esclusione molecolare), elettroforetiche (SDS-PAGE, UREA-PAGE, elettroforesi bidimensionale) e spettrometria di massa, ottenendo nuovi dettagli sui sistemi di organizzazione proteica in sistemi complessi, come le matrici alimentari.

Introduction

1.1 Protein structure

Proteins are complex natural macromolecules made of sequences of amino acids with an amide type of covalent chemical bond, called peptide bond. Each protein comprises a linear sequence of amino acids (primary structure) encoded by the genetic code.

The secondary structure of the protein consists in the spatial arrangement of the polypeptide chain through repetitive and regular structures stabilized by hydrogen bonds, α-helix and β-pleated sheets. In the α-helix motif, the hydrogen bonds are shared by amino acids that are close in the primary structure of the protein, with a right hand-spiral conformation; while the β-sheets involve faraway ones in a pleated conformation. These two regular structures can be present also in combined forms. The steric hindrance, due to the presence of too large amino acids R-groups (i.e tryptophan, tyrosine) or too small (i.e glycine) or proline, destabilizes the α-helices. Proline destabilizes α-helices, because of its irregular geometry, arising from its cyclic side chain, which blocks also the N atom in the main chain, preventing the formation of the hydrogen bond. The presence of the proline causes two H-bonds in the helix to be broken, since also the NH group of the following residue is prevented from forming a good hydrogen bond.

An important conformational features of the globular proteins are the reverse turns, β-turns and β-bends. They occur at “hairpin” corners, where the peptide chain suddendly changes direction. Such corners involve four amino acid residues, often including proline and glycine, which can be found in the unstructured regions of secondary structures.

The tertiary structure of a protein chain represents its three-dimensional organization, as a consequence of its own primary structure and involves the interactions among the amino acid residues, which are far away from each other.

The tertiary structure can be stabilized through different types of interactions, which determine a compact but flexible arrangement:

- hydrophobic or hydrophilic interactions, since the hydrophobic residues (R) are normally hidden within the inner core of the protein, to minimize the contact with the surrounding water molecules and the polar hydrophilic groups tend to set towards the aqueous medium;
- ionic attractions, between $-NH_3^+$ and $-COO^-$ groups of two different amino acids residues;

- hydrogen bonds, which derive from the attraction among residues groups (R) or that are established among peptide groups;
- disulfide bonds, the only involved covalent bonds, which are formed between -SH groups of the cysteine molecules, which, as a consequence of an oxidation reaction, give rise to a -S-S bond.

According to the tertiary structure, proteins can be divided into fibrillar or fibrous and folded or globular proteins. In the fibrous proteins, which as general features, are water-insoluble and have a structural role, the polypeptide chains is arranged within a single regular structure (α-helix or β-sheets), stabilized by intermolecular bonding (primarily hydrogen bonds and hydrophobic interactions).

The globular proteins are spherical proteins, where the regular structures are randomly mixed. They can have several functional roles and, as a consequence of the folding process, where the hydrophobic amino acids are bounded towards the inner part of the molecule, whereas the hydrophilic amino acids are set outwards, allowing dipole-dipole interactions with the solvent, they are mostly water-soluble. The globular protein folding process is complex but thermodynamically favored and the polypeptide chains are packed through the formation of intermolecular non-covalent bonds (hydrogen bonds, hydrophobic interactions). Generally, this type of tertiary structure, can be easily modified with changes in the conditions of the medium (i.e application of high temperatures, adding salts or through pH variations).

Besides these three structural levels, proteins occur also as molecular aggregates, which are arranged in an ordered geometric organization (quaternary structure). This is an example of a supramolecular architecture, which is characterized by the involvement of weak bonds, such as hydrogen bonds and van der Waals contacts or stronger linkages, such as ionic and covalent bonds. All of these levels of protein structure help to stabilize the folded-up, active conformation of a protein, together with the *solvation shell*, i.e the layer of solvent that surrounds a protein.

In an aqueous medium, this protein structure can be modified by a dynamic conformational changing, up to determine the native protein structure, the one characterized by the minimum energy state and the greater stability (Food Chemistry 4th revised and extended version, Springer).

1.2 Protein denaturation

Proteins, in their native state, are organized into well-defined, folded three dimensional structures (i.e globular, rodlike or mixture of rodlike and globular portions).

The occurrence of major changes in the native structure of any proteins, which leads to an unfolded state, without the alteration of the protein primary structure, is defined "*Protein denaturation*" (Tanford, 1968).

The denaturation event can be accomplished through the application of the heat treatment, modifying the pH, increasing the interface area, or adding solvents, salts or denaturing agents, which alter and or disrupt the electrostatic attractions, the hydrophobic and hydrogen bonds, which determine the protein folded state.

Protein denaturation can be a reversible event, when the polypeptide chain is stabilized in its unfolded state by the presence of the denaturing agent and the native conformation can be re-accomplished, since the the source of the unfolding event can be removed. The irreversible denaturation occurs when the unfolded protein, exposing the hydrophobic residues, which are normally hidden in the inner part of the protein, gives rise to aggregation events, through the formation of tight linkages, due to non-covalent and covalent bonds.

In particular, the heat treatment is used in order to improve the texture, the taste, the digestibility and the microbiological safety of food products (Boye et al., 1997), determining deep changes to the main constituents of the food matrices, as in the case of proteins.

With the progressive increases in temperature, the protein loses its secondary and tertiary structure, adopting an almost completely open configuration, random-coil like (Davis &William, 1998). The protein begins to open, as the temperature increases, leading the hydrophobic groups, initially oriented inwards, to be oriented towards the medium.

The conformational changes, caused by the loss of the native protein structure, through the weakening of intramolecular bonds (hydrophobic interactions, hydrogen bonds, electrostatic interactions and disulfide bridges) lead to a new organization of the protein structure.

1.3 Protein aggregation

The aggregation event is one of the consequences of the interaction among the heat-denatured proteins.

The term 'aggregates' is a common term used to refer to multimeric species, which are linked through covalent bonds or non-covalent interactions. It can follow different pathways depending on the environmental conditions, the applied stress and the native state of a protein (Mahler et al., 2009).

Protein aggregates can be categorized on the basis of different chemical-physical characteristics, defining, as general rule, two main forms, *soluble* or *insoluble aggregates*. These aggregates can be built up by *non-covalent* (made by weak interactions and/or hydrogen or

hydrophobic bonds) (Karshikoff, 2006) or *covalent* (made by disulfide or nondisulfide crosslinks) (Cromwell, Hilario & Jacobson, 2006) bonds and whose formation can be *reversible* or *irreversible*. The formation of reversible aggregates is often referred to the self-assembling protein machinery, carried on by the weak non-covalent interactions, highlighting an equilibrium between the monomeric and higher order forms, which shift according to the changes in the medium (i.e protein concentration or pH variations) (Cromwell et al., 2006; Mahler et al., 2009; Bouhallab & Croguennec, 2013). Irreversible protein aggregates formation is more complex and according to the model of Lumry-Eyring is a two-step event, where the protein undergoes first a reversible conformational change from its native organization to an aggregation-prone state and, then, arranges itself irreversibly to an aggregated state (Andrews & Roberts, 2007; Mahler et al., 2009).

As cardinal rule, the protein tends to minimize the stress factors, such as the unfolding and the exposure of the hydrophobic amino acid residues that increase the surface energy, or Gibb's free energy. The initial aggregation (pre-nuclear phase), driven by hydrophobic interactions and hydrogen bonds among the polar amino acid residues pushes the process. The steps that come early the pre-nuclear aggregates are unfavourable, owing to the loss of the rotational and translational entropy, which is larger than the energy gain, due to the bonds formation. However, as the aggregate size increases, the energy gain, due to the larger number of bonds per molecule formed overcomes the net entropy loss and the addition of monomers becomes thermodynamically favourable (Saha & Deep, 2014).

The change in Gibb's free energy, at particular sizes, is controlled by the ratio of the forward and reverse rate constants, referring to polymerization and depolymerization reactions, respectively (Ferrone, 1999).

Protein aggregation is the response to many factors, such as temperature and pH variations, protein concentration, the ionic strengh, the presence of ligands, including specific ions, but it is affected also by any mechanical stress or processing, which can influence the aggregation rate and the type of induced aggregates.

The study of protein aggregates has a great relevance, due to the key role that proteins may have, especially, in the biological, pharmaceuticals and medical fields.

The possibiliy to remove or not a protein aggregate helps to distinguish a "soluble" or an "insoluble" aggregate. The former is not visible and may not be removed by a filter with a pore size of 0.22 μm, the latter, indeed, is a visible particulate, which can be removed by filtration (Cromwell et al., 2006).

Several methods have been developed to study the size and shape, the molecular weight and the structure of protein aggregates, such as chromatographic (Unger, 1983) and electrophoresis

techniques (Shapiro et al., 1967; Laemmli, 1970), microscopic methods (Ma, Shieh, Qiao, 2006; Frederix et al., 2003), static or dynamic light scattering (Tsai, van Zanten, Betenbaugh, 1998; Qian, Mhatre, Krull, 1997) and spectroscopic analysis (Pelton & McLean 2000), but they can be real helpful according to what it is looking for, and mostly, they can work as combined techniques.

In the development of this thesis, size exclusion liquid chromatography (SE-HPLC) and the Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis (SDS-PAGE) were used to quantify and assess the size and the composition of 'soluble' protein aggregates. Size exclusion liquid chromatography (SE-HPLC) is a chromatographic method, which is applied to study large molecules and /or macromolecular complexes.

In particular, gel permeation chromatography (GPC/SEC) is the technique used to characterize the polymers molecular weight distribution. The molecules can be fractionated by their size through the elution in column, which retains the low molecular weigh polymers longer than the high molecular weigh ones. The sample is separated inside a column, a hollow tube tightly packed with extremely small porous beads, typically polymer or silica, designed to have pores of well defined size. One of the most important step is the dissolution of the polymer sample in a solvent, because even if a polymer can be described as long chains of linked monomers, they don't exist in this way in a solution. In this way, the molecules arrange themselves like a ball of string and behave like tiny spheres during the analysis. The size of the sphere is linked to the molecular weight, therefore, the high molecular weight polymers will form larger spheres. During the elution, if the polymers coils are larger than the biggest pores of the beads column, they cannot be retained and so they flow with the mobile phase, while if the polymers coil are smaller than the smallest pores of the column beads, they can enter into the pores, occupying all the stationary phase. This partitioning occurs repeatedly and small polymer coils that can enter many pores in the beads exit the column slowly, while large polymer coils that cannot enter the pores take less time to leave the column and polymer coils of intermediate size exit the column somewhere between these examples. The application of this technique allows to fractionate '*soluble*' aggregates with a molecular weight ranging from 5 to 1000 kDa. The too large oligomers escape the detection eluting with the void volume, while the '*insoluble*' aggregates are not analyzed through this technique due to the sample preparation procedures (centrifugation or the use of a precolumn) (Mahler et al., 2009).

The use of molecular weight standards allows to have indications on the differences with respect to the elution of the analyzed sample, together with some detectors that reacts directly to the molecular weight of the components when they leave the column and rely on the physical properties of the polymer, such as their ability to scatter the light or viscosity (Striegel et al., 2009).

The electrophoretic techniques, especially SDS-PAGE, were developed to discriminate the formation and the composition of non-covalent and covalent protein aggregates linked through disulfide bonds, using the ditiothreitol (DTT) or β-mercaptoethanol, as reducing agents.

This techinque was introduced by Shapiro et al., (1967) and allows the protein separation according to the molecular weight, without any influence of the protein shape along the electrophoretic separation (Lane, 1978). The individual charge of the protein is masked by the anionic detergent sodium dodecyl sulfate (SDS), which is able to interact with proteins in a constant ratio of 1.4g of SDS per gram of protein. The protein unfolding, as a consequence of the disruption of the secondary and tertiary structures and the cleavage of the disulfide bonds, then protecting –SH groups with alkylation with iodoacetamide, allows the SDS to bind the unfolded protein chains, by forming ellipsoids with identical central axes. The benefits of using SDS electrophoresis are closely related to its ability of solubilization almost all proteins; the SDS-protein complexes are highly charged and so they have a high electrophoretic mobility, with high resolution, especially with restrictive gels and the bands are easy to fix and to be analyzed with sensitive and efficient image analysis software (Electrophoresis in Practice, 4th revised and enlarged version, Wiley VCH).

A disadvantage of this technique is the detection of very large protein aggregates, with molecular weight higher than 250 kDa, which is limited, but the possibility of control the acrylamide/bis-acrylamide concentration with the use of gradient gels and/or specific buffers and the possibility of the combination of different electrophoretic techniques in 2D electrophoresis applications is helpful. These latter, as examples, can be performed using the immobilized pH gradient strips as first dimension and the SDS-PAGE, as orthogonal second dimension, to study every single element of the sample according to the isoelectric point and the molecular weight (Holland et al., 2011); a not-reducing alkaline urea page (AU-PAGE) or SDS-PAGE as first dimension coupled with a reducing SDS-PAGE, as second dimension, can be performed to study the monomers of the aggregates and other constituents (Patel et al., 2006; Chevalier & Kelly, 2010). All these electrophoretic techniques can be easily coupled to sensitive mass spectrometry analyses, giving new and greater perspectives for the investigation of protein aggregates through electrophoretic analyses.

Mass spectrometry has become a fundamental tool for the analysis of food proteins, with a wide range of techniques used to quantitatively and qualitatively analyze protein mixtures in food matrices. The MS-based and the new developed techniques, such as tandem mass spectrometry (LC-MS-MS) are powerful methods to assess the biochemical, technological and safety aspects of food products, including the analysis of the supramolecular complexes occurring in food matrices, which in turn affect the structural and rheological properties of the products (Mamone et al., 2009).

The study of protein aggregates has always been considered as crucial, especially in the biomedical field, because higher levels of non-native secondary structural elements with rich intra and inter-protein β−sheets, commonly referred to as amyloid fibrils (Eisenberg et al., 2006) were found to be related to the arise of several neurodegenerative diseases, i.e Alzheimer and Parkinson syndrome (McManus et al., 2016; Ross & Poirier, 2004).

Currently, this study has become an important topic also in the field of food science and technology.

1.3.1 Food Protein aggregation

Food proteins are considered as versatile biopolymers, whose functions and behaviour have been studied for a long time. The study of food protein assemblies gives important informations on the influence that these structures have on the structural and sensory properties of food (Giacomazza et al., 2016; Deuscher et al., 2017).

Specific protein assemblies, in normal conditions, are a distinctive property for some food matrices (i.e casein micelles in milk; the muscular protein organization in the sarcomere) that are modified when undergo specific processing and/or during the storage. These events lead to a new arrangement in an 'induced-assembling'.

The aggregation of food proteins differs from all other classes of protein systems (Ubbink et al., 2008), being strongly dependent on the characteristics of the medium.

Protein self-assembling in food systems occurs through the arrangement of different structures, such microspheres (Desfougères et al., 2010) together with the formation of three-dimensional, two-dimensional and one-dimensional protein aggregates or colloidal dispersions in solution (Mezzenga & Fisher, 2013).

The study of the aggregation in one dimension, for istance, is linked to self-assembling mechanism of food proteins, with the formation of fibrillar aggregates, whose study is becoming very important in the field of food technologies for their use as functional food ingredients but also because this arrangement is similar to the amyloid protein aggregates, occurring in *in vivo* test (Mezzenga & Fischer, 2013),

Food matrices are complex systems, in which more macro-constituents coexist and where the presence of other types of components, such as hydrocolloids or amphiphilic compounds, can influence the aggregation event into larger and more complex structures.

Moreover, the formation of these protein architectures could affect the functional and structural properties of proteins, through their ability to protect and/or release substances (Chapeau et al., 2016; Zacharova et al., 2016).

The ability to form these new structures, which can be defined as supramolecular, is a property of proteins, which entails a new recognition among the protein structures (Donald, 2008) according to the rules of supramolecular chemistry.

1.4 Supramolecular chemistry

Supramolecular chemistry was defined as "the chemistry of intermolecular binding, which collects structures and functions of entities formed by the association of two or more chemical species" (Lehn, 1988; 2004). In its simplest form, supramolecular chemistry deals with systems where a molecule, defined as *host*, through a non-covalent interaction or a form of complexation 'binds' a molecule, called *guest*, giving life to a *host-guest* supermolecule. This type of interaction affects the functionality (molecular recognition, transport, etc.) of the most common biological systems (Lehn, 1988).

Due to the lability of the non-covalent interactions, which bond the molecular entities of the supramolecular structure, supramolecular chemistry was defined also as a *dynamic chemistry* (Lehn, 2007).

This *dynamic* behaviour allows the supramolecular architectures to associate and dissociate reversibly, leading to the incorporation/decorporation and rearrangement of its molecular components. The possibility to transfer this *dynamic* feature also in the molecular chemistry, needs to intend the covalent bond as ‘dynamic’ as well, allowing a continuous reorganization and exchange of the building blocks of the supramolecular architecture (Lehn, 2007), whose properties are not simply the sum of its individual subunits (Deuscher et al., 2017).

In this perspective, the studies which have been carried out in recent years deepened the mechanism of protein assembling, evaluating the arrangement of different types of supramolecular structures (amyloid fibrillar networks, spheres, nanotubes) (Gosal et al., 2002; Gosal, Clark, Ross-Murphy, 2004; Ipsen & Otte, 2007; Desfougères et al., 2010).

The specific protein organization in supramolecular structures is strictly related to the physico-chemical conditions of the medium. Therefore, even if the self-assembling is based on the molecular recognition, the conditions and the composition of the medium, especially in the case of a food system, are a key element to set the size, shape and the characteristics of the supramolecular structure (Bouhallab & Croguennec, 2013).

The formulation and processing of foods are related to the protein content and the supramolecular protein organization, which provide for the functional properties of the product (Curtis, 2019).

The presence of several main constituents in the food matrix, such as, carbohydrates, lipids, minerals and the possible presence also of small solutes (ions, amino acids, fatty acids), can

influence the protein assembling machinery (Unterhaslberger et al., 2006; Nigen, Croguennec, Bouhallab, 2009). Therefore, in the light of the great variability of the protein structure and according to its stability, net charge, presence of hydrophobic and hydrophilic residues and the physico-chemical characteristics of the medium, a protein system can arrange into unlimited supramolecular structures (Bouhallab & Croguennec, 2013).

Supramolecular architectures can be roughly divided into two main groups: spontaneous and induced assemblies. The self-assembling machinery and the formation of "molecular assemblies", determine the formation of polymolecular systems characterized by the spontaneous association of an undefined number of components, which reorganize into larger structures, such as membranes and micelles, with a specific microscopic organization and macroscopic characteristics (Lehn, 1988), without any energy or denaturing agents contribution.

The self-assembling event is widely present in nature because it carries out many biological functions (structural, regulation, protection, transport). This type of ordered arrangement is based on low energy protein-protein interactions (hydrogen bonds, salt bridges, electrostatic interactions) and the supramolecular structure can change size (from nanometer to micrometre) and shape (fibres or sferical complexes), according to the conditions of the medium and, moreover, being based on the surface energy and attractive or repulsive interactions, it can be stable and/or reversible (Becker et al., 2012; McManus et al., 2016).

The "induced assemblies" occur when the protein system is destabilized as a result of denaturing and/or hydrolysis event, which, hence, entail, their re-organization. The application of the high temperatures, pH conditions and/or solvent change are the conditions that determine this protein arrangement (Gosal et al., 2002; Gosal et al., 2004; Lara et al., 2011; Diniz et al., 2014; Deuscher et al., 2017), which is often irreversible. The induced supramolecular assembling are indipendent from the primary structure of proteins, because different proteins can arrange into similar supramolecular structures (fibrils, spherulites, particulate aggregates, nanotubes) (Bouhallab & Croguennec, 2013).

Within this context, the aim of this thesis was the study of the induced protein assembling in three different protein systems: cereal products, meat products and in UHT-treated milk.

In these three matrices, the heat treatment was the common external stress agent. However, several other structural modifications, with subsequent protein rearrangement, can occurr pre and/or post the application of the heat treatment, such as during the extrusion or the storage of the product.

The assessment of the protein aggregates at molecular level, as the result of the new chemical and physical environmental conditions, can be a useful way to understand the mechanisms that drive the protein-protein and, consequently, aggregate-aggregate interactions, which could be

used, in turn, to better ponder on the technological properties and deepen the knowledge of what occurr, at higher scale levels, in complex food matrices.

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Session 1

Cereal Products

Protein organization in wheat-based products

Wheat is a key food being associated with the most important food uses. The agronomic adaptability, the ease of storage, the nutritional properties and the ability of its flour to produce a large variety of products are the main reasons why it is one of the main crops spread all over the world.

The first cereal proteins classification based on the solubility and the functionality was developed by Osborne (1908).

Osborne found that cereal proteins could be separated on the basis of their solubility in four main classes: albumins (soluble in water and dilute buffers), globulins (soluble in salt solutions), prolamins (soluble in 70 - 90% ethanol) and glutelins (soluble in dilute acid or alkali).

According to their functionality, they can be divided into non gluten-forming proteins (15-20% of the total protein) and proteins which can develop a viscoelastic mass (the gluten), i.e the gluten proteins.

The non-gluten proteins, albumins and globulins, occurr in the outer layers of the kernels and they are mostly monomeric proteins, with a phisiological role.

The gluten proteins, gliadins and glutenins, conferring the capacity of water absorption, cohesivity, viscosity and elasticity, are the main elements to determine the variety of end-uses derived from the wheat dough processing.

Glutenins and gliadins belong to the same storage-protein family, the prolamins, containing large amounts of glutamine and proline and occurring in the endosperm of the kernels, where they form a matrix around the strach ganules (Goesaert et al., 2005).

Gliadins are monomeric proteins with molecular weight around 28–55 kDa and can be classified according to their different primary structures into the α-/β-; γ- and ω-type (Wieser et al., 2007).

The glutenin fraction comprises aggregated proteins linked by interchain disulphide bonds; they have molecular weights ranging from about 500,000 Da to more than 10 million Da. Glutenin subunits (GS) can be divided into the high-molecular-weight (HMW-GS, MW 67–88 kDa) and low-molecular-weight (LMW-GS, MW 32–35 kDa) subunits (Wieser, 2007).

Inter-chain disulphide bonds stabilize the glutenin polymers, while hydrogen bonds are important in the stabilization of the interactions between the glutenin polymers and gliadins (Belton, 2005).

This protein network can be separated using solvents, such, as urea, which disrupt hydrogen bonding, while the use of reducing agents (such as 2-mercaptoethanol or dithiothreitol) can lead to

the breaking of the glutenin polymers, releasing the individual subunits (Shewry, 2009).

Temperature can affect the structural arrangement of the gluten proteins; indeed, during heating, glutenins become less extractable than gliadins (Schofield et al., 1983) which in turn tend to react with glutenin polymers decreasing their mobility (Redl et al., 1999).

The dough processing leads to the building of a network in which the polymers are aligned in the direction of the extension (Shewry, Black, Bewley, 2000) and where the self-assembling machinery of gluten proteins (Kuktaite et al., 2011) and the occurrence of several reactions (Delcour et al., 2012) determine the arrangement of a great protein architecture.

Gluten proteins polymerization is one of the main consequences of the heat processing.

The study of the size distribution of protein polymers, based on the approach of Gupta, Khan, MacRitchie (1993), was already used by several authors (Kuktaite, Larsson, Johansson, 2004; Lamacchia et al., 2007; Bruneel et al., 2010), evaluating the amount of the high molecular weight protein polymers and thus the optimal degree of protein polymerization, which was found to be good associated with the technological properties of both flour and pasta products.

Starting from these considerations, in this session the protein aggregation was assessed through the application of chromatographic and electrophoretic tecniques in different wheat-based pasta products:

1. replacing durum wheat flour with different percentages of einkorn flour;
2. evaluating the influence of the hydration method on durum wheat pasta enriched with durum wheat bran.

A brief draft of the paper on the research of the gluten arrangement in wheat-based biscuits incurring peanut proteins is also described.

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Publications

- la Gatta, B., Rutigliano, M., Rusco, G., Petrella, G., Di Luccia, A. (2017). Evidence for different supramolecular arrangements in pasta from durum wheat (Triticum durum) and einkorn (Triticum monococcum) flours". Journal of Cereal Science, 73, 76 - 83.

- la Gatta, B., Rutigliano, M., Padalino, L., Conte, A., Del Nobile, A., Di Luccia, A. (2017). The role of hydration on the cooking quality of bran-enriched pasta. LWT- Food Science and Technology, 84, 489 - 496.

Paper 1: Evidence for different supramolecular arrangements in pasta from durum wheat (Triticum durum) and einkorn (Triticum monococcum) flours.

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Abstract

The effects of the replacement of einkorn flour on pasta proteins aggregation were studied by sodium dodecyl sulfate polyacrylamide gel electrophoresis and size exclusion high performance liquid chromatography. Pasta was produced replacing durum wheat semolina with an increasing amount of einkorn flour (30, 50 and 100%). The polymeric protein structure of flours and related pasta and pasta mixture was determined by protein subunits composition and size of polymeric proteins. The unextractability of polymeric protein was related to the unextractable protein fraction and to the determination of –SH/-SS groups. Durum wheat semolina and einkorn flours increased their unextractable and polymeric fractions during pasta processing. The unexpected results derived from the areas of unextractable fractions and total and large unextractable polymeric fractions of 70/30 semolina/einkorn pasta mixture that were lower than those of 50/50 semolina/einkorn pasta mixture. Although the semolina flour contained more gluten proteins than einkorn flour, a higher aggregation rate was registered for 50/50 semolina/einkorn flour pasta. These results suggested that a different arrangement of gluten network occurred in pasta mixture and it was regulated by a self-assembling machinery influenced by the nature of HMW-GS. The 50/50 semolina/einkorn pasta mixture determined a supramolecular structure in the developing of its protein network.

1. Introduction

Gluten proteins generate a polymeric network through hydration and mechanical energy during dough manufacture. Polymeric aggregation occurs by sulphydryl/disulfide (SH/SS) interchange bonds between high and low molecular weight glutenins and it is stabilized by non covalent bonds (hydrogen and ionic); gliadins interact with glutenin network by non-covalent interactions (hydrogen, ionic and hydrophobic) and the stability of the intra-molecular SS bonds prevents them from becoming involved in SH/SS interchange reactions (Weiser, 2007). The increase of the gluten network size from polymeric protein aggregates to supramolecular structures has been demonstrated to occur by a self-assembling mechanism in a hierarchical fashion (Kuktaite et al., 2011). Protein self-organization into elastomeric or amyloid-like fibrils is favoured by proline and glycine (Rauscher et al., 2006), in fact, HMW-GS contains repeating sequences of proline, glycine and glutamine, suggesting that this novel structure is dependent on the interaction of these three amino acids (Raucher et al., 2006). The high levels of glutamine residues, located mainly in their repetitive domains, formed hydrogen bonds predominantly between these residues favouring the formation of the gluten network and contributing to its viscous and elastic properties as proposed by Belton (1999).

In pasta processing, extrusion and drying temperatures cause protein denaturation through dissociation and unfolding assisted by sulphydryl/disulfide interchange reactions this phenomenon allows the gliadins and glutenins to re-polymerize (formation of intermolecular covalent bonds), which yields high tensile strength/Young's modulus (Ullsten et al., 2011). At higher temperatures gliadins embed the supramolecular glutenin network as a result of SH/SS interchange reactions (Schofield et al., 1983). However additional cross-links between other amino acids may also occur in gluten proteins, such as dityrosine bond (Tilley et al., 2001), β-elimination of cystine forming dehydroalanine (DHA) (Rombouts et al., 2010). The presence of tyrosyl and thiyl free radicals could suggest the possibility of their combination forming thiyl-tyrosyl bonds (Takasaki and Kawakishi, 2000; Lamacchia et al., 2011). Furthermore, heating gluten proteins could lead to isopeptide bonds (Rombouts et al., 2011). The sulphydryl/disulfide interchange reactions and self-organization of the gluten network with the formation of new covalent links reinforces the supramolecular structure. The polymerization, as a result of the gluten proteins network, can be revealed by measuring the contents of extractable proteins in dilute sodium dodecyl sulphate (SDS) solution, while the level of unextractable proteins, sonicated in the same solution, represents a measure to establish the degree of protein polymerization. The size of these polymeric structures was revealed through size exclusion high performance liquid chromatography (SE-HPLC). The chromatographic results showed a different assessment of supramolecular architecture of

extractable and unextractable proteins according to the nature of cereals, pseudo-cereals or other raw matter source as well as their mixtures (Lamacchia et al., 2010; Lamacchia et al., 2011).

Einkorn (*Triticum monococcum L. subsp. monococcum*) is a cereal with an high-nutritional value, especially for its high protein and antioxidants content (Brandolini et al., 2008). Moreover, this cereal better suits to the formulation of products for its high content of dietary fibre, carotenoids and tocols (Corbellini et al., 1999; Brandolini et al., 2008). In an electrophoretic study of einkorn gluten proteins Corbellini et al. (1999) observed that SDS-PAGE patterns of the reduced glutenin fractions did not show differences between the lines in HMW subunit (HMW-GS). Significant differences, instead, were observed in LMW subunit (LMW-GS) bands, in particular two LMW-GS bands were strictly correlated with high bread making potential. Additionally, Wieser et al. (2009) found that the total gluten proteins (gliadins + glutenins) content of einkorn flour was similar or even higher compared to common wheat and spelt and that einkorn flour was characterized by a high ratio of gliadins to glutenins and by a low content of high-molecular weight glutenin subunits. As known the gluten proteins content, ratio of gliadins to glutenins and/or the nature of gluten proteins determine the dough properties and baking performance of common wheat flours (Kuktaite et al., 2004). In this context, our study on gluten proteins aggregation from semolina (*Triticum durum*) and einkorn (*Triticum monoccoccum ssp. monococcum*) mixtures is included.

The aim of this paper was to study the effect of semolina replacement with increasing amount (30, 50 and 100%) of einkorn flour on pasta protein aggregation in supramolecular structures by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and size-exclusion high-performance liquid chromatography (SE-HPLC).

2. Material and Methods

2.1 Pasta samples

Durum wheat semolina and einkorn (*Triticum monococcum*) whole flour were found at a local mill (Casalnuovo Monterotaro, Foggia, Italy) and at a mill located in Umbria (Sigillo, Perugia, Italy), respectively.

The durum wheat semolina was the type of flour that commonly is used for pasta production, while the einkorn flour was the biological type, organic stone ground.

Four types of macaroni were produced: 100% durum wheat semolina, 100% einkorn and pasta where the 30% and 50% of semolina was replaced with einkorn flour. Macaroni were produced with these operating conditions: semolina was mixed with water by a rotary shaft-mixer (Namad, Rome, Italy) at about 25°C for 20 min to obtain a dough with a moisture content ranging from 29% to 31%. This difference was due to the percentage of semolina – einkorn mixture. The dough was

extruded with a 60VR extruder (Namad, Rome, Italy). All the different types of pasta were produced in the same conditions (temperature 50 ± 5°C; kneading time 15 min; vacuum degree 700 mmHg). A Bronze die-plate was used. Macaroni were dried at 55°C for 12 h (SG600; Namad, Rome, Italy).

2.2 SDS-PAGE (sodium dodecyl sulphate-polyacrilamide gel electrophoresis) analysis

Total proteins from semolina, einkorn flour and milled macaroni (1g) were extracted using 10 ml of an extraction buffer containing Tris-HCl 0.0625 M pH 6.8, SDS 2%, Glycerol 10% (v/v). Samples were left in contact with the extraction buffer for 2 h, and then they were centrifuged at 13000xg for 10 min at 25°C. The supernatants, containing proteins, were carefully removed and stored at -20°C until use. To separate the extracted proteins, SDS-PAGE was performed on a 12% gel both under reducing conditions with dithiotreitol (DTT) 5% (w/v) and non-reducing conditions, using a horizontal electrophoresis system Hoefer SE 600, (GE Healthcare, Milan, Italy). SDS-PAGE analysis was carried out according to Laemmli (1970), at 25 mA for 3h at room temperature. The gels were stained with 0.25% w/v Coomassie Brilliant Blue (CBB) overnight.

2.3 Size exclusion-HPLC analysis

Proteins from semolina, einkorn flour and milled macaroni were extracted with a two-step extraction procedure followed by Gupta et al., (1993) and Lamacchia et al., (2010). The first step extracts the SDS-extractable proteins (proteins soluble in sodium dodecyl sulphate (SDS)), whilst the second extraction contains the SDS-unextractable proteins (proteins soluble only after sonication). "Total" proteins were extracted from ground samples (1g) dissolved in the same buffer used in the two steps described above, shaken, vortexed and sonicated for 30 s and then centrifuged (10 min, 10,000g, 25 °C).

SE-HPLC was performed using a liquid chromatograph Agilent 1100 Series system (Santa Clara, CA, USA) equipped with a Phenomenex Biosep SEC-S4000 column (300 x 7.8mm, Phenomenex, Torrence, CA, USA). Each sample (20 µl) was injected on the column and the eluted proteins were monitored at 214 nm. The mobile phase was 50% acetonitrile containing 0,1% trifluoroacetic acid with a flow rate of 0,7 ml/min. The SE-HPLC column was calibrated using protein standards with a range of molecular weights (KDa) as follows: ribonuclease A (13.7), chymotripsinogen (25.0), ovalbumin (43.0), bovine serum albumin (67.0), aldolase (158), catalase (232), ferritin (440) and thyroglobulin (669).

The proportion of unextractable polymeric proteins on total proteins was calculated as [peak 1+2 area (SDS-unextractable)/peak 1+2 area (total proteins TP)] x100 and it was indicated as total

unextractable protein fraction (tUPF), while the proportion of large unextractable polymeric fraction (lUPF) was calculated as [peak 1 area (SDS-unextractable)/peak 1 area TP] x100 and indicated as large unextractable fraction (lUPF).

2.4 Determination of thiol and disulphide groups

The protein disulfide and sulphydryl content in the flours and pasta samples was estimated by a colorimetric determination of free SH groups, using a solid phase assay $NTSB^{2-}$, according to the method of Chan and Wasserman, (1993).

2.5 Statistical analysis

The results were compared by one-way variance analysis (ANOVA). A Duncan's multiple range test, with the option of homogeneous groups ($p<0.05$), this was used to determine the significance between samples. STATISTICA 7.1 for Windows (StatSoft, Inc, Tulsa, OK, USA) was used for this purpose.

3. Results

The einkorn and semolina flour were primarily characterized by electrophoretic analysis compared with 100% pasta and pasta mixtures to better understand polymeric aggregation phenomena occurring during pasta production.

3.1. Electrophoretic profiles of flours and pasta

Protein composition of durum wheat semolina, einkorn flour, 100% semolina pasta, 100% einkorn pasta and of the two different pasta mixtures, 70% of semolina–30% of einkorn flour and 50% of semolina–50% of einkorn flour was studied by SDS-PAGE under reducing (Figure 1A) and nonreducing conditions (Figure 1B).

Semolina and einkorn flour showed an electrophoretic profile which included protein bands ranging between 100 e 10 kDa (Figure 1A). High Molecular Weight Glutenin Subunits (HMW-GS) were included between 116 and 67 kDa. It was possible to identify 1Ax1 HMW-GS in the electrophoretic pattern of einkorn flour, while semolina pattern showed 1Ax2 and 1Bx7 subunits, according to Shewry (2009). In this range of molecular weight, it is possible to reveal also ω-gliadins (Figure 1A). Bands included between 67 and 10 kDa contained mainly Low Molecular Weight Glutenin Subunits (LMW-GS), gliadins (α, β e γ) and low molecular weight globulin and albumins.

We observed more bands at level of ω-gliadins and D-type LMW-GS in einkorn pattern, on the other hand the glutenin subunit D-LMW originated by a mutation in one or more genes encoding ω-

gliadins. Considering that einkorn is usually richer in gliadins, we can assert that the most of these bands belong to ω-gliadins (Weiser et al., 2009). However, the overlapping of LMW-GS and gliadins bands made it difficult to have a clear distinction amongst the different fractions, since homology of sequences in these two classes of proteins make them electrophoretically unresolved.
At less than 20 kDa albumins and globulins fraction was revealed both in einkorn and in semolina flour, with evident differences in the number and mobility of the bands.

In Figure 1A, the electrophoretic profiles under reducing conditions of the different types of pasta are shown too. Pasta made with both 100% semolina (lane 3) and 100% einkorn flour (lane 4) showed a protein pattern very similar to that of the respective flours (lane 1, semolina and lane 2, einkorn flour) for both number and intensity of bands. However, there were some differences in 100% einkorn flour to its respective pasta at level of HMW-GS for the absence of minor protein bands. The composite pasta was produced replacing semolina with einkorn flour (lane 5, 70/30 semolina/einkorn pasta mixture; and lane 6, 50/50 semolina/einkorn pasta mixture) and showed hybrid protein profiles between semolina and einkorn flour including the presence of 1Ax2, 1Ax1 and 1Bx7 HMW-GS. In addition, it was possible to observe that the protein bands intensity of composite pasta varied concurrently to the percentage of added einkorn flour, the protein profile of 50/50 semolina/einkorn pasta mixture actually showed a diminution of band intensity of 13,2% with respect to 70/30 semolina/einkorn pasta mixture in reducing condition, whereas it showed an increase of only 3,1% in nonreducing condition. This result suggested a lower extractability of proteins probably due to the formation of larger unextractable polymeric proteins that indicated a supramolecular structure of glutenin network. In fact, in nonreducing conditions, electrophoretic patterns of flours and pastas showed the absence of bands above 75 kDa corresponding to HMW-GS, suggesting the formation of a supramolecular structure of polymeric proteins above 250 kDa as indicated by molecular weight standard (Fig. 1B). The bands below 75 kDa could correspond mainly to the gliadin fraction since the gluten network is responsible to cross-linking of HMW and LMW-GS.

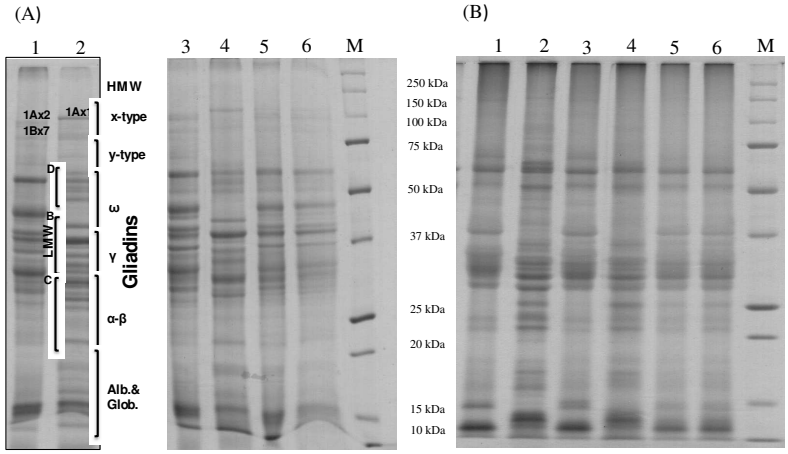


Figure 1 A. SDS-PAGE of total protein samples under reducing conditions. Lane 1: durum wheat semolina; lane 2: einkorn flour; lane 3: 100% durum wheat semolina pasta; lane 4: 100% einkorn flour pasta; lane 5: 70% semolina-30% einkorn pasta; lane 6: 50% semolina-50% einkorn pasta; M: molecular marker.
Figure 1 B. SDS-PAGE of total protein samples under unreducing conditions. Lane 1: durum wheat semolina; lane 2: einkorn-flour; lane 3: 100% durum wheat semolina pasta; lane 4: 100% einkorn flour pasta; lane 5: 70% semolina-30% einkorn pasta; lane 6: 50% semolina-50% einkorn pasta; M: molecular marker. (From la Gatta et al., (2017) Journal of Cereal Science, 73, 76 – 83).

3.2 Effect of the replacement of semolina with einkorn flour on pasta polymeric protein and supramolecular structure.

3.2.1 SE-HPLC profiles of flours and pasta

Figure 2 shows the typical chromatographic profiles of total proteins and SDS-unextractable proteins, obtained by size exclusion HPLC fractionation from semolina (Figure 2A) and *Triticum monococcum* flour (Figure 2B). The fractionation of flours and pasta showed four peaks identified in agreement with Tosi et al., (2005): the peak 1 represents high and low molecular weight glutenin (named large polymeric proteins, LPP), peak 2 represents mainly glutenin with a low molecular weight (named small polymeric proteins, SPP), a large peak 3 represents mainly monomeric gliadins proteins (named large monomeric proteins, LMP), a smaller peak 4 represents albumins and globulins (named small monomeric proteins, SMP), which was possible to reveal in einkorn flour and relative pasta chromatograms but not in semolina flour and in 100% semolina pasta.

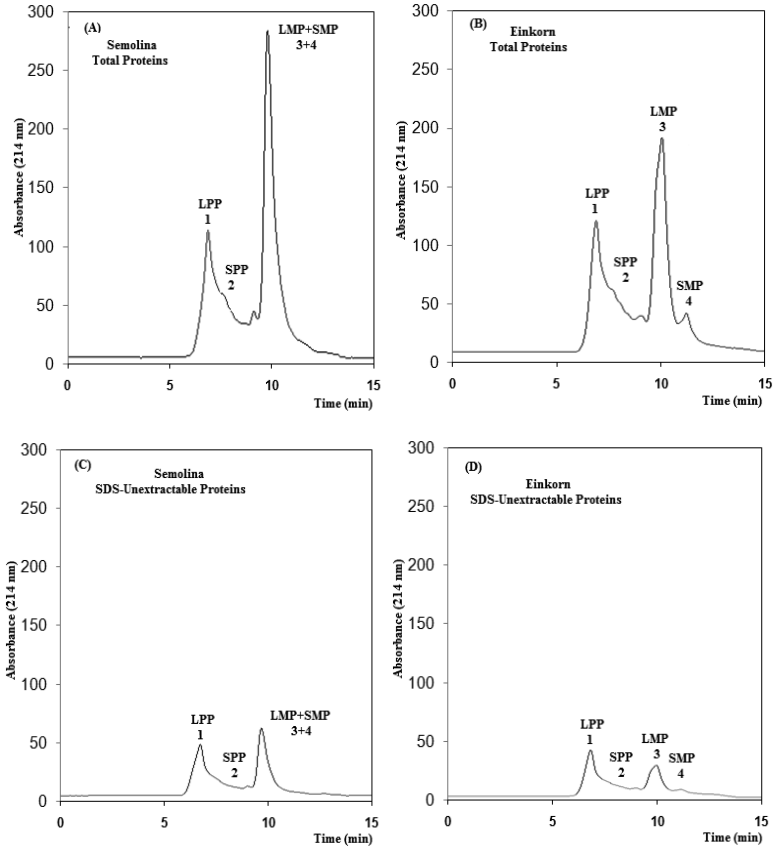


Figure. 2. SE-HPLC profiles of total and SDS-unextractable proteins. Total proteins of durum wheat semolina (A) and einkorn flour (B) and SDS- unextractable of durum wheat semolina (C) and einkorn flour (D). (From la Gatta et al., (2017) Journal of Cereal Science, 73, 76 – 83).

Although the SE-HPLC elution profile of einkorn flour and durum wheat semolina showed the same peaks, the area of the peaks themselves varied significantly between the durum wheat semolina and the einkorn flour (Table 1).

In a decreasing order, total proteins peaks areas were proportionally higher for 100% einkorn pasta, 70/30 semolina/einkorn pasta mixture and 100% semolina pasta than the remaining samples, with the lowest values for the two flour samples (Table 1). This means that a higher extractability of proteins was obtained for pasta samples, except for 50/50 semolina/einkorn pasta mixture. Surprisingly, total polymeric proteins distribution, TPP (derived from LPP+SPP), showed the same decreasing order of total peak areas, although the amount of TPP was higher in einkorn products, in percentage terms. Another information was given by LPP/SPP ratio, that showed a higher amount of SPP in semolina flour and in 70/30 semolina/einkorn pasta mixture, with 0.96 and 0.93, respectively. In the case of 100% semolina pasta and 50/50 semolina/einkorn pasta mixture, the peak areas of LPP and SPP were very similar with a consequent LPP/SPP ratio of about 1. Finally, the LPP/SPP ratio was higher than 1 in einkorn flour and in 100% einkorn pasta.

To compare monomeric proteins (Total Monomeric Protein, TMP), we considered the sum of LMP and SMP area peaks for each sample, since the SMP peak was not detectable in semolina flour and

100% semolina pasta chromatograms (Fig.2 and Table 1). The lowest values of TMP proteins were found, in a decreasing order, for semolina flour, 50/50 semolina/einkorn pasta mixture and einkorn flour, while the highest values were, in a decreasing order, those of 70/30 semolina/einkorn pasta mixture, 100% semolina pasta and 100% einkorn pasta. Considering, the ratio of polymeric and monomeric proteins (TPP/TMP), we observed a higher proportion of monomeric proteins in all samples, however einkorn flour and 100% einkorn pasta showed a higher ratios linked to the highest amount of LPP for these two samples than the other samples, following the decreasing order: 70/30 semolina/einkorn pasta mixture = 50/50 semolina/einkorn pasta mixture > semolina flour = 100% semolina pasta.

The polymeric distribution for SDS-unextractable proteins (data not shown) showed that TPP were higher than TMP in all samples except for 70/30 semolina/einkorn pasta mixture. The LPP/SPP values were the highest for 50/50 semolina/einkorn pasta mixture and 100% pasta einkorn, as also TPP/TMP ratio, however it needs also to be noted that einkorn flour showed a notable content of polymeric fraction. In this latter case, we noted that the higher ratios TPP/TMP were linked both to a lower amount of TMP and to a high value of LPP, as shown also by the LPP/SPP ratio. Therefore, despite the lower gluten content in einkorn flour it is observed a higher proportion of polymeric protein fraction.

The overall results did not seem always consistent and sometimes contradictory, indicating that different assessments are involved in supramolecular architecture of flour and pasta polymeric protein network. However, it was evident a remarkable protein aggregation of einkorn flour, that could determine a self-assembling and/or self-organization machinery by its HMW-GS and LMW-GS.

Table 1 Area of the SE-HPLC peaks of total proteins. LPP: large polymeric proteins; SPP: small polymeric proteins; LMP: large monomeric proteins; SMP: small monomeric proteins of flours and pasta samples. TPP: total polymeric proteins (LPP+SPP); TMP: total monomeric proteins (LMP+SMP). (From la Gatta et al., (2017) Journal of Cereal Science, 73, 76 – 83).

		Semolina flour	**Einkorn flour**	**Semolina Pasta 100%**	**Einkorn Pasta 100%**	**Pasta mixture Semolina/Einkorn 70/30**	**Pasta mixture Semolina/Einkorn 50/50**
LPP	mAu·s	4427.35±69.79^{a}	5448.90±24.32^{b}	6478.75±100.76^{c}	9392.35±116.04^{d}	7317.2±24.18^{e}	5429.25±19.16^{b,f}
	%	*20.46*	*25.37*	*21.45*	*27.58*	*22.38*	*23.98*
SPP	mAu·s	4632.95±7.42^{a}	4812.05±115.47^{a,b}	6201.3±189.79^{c}	7282.15±81.53^{d}	7839.4±43.98^{e}	5018.2±75.80^{c,b}
	%	*21.41*	*22.40*	*20.53*	*21.39*	*23.97*	*22.17*
LMP	mAu·s	12582.65±172.32	9143.35±12.52	17526.95±284.61	14238.85±43.06	15240.85±6.43	10433.35±81.39
	%	*58.14*	*42.57*	*58.02*	*41.82*	*46.61*	*46.09*
SMP	mAu·s	ND	2076.5±1.5	ND	3137,45±15.34	2303,55±37.26	1755.25± 8.56
	%	/	*9.67*	/	*9.21*	*7.04*	*7.75*
Total peak areas	mAu·s	**21642.95±234.69^{a}**	**21480.8±150.76^{a}**	**30207±195.59^{b}**	**34100.8±185.26^{c}**	**32701±111.86^{d}**	**22636.05±184.91^{e}**
	%	100%	100%	100%	100%	100%	100%
TPP	mAu·s	**9060.30±62.37^{a}**	**10260.95±139.80^{b}**	**12680.05±89.02^{c}**	**16674.50±197.57^{d}**	**15156.6±68.17^{e}**	**10447.45±94.96^{b,f}**
	%	*41.86*	*47.76*	*41.98*	*48.97*	*46.35*	*46.15*
TMP		**12582.65±172.32^{a}**	**11219.85±10.96^{b}**	**17526.95±284.61^{c}**	**17376.30±58.41^{c,d}**	**17544.40±43.70^{c,d,e}**	**12208,60±89.94^{a}**
	%	*58.14*	*52.24*	*58.02*	*51.03*	*53.65*	*53.85*
LPP/SPP		0.96	1.13	1.04	1.29	0.93	1.08
TPP/TMP		0.72	0.91	0.72	0.96	0.86	0.86

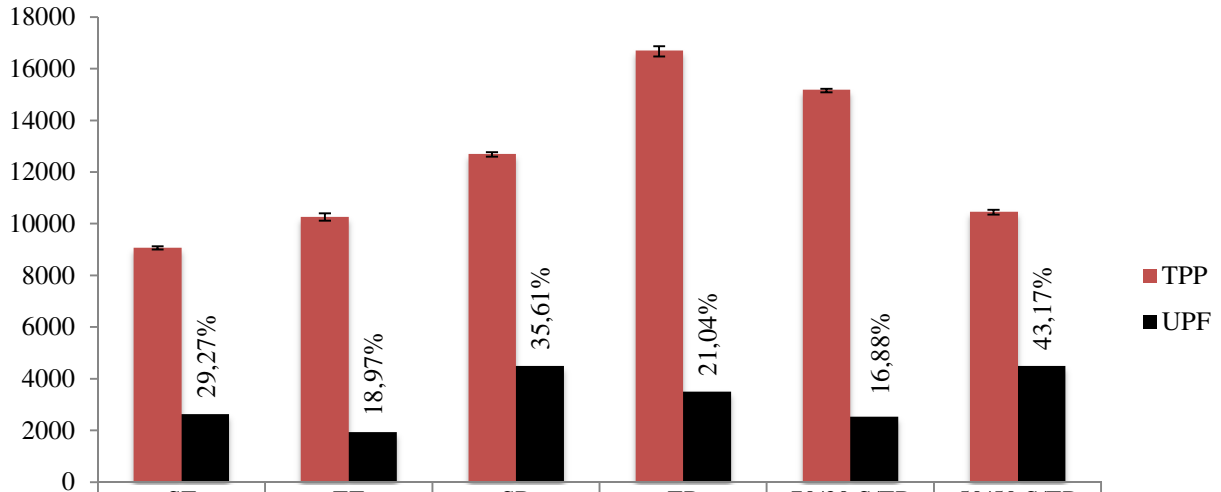
ND=Not Detectable
a, b, c, d, e and f=P<0.05

3.2.2 Total Unextractable Polymeric Fraction (UPF), large Unextractable Polymeric Fraction (lUPF) and content of thiol and disulphide groups in flours and pasta

The effects on protein polymeric structure induced by the interaction between durum wheat semolina and einkorn proteins were studied by evaluating the proportion of tUPF, lUPF and the concentration of –SH free and S-S groups in each sample.

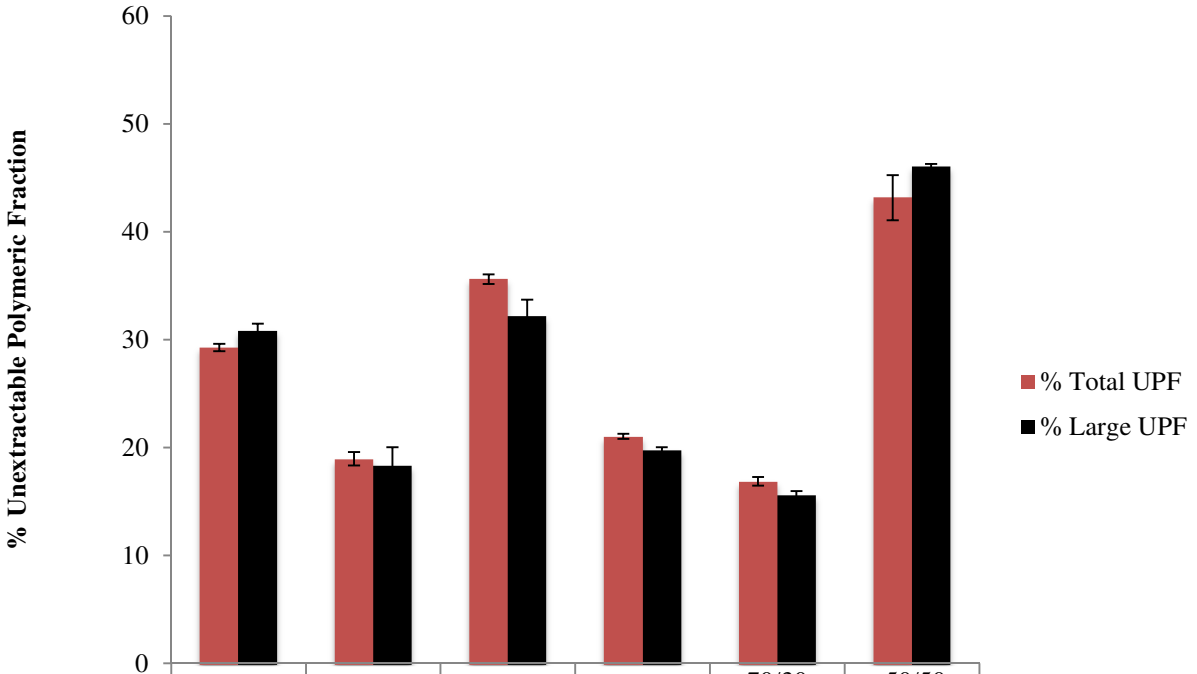
On the basis of electrophoretic and chromatographic results, we first evaluated the impact of UPF (unextractable polymeric fraction) on TPP (total polymeric fraction) (Fig. 3). The highest incidence of UPF on TPP was found in the case of the 50/50 semolina/einkorn pasta mixture (43.17%), justifying its minor extractability (Table 1) and suggesting the presence of a larger polymeric structure. By contrast, the lower values were related to einkorn flour (18.97%) and 70/30 semolina/einkorn pasta mixture (16.88%), while the values of, 100% semolina pasta (35.61%) and 100% einkorn pasta (21.04%) were higher than the relative flours (29.27% and 18.97%, respectively), as expected. These results confirmed a different arrangement of the supramolecular structure of the polymeric network and, consequently, the great aggregative properties of einkorn gluten proteins.

To better define the polymeric aggregation in flours and pasta samples, we calculated the percentage of tUPF (total unextractable polymeric fraction) and lUPF (large unextractable polymeric fraction) obtained by SDS-unextractable proteins and total protein peaks area values after SE-HPLC fractionation (Fig. 2) according to Kuktaite et al. (2004), but modifying relationships to better show supramolecular aspects.



	SF	EF	SP	EP	70/30 S/EP	50/50 S/EP
TPP	9060,3	10260,95	12680,05	16674,5	15156,6	10447,45
UPF	2651,80	1946,75	4514,95	3507,85	2557,85	4509,90

Figure 3. Evaluation of the incidence of UPF (unextractable polymeric fraction, peak 1 + peak 2 area) on TPP (total polymeric fraction, peak 1 + peak 2 area). SF: durum wheat semolina; EF: einkorn flour; SP:100% durum wheat pasta; EP:100% einkorn pasta; 70/30 S/EP: 70% semolina -30% einkorn pasta; 50/50 S/EP: 50% semolina-50% einkorn pasta. (From la Gatta et al., (2017) Journal of Cereal Science, 73, 76 – 83).



	SF	EF	SP	EP	70/30 S/EP	50/50 S/EP
% Total UPF	29,27	18,97	35,61	21,04	16,88	43,16
% Large UPF	30,80	18,36	32,16	19,80	15,63	46,00

a, b, c, d, e and f=P<0.05
A, B, C, D e F=P<0.05
*=P<0.05

Figure 4. Percentage of Total and Large UPF. SF: durum wheat semolina; EF: einkorn flour; SP:100% durum wheat pasta; EP:100% einkorn pasta; 70/30 S/EP: 70% semolina -30% einkorn pasta; 50/50 S/EP: 50% semolina-50% einkorn pasta.
The letters a,b,c,d,e,f indicate the difference among the percentages of Total UPF; the letters A,B,C,D,E indicate the difference among the percentages of Large UPF; * indicate that total UPF and large UPF are statistically different. (From la Gatta et al., (2017) Journal of Cereal Science, 73, 76 – 83).

The tUPF was the highest in 50/50 semolina/einkorn pasta mixture (43.16%) and the lowest in 70/30 semolina/einkorn pasta mixture, (16,88%). The trend of tUPF followed the order: 50/50 semolina/einkorn pasta mixture > 100% semolina pasta > semolina flour > 100% einkorn pasta > einkorn flour > 70/30 semolina/einkorn pasta mixture (Fig.4).

The lUPF followed the same trend of tUPF, however, it can be noted that lUPF had a higher engraving of tUPF in the case of semolina flour and 50/50 semolina/einkorn pasta mixture than in the other samples (Fig. 4).

As expected, Figure 4 shows a significant increase of the tUPF ($p<0.05$) from the durum wheat semolina to the 100% semolina pasta (respectively from 29,27 to 35.61%), similarly they significantly increase ($p<0.05$) from einkorn flour to 100% einkorn pasta (respectively from 18.97% to 21,04); on the contrary, this increase is not significant for the lUPF. The differences between total UPF and large UPF were not significant except for the total UPF and Large UPF of 100% einkorn pasta ($p<0.05$). Pasta mixtures showed a notable difference for total and large UPF, that

increased from 70/30 semolina/einkorn pasta mixture (respectively 16,88% and 15,63%) to 50/50 semolina/einkorn pasta mixture (43,16% and 46,00%).

The increase of tUPF from flours to pasta is due to both pasta making and drying temperatures (50-60 °C). These events induce interactions amongst different class of proteins (gluten HMW and LMW subunits; albumins and globulins; albumins/globulins with gluten proteins polymer) leading to the formation of larger aggregates or a supramolecular structure, which often are unextractable in phosphate buffer without sonication (Lamacchia et al., 2011). However, it must be noted that in the case of semolina flour and 100% semolina pasta, lUPF and tUPF have an opposite behavior. The lUPF tends to have a higher engraving with respect to tUPF in semolina than on 100% semolina pasta (Fig. 4), while einkorn flour and 100% einkorn pasta followed the same trend.

Summarizing, the 50/50 semolina/einkorn pasta mixture exhibited in the SDS-unextractable fraction a higher amount of LPP (32,72%), higher tUPF (43,16%) and lUPF (46,00%) as well as a lower extractability; by contrast the 70/30 semolina/einkorn pasta mixture exhibited in SDS-unextractable fraction (data not shown) a lower amount of LPP (21,72%), lower tUPF (16,88%) and lUPF (15,63%) as well as a higher extractability. So these were the most contradictory results that could find an explanation in recent studies, these results have showed that glutenins aggregate by self-assembling (Mackintosh et al., 2009) and are dependent on the interaction of repeating sequences of proline, glycine and glutamine (Rauscher et al., 2006). Kuktaite et al., (2011) found highly polymerized gluten proteins pattern with intimately mixed glutenins and gliadins linked through SH/SS interchange reactions and highlighted the complexities of the supramolecular structures and conformations of wheat gluten polymeric proteins.

To evaluate the influence of SH□SS interchange reactions on polymer formation we determined the concentration of free thiols (-SH) and disulfide (S-S) groups, computing also total cysteine and SS/SH ratio, as reported in Table 2. Durum wheat semolina and einkorn flour showed similar comparable concentrations, albeit these were significantly different ($P<0.05$). In 100% pasta semolina and 100% einkorn pasta free thiol decreased significantly ($P<0.05$) whereas a significant increasing ($P<0.05$) of disulfide group and a higher SS/SH ratio was observed. In this case, the differences in thiol groups could be ascribed to differently bonded groups, such as cysteine tyrosine crosslink (5-S-cysteinyldopa), due to the oxidizing effect of free radicals (Takasaki and Kawakishi, 2000; Lamacchia et al., 2011).

Pasta made replacing semolina with 30% of einkorn flour showed the highest ratio SS/SH demonstrating a tangible diminution of free SH with a concomitant increase of S-S bonds. This is the case in which SH/SS interchange was the predominant reaction. Differently was the case of pasta made replacing semolina with 50% of einkorn flour, where it was only observed a significant

diminution of S-S bonds and the lowest ratio of SS/SH, suggesting, in this case, an oxidazion of S-S bonds to give rise to 5-S-cysteinyldopa crosslink. As a matter of fact, the highest value of lUPF and tUPF confirmed the formation of other different covalent bonds from S-S ones but, somehow, involving sulfide groups (Fig. 4).

Table 2. Effect of pasta processing on contents in free thiol (SH), disulfide groups (SS) and Total Sulfhydryl. Mean ± standard error for three replicates. Values are micromoles per gram of sample. (From la Gatta et al., (2017) Journal of Cereal Science, 73, 76 – 83).

Samples	Free Thiol (µmol/g prot)	Disulfide group (µmol/g prot)	Disulfide/ Free Thiol (SS/SH)	Total Sulfhydryl (µmol/g prot))
Semolina	51.80 ± 0.35a	87.14 ± 1.02A	1.68	226.07 ± 1.72
Einkorn Flour	54.38± 0.57b	93.34± 0.86B	1.72	241.06 ± 2.29
100% Semolina Pasta	46.85 ± 0.70c	87.06 ± 1.67A	1.86	220.97 ± 3.81
100% Einkorn Pasta	49.77 ± 0.92d	98.99 ± 5.08C	1.99	247.74 ± 10.04
70% Semolina Pasta 30% Einkorn Pasta	47.01 ± 0.13c,e	110.86 ± 0.24D	2.34	268.74 ± 0.57
50% Semolina Pasta 50% Einkorn Pasta	55.63 ± 1.39b,f	82.04 ± 1.47E	1.48	219.72 ± 3.44

a, b, c, d, e and f=P<0.05
A, B, C, D e E=P<0.05

4. Discussion

The production of 100% einkorn pasta, 70/30 and 50/50 semolina/einkorn pasta mixtures suggested a consistent protein aggregation of einkorn gluten proteins. As demonstrated, HMW patterns showed the glutenin subunit, 1Ax1 in einkorn and 1Ax2 and 1Bx7 in wheat durum semolina, which are desirable subunits for bread-making quality (Popineau et al., 2011). The amino acid sequence between the two subunits encoded by chromosome lAx is very similar differing from each other only by seven single amino acid substitutions (all except one in the repetitive domain), and in the insertion of a single hexapeptide and adjacent hexa and tripeptides in 1Ax1. The clear effects of these changes are the increase in the numbers of glycine, glutamine and proline residues (derived from the inserted repeat peptides), with little changes in other residues. Rauscher et al., (2006) highlighted the role of proline and glycine in the control of protein self-organization into elastomeric or amyloid-like fibrils. Since HMW-GS contains repeating sequences of proline, glycine and glutamine, this novel structure could be dependent on the interaction of these three amino acids and could shed light on the relationship between sequence structure and elastomeric qualities of these intriguing proteins. To confirm it, the presence of HMW 1Ax1 in einkorn and 1Ax2 in semolina could increase glutenin aggregation, which in turn increased the unextractable protein fraction and it was correlated with the good quality of the final product structure (Gupta et al., 1993; Popineau et al., 2001). A supramolecular architecture can originate in a process of reticulation of gluten proteins (Mackintosh et al., 2009). Therefore, the arrangement of the polymeric structure in a supramolecular architecture of the different samples of flour and pasta can determine their rheological and cooking properties (Bruneel et al., 2010).

From an overall observation of polymeric fractions, flour and pasta showed the expected results, increasing the polymeric fraction from flour to pasta; 70/30 and 50/50 semolina/einkorn pasta mixtures showed unexpected results (Table 1). As demonstrated, the content of polymeric fractions should mediate that of 100% semolina and that of 100% einkorn pasta, but 70/30 semolina/einkorn pasta mixture showed the lowest content of polymeric fraction, whereas 50/50 semolina/einkorn pasta mixture showed the highest one. In the unextractable fraction of the 50/50 semolina/einkorn pasta mixture, the highest LPP/SPP ratio and the highest value of UPF on total polymers (43%) indicated that larger polymers were aggregated in a supramolecular network structure, induced by the drying process. In this stage, the largest polymeric structures were self-assembled by expected SS bonds but also by covalent bonds deriving by sulphydryl radicals, due to the observation of a decrease of the disulfide bonds (Table 2). However, we didn't exclude the formation of other covalent bonds. In the 70/30 semolina/einkorn pasta mixture, the unextractable polymeric fraction (Fig.3) has bearing only for about 17% of the total polymers and the proportion of tUPF and lUPF

was the lowest, because both fractions were the lowest (Fig. 4). Moreover, although the proportion of semolina was 70%, tUPF and lUPF were closer to einkorn flour and 100% einkorn pasta (Fig. 4). In this case, the LPP/SPP ratio decreased from total polymeric fraction (0,93) to unextractable polymeric fraction (0,81) indicating that SPP constituted the more important fraction in the aggregation event through disulphide bonds, as demonstrated by the highest ratio SS/SH (Tab. 3). A more recent work by D'Agnello et al. (2016), analyzing semolina and einkorn flours, related pasta and pasta mixtures 70/30 and 50/50 semolina/einkorn, asserted that in 50/50 semolina/einkorn pasta mixture larger polymeric structures formed than in 70/30 semolina/einkorn pasta mixture. They justified this lower aggregation in 70/30 semolina/einkorn pasta mixture by a high content of ω-gliadin, albumin and globulin, although these data were contradictory because, in 70/30 semolina/einkorn pasta mixture, there was a lower content of einkorn and therefore less ω-gliadin, albumin and globulin than in 50/50 semolina/einkorn pasta mixture. Moreover, our percentage data of SMP were very different compared to those of the authors, because they considered, as SMP peaks, the interacting peaks of sample buffer (Bean and Lookhart, 2001), which were not considered in our figure 2.

Our results strongly indicated that during dough and pasta production a different structural arrangement occurred in einkorn and semolina gluten proteins. Taking into account that the percentage of TPP increased in the unextractable fraction and that TPP/TMP was higher in einkorn flour, in 100% einkorn pasta and 50/50 semolina/einkorn pasta mixture, than in 70/30 semolina/einkorn pasta mixture, we can suggest that the protein aggregation can have a different self-assembling through cross-linking of polymeric proteins according to their nature and content. The polymeric aggregation influenced the size and solubility of the protein (Domenek et al., 2002) therefore, the more the unextractable fraction increased, the larger supramolecular structure was. The temperature reached in pasta processing gave rise, mainly in einkorn pasta, to the aggregation of albumin and globulin by coagulation (Shomer et al., 1995) and gliadin (Shomer et al., 1998) as well as self-assembling (Kuktaite et al., 2011) forming specific ultrastructure, fibrils like. In einkorn HMW 1Ax1 caused a different arrangement of polymeric proteins and probably the highest content of albumin and globulin caused a wider gluten polymerization through SH-SS interchange (Lagrain et al., 2011), that in turn made the the gluten network much larger, while the lowest SS/SH ratio showed other covalent bonds formation inside the intra gluten network. From our results also emerged the importance of the relative ratio amongst gluten protein species, which generate supramolecular structure by self-organization. All of this suggested that the architecture of polymeric protein network depends on the nature and proportion of the different classes of proteins and that the supramolecular self-assembling occurs in a hierarchical fashion. These results indicated

that in pasta processing and, mainly in pasta mixtures, when there are two or more components, the complexity of gluten network assessment does not always permit to predict supramolecular polymeric structures.

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Paper 2: The Role of Hydration on the Cooking Quality of Bran-Enriched Pasta

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Abstract

Hydration of multi component systems, such as pasta enriched with wheat bran, is a complex phenomenon due to water competition in the mixing. The influence of hydration method on the quality of wheat pasta loaded with wheat bran is addressed in this work. In particular, spaghetti in mixture with wheat bran were produced according to two different hydration methods: the durum wheat semolina and bran were first mixed together and then hydrated (-BT); durum wheat semolina and bran were separately hydrated and then mixed together (-BS). Two different concentrations of wheat bran, 20 g/100 g and 25 g/100 g, were prepared. Size exclusion-HPLC analysis was performed to investigate conformational polymeric changes of how the gluten network is affected by the hydration procedure. The sensory properties and the cooking quality of the samples were also assessed. Results suggest that the hydration method markedly affects the formation of disulphide bonds between simultaneous or separated constituent hydration, suggesting that separate hydration increases the number of disulphide bonds and the strength of the gluten network. As a matter of fact, samples obtained by separated hydration recorded a significant improvement of both sensory attributes and cooking quality parameters as compared to samples obtained by simultaneous hydration.

1. Introduction

Recently, a growing demand for healthy food, such as products with high fiber content and low calories, has been observed (Anderson et al., 2009; Krishnan & Prabhasankar, 2012). Traditionally, pasta is manufactured from durum wheat semolina, known to be a good source of low glycaemic index carbohydrates (Liljeberg & Bjork, 2000; Bjork, Liljeberg, & Ostman, 2000). Because the FDA considers pasta as a good vehicle for nutrients enrichment to increase its nutritional value and lower the caloric value by incorporating dietary fiber sources into pasta formulation is feasible (Chillo, Laverse, Falcone, & Del Nobile, 2008; Fuad & Prabhasankar, 2010; Bustos, Perez, & Leon, 2011; Rakhesh, Fellows, & Sissons, 2015). From a histological structure point of view, a complex biological material with a different chemical composition constitutes the durum wheat bran. Although wheat bran mainly consists of non-starch carbohydrates, which is not homogenously distributed and contains high levels of starch, protein, lipids, lignin and various minor components (Hemdane et al., 2016). Wheat bran can change the rheological and extrusion properties of the dough by causing discontinuity and a lack of homogeneity in the dough, compromising the formation of a cohesive network of gluten polymeric proteins that ultimately reduces the dough strength and mixing stability (Manthey & Schorno, 2002). As a matter of fact, dough development and quality are affected by wheat bran content due to the dilution of the gluten proteins, hindering proper gluten development by physically blocking the proper contact between the flour particles (Kaur, Sharma, Nagi, & Dar, 2012; Sobota, Rzedzicki, Zarzycki, & Kuzawinska, 2015). It was also found that the non-starch carbohydrates might cause a detrimental effect, interfering with proper gluten agglomeration. It has been suggested that the arabinoxylans interfere with gluten proteins, mainly by covalent binding of ferulic acid with tyrosine residues in gluten proteins, in turn affecting the formation of the gluten network (Piber & Koehler, 2005). This was substantiated by the observation that addition of ferulic acid could prevent oxidative cross-linking during gluten formation (Wang, Oudgenoeg, van Vliet, & Hamer, 2003). Taking into account these findings, it appears clear that the strength of the gluten network can change in relation to the elements of discontinuity.

The durum wheat semolina hydration level is another key factor to be considered to obtain a strong gluten network (Yalla & Manthey, 2006; Jacobs, Hemdane, Dornez, Delcour, & Courtin, 2015). A level of hydration, different from the optimal one, brings about the formation of a weaker gluten network and, consequently, a decrease in the sensory qualities of pasta. When the durum wheat semolina is the only constituent, it is easy to control its optimal hydration level. However, when semolina is mixed with other constituents, such as durum wheat bran, its hydration level is no longer under control due to the competition for the water molecules. In these cases, the adopted

hydration methods can modify the hydration levels of the constituents, which in turn can affect the strength of gluten network formed via the extrusion process and, consequently, the sensory quality of pasta (Bock et al., 2015).

The aim of the present study was to determine the impact of different hydration process on the gluten network formation in durum wheat spaghetti fortified with 20 g/100 g and 25 g/100 g of durum wheat bran. Size exclusion-HPLC analysis was used to determine the change of polymeric protein distribution as the pasta and the raw matter are affected by the hydration process. In addition, the effects of different hydration methods on sensory characteristic and cooking quality of pasta were also assessed.

2. Materials and Methods

2.1. Raw material

Durum wheat seeds (*cultivar* PR22D89) were provided from the C.R.A. (Agricultural Research Council, Foggia, Italy). The durum wheat was milled in an experimental mill (roller mill Mod MLU202 Buhler). The bran was ground to fine flour on a Tecator Cyclotec 1093 (International PBI, Hoganas, Sweden) laboratory mill (1-mm screen - 60 mesh). The durum wheat semolina was characterized by 13 g/100 g of protein content, on dry matter, and by quality parameters: W 189 and P/L 2.1.

2.2. Spaghetti preparation

Spaghetti was produced with durum wheat semolina by using the following operating conditions: semolina was mixed with water using a rotary shaft mixer (Namad, Rome, Italy) at 25 °C for 20 min to obtain a dough with 30 g/100 g moisture content. The bran was added at two concentrations: 20 g/100 g and 25 g/100 g, specifically, the bran was hydrated separately (20-BS and 25-BS) or together with durum wheat semolina (20-BT and 25-BT). The amount of water added has been modified according to the fibre-semolina dough to account for the higher water absorption capacity of the fibres (Table 1).

In addition, for both the formulations, 20-BS and 25-BS, the bran and semolina were mixed separately with water at 25 °C for 10 min. Then once hydrated, semolina and bran were mixed together at 25 °C for 10 min. Spaghetti without any enrichment (100 g/100 g semolina pasta) were also manufactured and used as the reference sample (semolina pasta). The doughs obtained were extruded with a 60VR extruder (Namad) and dried in a dryer (SG600; Namad), as described by Padalino et al. (2013).

Table 1 – Spaghetti preparation (From la Gatta et al., (2017) LWT- Food Science and Technology, 84, 489 – 496).

	Semolina (g)	Water (ml)	Bran (g)	Water (ml)	Total Water (ml)
CTRL	2500	750	-	-	750
20-BT	2000	-	500	-	850
25-BT		-	625	-	900
20-BS	2000	450	500	600	1050
25-BS	1875	400	625	750	1150

2.3. Size exclusion-high performance liquid chromatography (SE-HPLC) analysis

Proteins from semolina, bran and milled spaghetti (1g) were extracted following the two-step extraction procedure (Gupta, Khan, & MacRitchie, 1993). The first step extracts the SDS-extractable proteins (proteins soluble in sodium dodecyl sulphate (SDS), whilst the second extraction contains the SDS-unextractable proteins (proteins soluble only after sonication). The protein extraction and HPLC analysis were performed in triplicate.

SE-HPLC was performed according to Johansson, Prieto-Linde, & Jönsson, (2001), using a liquid chromatograph Agilent 1100 Series system (Santa Clara, CA, USA) equipped with a Phenomenex Biosep SEC-S4000 column (300 x 7.8mm, Phenomenex, Torrence, CA, USA). The SE-HPLC column was calibrated using protein standards with a range of molecular weights (kDa) as follows: Vitamin B_{12} (1.35), Myoglobin (17.0), ovalbumin (44.0), γ-globulin (158.0) and thyroglobulin (670).

The percentage of unextractable polymeric proteins (UPP) was calculated as described by Kuktaite, Larsson, & Johansson, (2004).

2.4. Determination of protein content

Nitrogen content of flours, pasta mixtures and of the residues obtained after the extraction of the unextractable polymeric proteins was estimated by the Kjeldahl method and was converted into protein by using a factor of 5.70. The analyses were carried out by an automatic digestion unit and through an automatic distillation and titration system (VELP Scientifica Srl, Usmate, Monza-Brianza – Italy). Three measurements for each sample were performed.

2.5. Determination of free thiol and total sulphydryl group content

Accessible free thiols and total sulphydryl group content in the flours and pasta samples were estimated by a colorimetric determination of the free SH groups, using a solid phase assay $NTSB^{2-}$, according to the method of Chan and Wasserman, (1993). The determinations of accessible free thiols and total sulphydryl group were performed in triplicate.

2.6. Sensory analysis

Dry spaghetti samples were examined by a panel of 15 trained tasters (seven men and eight women, aged between 28 and 45 years) in order to evaluate the sensory attributes. The panelists were selected in a preliminary session and were experienced in the products and terminology (ISO 11036, 7304). In these specific trials, panelists were asked to indicate color, homogeneity and resistance to breaking of uncooked spaghetti and elasticity, firmness, bulkiness, adhesiveness, color, odor and taste of cooked spaghetti. For the evaluation, a nine-point scale, where 1 corresponded to *extremely unpleasant*, 9 to *extremely pleasant* and 5 to the *threshold acceptability*, was used to quantify each attribute. On the basis of the above-mentioned attributes, panelists were also asked to score the overall quality of both cooked and uncooked samples, using the same nine-point scale (Padalino et al., 2013).

2.7. Cooking quality

The optimal cooking time (OCT) of pasta and the cooking loss (the amount of solid substance lost into the cooking water), were both evaluated according to the AACC approved method 66-50. The swelling index and the water absorption of cooked pasta (grams of water per gram of dry pasta) were determined according to the procedure described by Padalino et al. (2013). Three measurements for each sample were performed.

2.8. Statistical analysis

Experimental data were compared by one-way analysis of variance (ANOVA). A Duncan's multiple range test, with the option of homogeneous groups ($P < 0.05$), was carried out to determine significant differences between spaghetti samples. STATISTICA 7.1 for Windows (StatSoft, Inc, Tulsa, OK, USA) was used for this aim.

3. Results and Discussion

3.1. Size exclusion-HPLC analysis

In Fig. 1 is shown the total polymeric protein distribution extracted from durum wheat semolina and durum wheat bran. The fractionation of semolina, bran and spaghetti samples showed four peaks identified in agreement with Tosi et al., (2005) as: peak 1, included large polymeric proteins, LPP; peak 2, included small polymeric proteins, SPP; a large peak 3, included large monomeric proteins, LMP and a smaller peak 4, represents small monomeric proteins, SMP. The most remarkable differences between semolina and wheat bran were a higher content of LPP in semolina, with an average molecular weight of about 500 kDa and the absence of SPP peak in wheat bran, with an average molecular weight of about 140 kDa. Of the three detected fractions in this work (total proteins, unextractable and soluble) we referred to the unextractable fraction that was used to establish the polymeric protein assessment of the gluten network (la Gatta, Rutigliano, Rusco, Petrella, & Di Luccia, 2017).

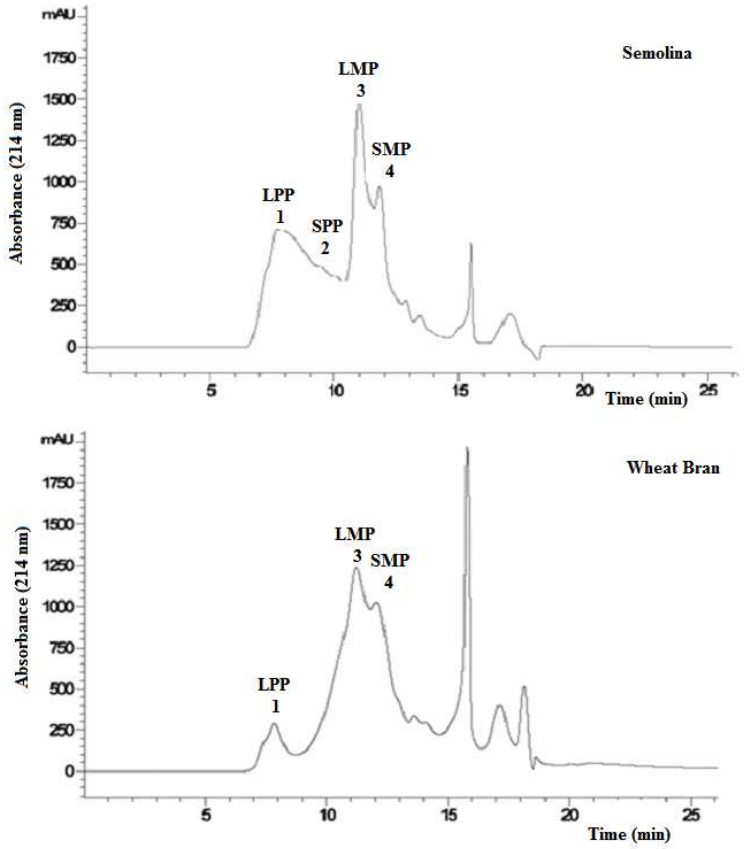
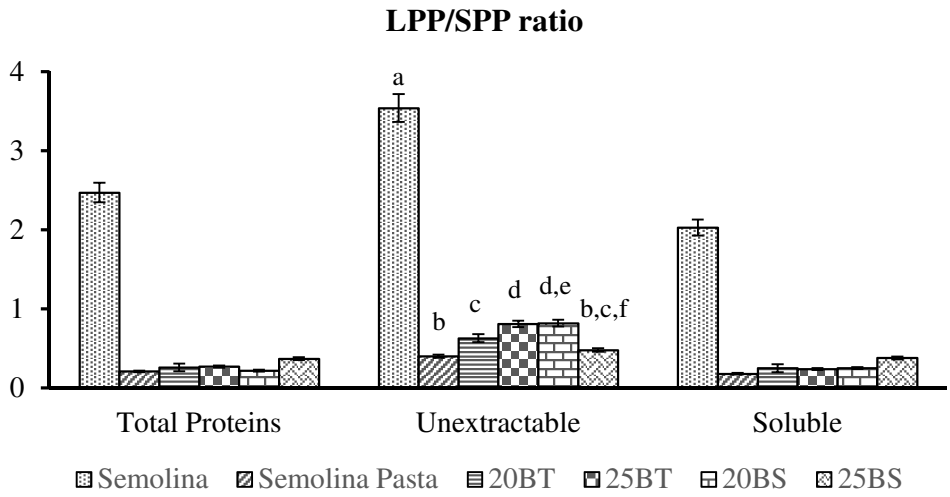
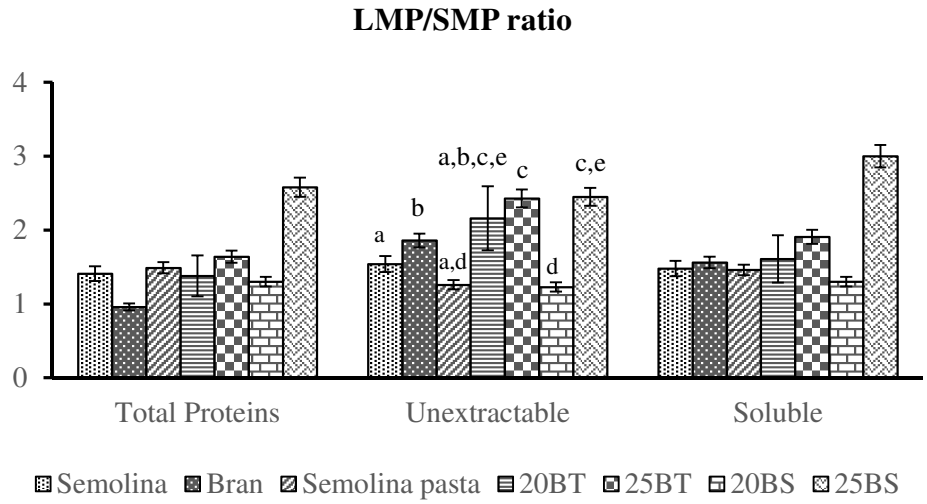


Figure 1. Size exclusion – high performance liquid chromatography profiles of total polymeric proteins from semolina and durum wheat bran. Peak 1, included high and low molecular weight glutenin (large polymeric proteins, LPP); peak 2, included mainly glutenin with a low molecular weight (small polymeric proteins, SPP); a large peak 3, included monomeric gliadins proteins (large monomeric proteins, LMP) and a smaller peak 4, represents albumins and globulins (small monomeric proteins, SMP). (From la Gatta et al., (2017) LWT- Food Science and Technology, 84, 489 – 496).

In Fig. 2 are shown the variations of the LPP/SPP, LMP/SMP and TPP/TMP ratios, calculated by the areas of each fractionated peak by SE-HPLC (Table 1 S1). The most relevant results were perceived from the ratios related to the polymeric protein fractions, especially in the unextractable fraction that was the best suited to define gluten network arrangement (Bruneel, Pareyt, Brijs, & Delcour, 2010). The ratios referred to the polymeric protein fractions (LPP/SPP) of the semolina were significantly higher ($P < 0.05$) than the pasta samples probably due to the insolubility of gluten proteins determined by extrusion and exsiccation process (Petitot, Abecassis, & Micard, 2009). With regard to the monomeric proteins, the LMP/SMP ratio showed higher values for pasta mixture 25BT and 25BS, whereas the lowest ones were the ratios of semolina pasta and pasta mixture 20BS.





The contribution of polymeric and monomeric proteins is well expressed by TPP/TMP ratio and the highest value was detected for pasta mixture 20-BS (2.31).

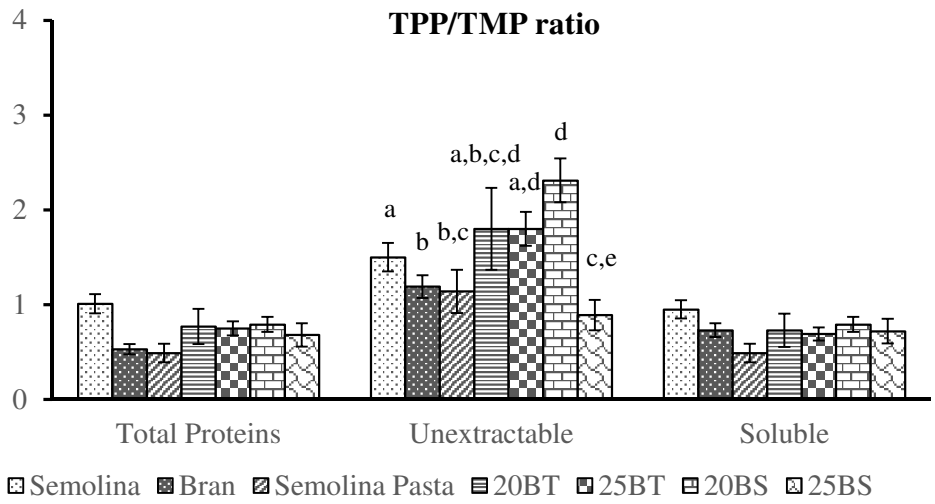


Figure 2. The variations of the LPP/SPP, LMP/SMP and TPP/TMP ratios (LPP: large polymeric proteins; SPP: small polymeric proteins; LMP: large monomeric proteins; SMP: small monomeric proteins; TPP: Total polymeric proteins; TMP: total monomeric proteins) in Semolina, wheat bran, semolina pasta and pasta mixtures with different wheat bran concentration (20 and 25 g/100 g) and with different hydration procedures: together BT and separately BS. (From la Gatta et al., (2017) LWT- Food Science and Technology, 84, 489 – 496).

An overall view of the samples, the LPP/SPP ratio showed lower values in the unextractable fraction than semolina did and they exhibited also a higher value of the TPP/TMP ratio with respect to the other two fractions (total proteins and SDS-soluble proteins), suggesting a more complex polymeric structure composed of a larger amount of SPP with respect to the LPP. However, the LPP/SPP ratio was lower than 1 for all samples, denoting, therefore, the fraction of SPP was higher than LPP.

Analyzing these results in term of polymeric proteins, accounting for protein reticulation degree, it seems that the network was built with a higher content of SPP (LPP/SPP<1) for all samples, except for the semolina, where LPP were the most represented (LPP/SPP>2). This could mean that durum wheat bran content and the hydration procedure affect the formation of polymeric protein reticulation in a different way. In a recent publication, Jacobs et al. (2015) demonstrated that bran water binding was influenced by particle size through macro- (void space between bran particles), micro- (space between pericarp cell) and nanoscale (cell wall matrix and cellulose structure) mechanisms. These authors observed that at molecular level macroscale involved strong water binding, whereas micro- and nanoscale involved a weak water binding. Moreover, bran is rich of polysaccharides that can bind water through formation of hydrogen (Chaplin 2003). On the basis of these findings, we could explain the results reported in Table 1 hypothesizing that in the case of concurrently hydration, the water uptake by wheat bran was in competition with starch and gluten protein of semolina determining an equilibrium in water sharing at macro-, micro, and nanoscale, requiring less water in hydration process. Alternatively, in the case of unconstrained condition through separating hydration, the reduction of the competition force allowed to a complete

hydration of bran and semolina constituents that each of them retain their own amount of water requiring more water for the hydration process.

Moreover, Bock et al. (2015) established that bran, redistributing available water, promotes partial dehydration of gluten and increases β-sheet structures inducing more interaction protein-protein than protein-water. The assumption of β-sheet form allows to the gluten to preserve its elastic properties during mixing and extrusion (Wellner et al. 2005) despite the discontinuous system deriving from the bran adding (Bock et al., 2015). Dynamic water interaction among matrix components, due to the bran incorporation, and working mechanisms affected significantly supramolecular assessment of gluten network that in turn it is reflected on final structure of pasta mixtures.

Table 1 S1. Polymeric Protein Total area obtained from size exclusion liquid chromatography (SE-HPLC). (From la Gatta et al., (2017) LWT- Food Science and Technology, 84, 489 – 496).

Total Polymeric Proteins		Semolina	Bran	100% Semolina Pasta	Pasta B (80%semolina-20%bran TM)	Pasta C (80%semolina-20%bran SM)	Pasta D (75%semolina-25%bran TM)	Pasta E (75%semolina-25%bran SM)
LPP	mAu	79164.2 ± 1408.56	15239.8 ± 663.97	8381.4 ± 48.22	15429.65 ± 247.42	13417.7 ± 709.23	19589.95 ± 500.00	15440.2 ± 323.57
	%	*35.85%*	*9.2%*	*5.63%*	*9.12%*	*8.02%*	*9.00%*	*10.88%*
SPP	mAu	32028.15 ± 227.05	42133.1 ± 962.51	40303.55 ± 542.14	58358.65 ± 121.69	60574.95 ± 77.71	73570.7 ± 1593.96	42039.15 ± 83.79
	%	*14.50%*	*25.43%*	*27.10%*	*34.47%*	*36.20%*	*33.84%*	*29.63%*
LMP	mAu	64083.5 ± 329.79	53193.9 ± 140.01	59913.6± 1188.36	55368.65 ± 156.20	52823.6 ± 621.97	77214.55 ± 626.85	60807.15 ± 663.20
	%	*29,03%*	*32.11%*	*40.24%*	*32.71%*	*31.57%*	*35.50%*	*42.86%*
SMP	mAu	45518.8 ± 441.94	55096.85 ± 36.27	40282.75 ± 1685.95	40116.9 ± 35.07	40526.75± 588.52	47105.5 ± 46.24	23595.1 ± 38.61
	%	*20,62%*	*33.26%*	*27.03%*	*23.70%*	*24.21%*	*21.66%*	*16.63%*
Total Area	mAu	220794.7 ± 863.87	165663.7 ± 1730.22	148881.3 ± 3.68	169273.9 ± 177.84	167343 ± 664.96	217480.7 ± 1420.86	141881.6 ± 1109.17
	%	*100%*	*100%*	*100%*	*100%*	*100%*	*100%*	*100%*

Table 2 S1. Unextractable polymeric protein fraction area obtained from size exlusion liquid chromatography (SE-HPLC). (From la Gatta et al., (2017) LWT- Food Science and Technology, 84, 489 – 496).

Unextractable Polymeric Proteins		Semolina	Bran	100% Semolina Pasta	Pasta B (80%semolina-20%bran TM)	Pasta C (80%semolina-20%bran SM)	Pasta D (75%semolina-25%bran TM)	Pasta E (75%semolina-25%bran SM)
LPP	mAu %	20294.95 ± 42.92 *46.72%*	9725 ± 529.06 *18.96%*	3177.75 ± 37.26 *15.35%*	8883.7 ± 875.26 *24.74%*	12637.75 ± 148.00 *31.31%*	11036.1 ± 30.69 *28.66%*	5559.3 ± 95.32 *15.34%*
SPP	mAu %	5760.1 ± 649.55 *13.26%*	18178.25 ± 52.54 *35.44%*	7851.85 ± 608.18 *37.90%*	14201.5 ± 1070.28 *39.55%*	15474.3 ± 435.29 *38.34%*	13700.9 ± 362.89 *35.58%*	11489.2 ± 1421.28 *31.72%*
LMP	mAu %	10530.55 ± 470.58 *24.24%*	15198.45 ± 73.47 *29.63%*	5395.25 ± 87.89 *26,04%*	8748.45 ± 63.85 *24.36%*	6757.85 ± 1052.95 *16.75%*	9755.25 ± 1064.12 *25.33%*	13616.95 ± 118.02 *37.60%*
SMP	mAu %	6854 ± 512.37 *15.78%*	8188.85 ± 44.19 *15.97%*	4289.9 ± 415.92 *20.71%*	4077.95 ± 539.73 *11.35%*	5490.7 ± 701.59 *13.60%*	4016.8 ± 377.60 *10.43%*	5556.7 ± 117.52 *15.34%*
Total Area	mAu %	43439.6 ± 1589.58 *100%*	51290.55 ± 610.87 *100%*	20714.75 ± 242.89 *100%*	35911.6 ± 1341.95 *100%*	40360.6 ± 2237.84 *100%*	38509.05 ± 1109.52 *100%*	36222.15 ± 1752.14 *100%*

In Table 2 are shown the large unextractable polymeric proteins (lUPP) and the protein percentages that were present in the residue after the last extraction the unextractable fraction. It was observed that the arrangement of the gluten network and its composition is an unpredictable factor in the pasta mixtures, according to la Gatta, et al., (2017). In our case, comparing the different samples, the gluten network depended on wheat bran content in the mixture that brings out also a critical hydration loading 25 g/100 g of bran in the mixture. In this latter case, the hydration procedures showed conflicting results, suggesting a gluten polymeric network that included more SPP and LMP, as showed also by LMP/SMP and LPP/SPP ratios (Fig. 2). The contrasting results can be justified by the bran content in the mixture and by the hydration system that affected significantly the self-assembly/self-organization machine that generated supramolecular structure of gluten network with different LPP/SPP, LMP/SMP and TPP/TMP ratios. In fact, the sample 25-BS, in which semolina and bran were separately hydrated, showed a more complex gluten network arrangement with the polymeric proteins (LPP and SPP) and with the large monomeric proteins (LMP). In this respect, the assessment of the gluten network of semolina pasta must be separated from those of the semolina-wheat bran pasta mixtures. The proteins in the residue of 25-BT and 25-BS were similar but their nature was different, as demonstrated by the differences in the lUPP. These differences were explained in Table 2, where the weight of the residues was comparable whereas the protein percentages were significantly different ($P < 0.05$) amongst raw matter (semolina and wheat bran), the couple of 20 g/100 g bran composite pasta (BT and BS) and the couple of 25 g/100 g bran composite pasta (BT and BS). These differences in the protein percentages were due to a higher content of proteins linked to the bran cell wall, whereas in semolina pasta, this was due to the protein polymeric network of a higher molecular weight (Mackintosh et al., 2009), which was not extractable, because of non reducible covalent bonds, such as dityrosine (Tilley et al., 2001) and thiol-tyrosine (Takasaki & Kawakishi, 1997). All these results demonstrated a different building in the assessment of polymeric structures when different raw matters are mixed for pasta production, as already demonstrated by la Gatta et al., (2017).

Table 2 -Weight and protein content of the residues after the extraction related to the unextractable proteins and percentage of lUPP. (From la Gatta et al., (2017) LWT- Food Science and Technology, 84, 489 – 496).

Samples	Weight of residues after last extraction (g)	Protein content of residue (%)	lUPP (%)
Semolina	0.74±0.04[a]	0.83±0.1[a]	22.34
Wheat bran	0.77±0.05[a,c]	1.10±0.28[a]	35.82
Semolina pasta	0.94±0.03[b]	4.51±0.14[b]	30.68
20-BT Pasta	0.80±0.03[a,c]	3.31±0.31[c]	41.48
25-BT Pasta	0.83±0.06[a,c]	5.48±0.59[b,e]	45.65
20-BS Pasta	0.84±0.03[c]	3.07±0.29[c,d]	46.49
25-BS Pasta	0.95±0.02[b,d]	5.78±0.34[e,f]	29.34

a, b, c, d, e, f = $P < 0.05$
(lUPP: large unextractable polymeric proteins)

3.2 Determination of accessible free thiol and disulphide content

Table 3 shows the content of thiols and disulfide content in semolina, bran and composite pastas; as expected, the disulphide content increased from semolina to relative pasta, while it was evident the different behaviour of composite pastas. These results suggested that the hydration procedure affects the formation of disulphide bonds, increasing from simultaneously to separating hydration. Further suggestions on the influence of different hydration procedure on gluten network structures came from the observation that the content of thiol groups (-SH) of semolina pasta did not differ significantly from 20 BS, whereas disulfide bonds (S-S) did not differ significantly between semolina pasta and pasta composites mixed together, confirming, in this latter case, the similarity between their architectural structures.

Table 3. Effect of pasta processing on contents in free thiol (SH), disulfide groups (SS) and their ratios. Mean ± standard error for three replicates. Values are nanomoles per milligram of protein. The asterisks indicate the statistical significance (P<0.05) between wheat bran and semolina pasta. (From la Gatta et al., (2017) LWT- Food Science and Technology, 84, 489 – 496).

Samples	Free Thiol (nmol/mg prot)	Disulfide group (nmol/mg prot)	Disulfide/Free Thiol (SS/SH)
Semolina	39.05 ± 0.57[a]	58.06 ± 0.34[a]	1.49
Wheat Bran	36.44± 2.01[a*]	41.28± 1.80[b]	1.13
Semolina Pasta	44.39 ± 2.93[a,e*]	72.00 ± 5.73[c,e]	1.62
20 BT Pasta	51.74 ± 0.57[b,f]	74.29 ± 4.94[c,d]	1.43
25 BT Pasta	58.28 ± 1.48[b,c]	69.06 ± 3.39[c]	1.18
20 BS Pasta	41.18 ± 5.56[a]	82.46 ± 2.22[d,e,f]	2.00
25 BS Pasta	48.47 ± 0.34[d,e,f]	91.34 ± 7.40[f]	1.85

a, b, c, d, e, f = P < 0.05

3.3. Sensory and cooking quality

As reported beforehand, bran supplementation in pasta may cause a weakening of the gluten protein network and may have a detrimental effect on its cooking and sensory quality. Table 4 shows the sensory properties of uncooked and cooked dry spaghetti of semolina pasta and pasta enriched with 20 g/100 g and 25 g/100 g durum wheat bran. Data on the dry uncooked pasta highlighted that for semolina pasta the overall quality score was significantly higher than that of the other samples. In particular, the semolina pasta presented the highest scores for colour and break resistance and pleasant yellow colour, whereas the samples fortified with bran had a darker colour, mainly those with greater bran amount. These results are in agreement with Sobota et al. (2015), which observed that the semolina pasta was characterized by the brightest and the most uniform colour. On the contrary, samples with the addition of bran were darker and their colour was less uniform and glossy. The samples showed a noticeable decline in the homogeneity score as the bran content increased. Regarding the break resistance, samples enriched with bran recorded the lowest score. It is conceivable that bran fibers interfere with the continuity of the gluten matrix causing weakening of the gluten network, which in turns reduces the mechanical strength and the cooking quality of the bran supplemented pasta (Padalino et al., 2013).

Concerning the cooked spaghetti, the samples enriched with bran recorded the lowest overall quality score, this was mainly due to the poor elasticity and low firmness (Table 4). These results may be associated to the fact that bran particles as high-fibre cereal material interfere with the formation of a continuous and regular gluten matrix during the making of the pasta dough. During

cooking, water penetrates the pasta structure more easily and the uncovered starch granules are more susceptible to leaching (Aravind, Sissons, Egan, & Fellows, 2012). As a consequence, pasta enriched with bran, mainly those with high bran content, were more adhesive than semolina pasta. As for the colour, the spaghetti made exclusively from durum wheat appeared to have a pleasant yellow colour, which indicated good quality. On the contrary, the samples enriched with bran appeared to have an intense brown colour, suggesting a lower acceptable quality. Beyond that, the acceptability score was negatively affected due to the taste of enriched pasta. In fact, incorporation of bran resulted in significant deterioration of pasta taste as compared to the semolina pasta. Also, Sobota et al., 2015 found that the specific aftertaste of the bran that lingers in the mouth gave lower taste score in the pasta sensory analysis than the semolina pasta but the addition of the bran did not influence negatively the odour of the pasta.

Regarding the influence of the hydration method on the sensory quality of cooked and uncooked pasta the data shown clearly indicate that there was an increase in the spaghetti whole sensory quality when pasta constituents were separately hydrated. In the case of uncooked pasta, color and resistance to break were both positively affected by separate hydration. As concerns the sensory quality of cooked pasta, separate hydration of durum wheat semolina and bran positively affected the score of all the attributes directly related to the pasta structure, such as elasticity, firmness, adhesiveness and bulkiness. This may be directly related to the fact that hydrating the constituents separately improves the strength of the gluten network. This hypothesis is consistent with the data reported beforehand on size exclusion-HPLC analysis and disulfide bonds content, where an increase in the number of detected S-S bonds was found when constituents were separately hydrated. As discussed earlier a separate hydration of durum wheat semolina ad bran allows to optimize the semolina hydration level; whereas, when the main constituents are simultaneously hydrated they both compete for water, this may lead to an incomplete semolina hydration and in a weakening of the gluten network (Yalla & Manthey, 2006).

Data regarding the cooking performance of spaghetti are shown in Table 5 these data highlight that bran incorporation in pasta determined a reduction of the optimum cooking time (OCT) as compared to the semolina pasta. This is due to the physical disruption of gluten matrix by bran particles, which provide a preferential pathway for water absorption into the whole-wheat spaghetti strand, reducing the cooking time (Kaur, et al., 2012). According to Kaur et al. (2012), the incorporation of bran, mainly at high amounts, causes a noticeable rise in the leaching out of solids from pasta into the cooking water. This could be attributed to the changes in the gluten protein network, responsible for retaining the amylose fraction during the cooking process because of the interference of dietary fibres contained in the bran (Table 5). Earlier, Tudorica, Kuri, & Brennan,

(2002) found that the rise in cooking loss could be due to the disruption of protein–starch matrix by the bran fiber and uneven distribution of water within the pasta matrix due to the competitive hydration tendency of the bran fiber. This in turn exposes the starch granules to swelling and rupture. It is worth noting that the data on cooking loss were in agreement with those obtained from the sensory analysis. In fact, as compared to the semolina pasta, the bran fortified pasta had lower score for the adhesiveness attribute (Table 4).

Regarding the swelling index and the water absorption no statistically significant differences were observed between samples (Table 5), even though there was a slight decrease in these parameters when the bran was added to the formulation. A possible explanation might be related to the fact that bran competes for water with starch, reducing the total amount of absorbed water (Brennan, et al., 2008; Laureati, Conte, Padalino, Del Nobile, & Pagliarini, 2016). In fact, the samples 20-BS and 25-BS showed a significant improvement of the cooking quality as compared to the 20-BT and 25-BT, confirming the data reported on pasta sensory quality. Data on pasta cooking quality seem to further support the idea that a separate hydration of durum wheat semolina and bran improves the quality of pasta. This may be strictly related to the formation of a higher number of S-S bonds, which contribute to the increase of the strength of the gluten network.

Table 4. Sensory characteristics of uncooked and cooked dry pasta samples.(From la Gatta et al., (2017) LWT - Food Science and Technology, 84, 489 – 496)

	Uncooked Spaghetti				Cooked Spaghetti							
	Colour	Homogeneity	Break Resistance	Overall Quality	Elasticity	Firmness	Bulkiness	Adhesiveness	Colour	Odour	Taste	Overall Quality
Semolina pasta	7.30±0.21^{a}	7.25±0.25^{a}	6.20±0.21^{a,b}	7.68±0.23a^{c}	7.46±0.21^{a}	7.20±0.24^{a}	7.22±0.20^{a}	7.40±0.24^{a}	7.52±0.29^{a}	7.80±0.28^{a}	7.70±0.27^{b}	7.56±0.30^{a}
20-BS	6.50±0.19^{b}	6.05±0.29^{b}	6.28±0.29^{a}	7.12±0.28^{b}	6.50±0.27^{b}	7.10±0.20^{a}	6.94±0.25^{a}	6.40±0.30^{b}	7.50±0.27^{a}	7.50±0.27^{a,b}	7.06±0.29^{b}	6.50±0.27^{b}
20-BT	6.28±0.25^{b}	5.75±0.27^{b}	5.78±0.29^{b,c}	6.02±0.28^{c}	5.50±0.27^{c}	6.28±0.29^{b}	6.04±0.25^{b}	5.96±0.30^{b,c}	6.50±0.27^{b}	7.20±0.27^{a}	7.20±0.29^{b}	5.50±0.27^{c}
25-BS	6.05±0.29^{b,c}	5.25±0.25^{c}	5.70±0.26^{c}	5.96±0.29^{c}	6.06±0.21^{b}	6.08±0.29^{b}	6.05±0.30^{b}	6.20±0.25^{b}	6.80±0.27^{b}	7.25±0.27^{a}	7.08±0.28^{b}	6.02±0.22^{b}
25-BT	5.75±0.30^{c}	5.00±0.25^{c}	4.76±0.21^{d}	5.28±0.25^{d}	5.06±0.29^{c}	5.10±0.20^{c}	5.74±0.30^{b}	5.54±0.25^{c}	6.76±0.21^{b}	7.08±0.29^{a}	6.76±0.21^{b}	5.08±0.28^{c}

a, b, c, d, = $P < 0.05$

Table 5. Cooking quality of cooked dry pasta samples (From la Gatta et al., (2017) LWT- Food Science and Technology, 84, 489 – 496).

	OCT (min)	Cooking Loss (%)	Swelling Index (g water per g dry Spaghetti)	Water Absorption (%)
Semolina pasta	11.30	6.04±0.29^{d}	1.96±0.07^{a}	154±4.29^{a}
20-BS	9.00	6.12±0.20^{c,d}	1.90±0.08^{a}	149±8.73^{a}
20-BT	10.00	6.51±0.12^{b,c}	1.92±0.03^{a}	151±2.41^{a}
25-BS	11.00	6.82±0.13^{b}	1.77±0.02^{b}	136±1.82^{b}
25-BT	10.30	7.56±0.30^{a}	1.88±0.06^{a}	146±5.86^{a}

a, b, c, d, = $P < 0.05$

4. Conclusions

Bran loaded durum wheat pasta and the hydration method affect gluten network formation that in turn reflects itself on the sensory properties and the cooking quality. The studied multicomponent system has showed three crucial factors: bran properties, its content and hydration systems. Bran properties and its content cause a physical discontinuity in the gluten network and a competitive hydration in simultaneous and separate procedures. These different procedures, affect the architecture of supramolecular gluten network according to the self-assembly/self-organization, changing the proportion of polymeric and monomeric proteins and increasing disulphide bonds in the formation of network. The modifications in the supramolecular structure and in the conformational arrangements that occurr in separate hydration of bran and semolina positively affects the cooking and sensory quality of pasta. This may be directly related to the fact that separate constituent hydration improves the strength of gluten network via the formation of a greater number of S-S bonds. Thus, a separate hydration of durum wheat semolina and bran allows to optimize the semolina hydration level; whereas, when the main constituents are simultaneously hydrated they both compete for water, this may lead to an incomplete semolina hydration and to different supramolecular structure of gluten network.

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Draft article. Evidence for a different protein network arrangement and detection of the immunoreactivity in baked wheat-based biscuits incurring allergens

During the training period as PhD student at the Manchester Institute of Biotechnology (MIB), with the tutor Professor Clare Mills, the influence of the baking on the protein solubility and organization in wheat-based baked products was assessed.

The research was carried out including allergens (peanut proteins) in the formulation of biscuits and muffins and studying how these may influence the organization of the gluten network, considering how this latter can modify their bioavailability.

The collaboration with Prof. Mills is still ongoing and a brief state of the art, the description of the methodology and the preliminary results of the research on biscuits are shown in this chapter.

1. State of the art

1.1 Wheat-based cereal products: biscuits characteristics

Wheat is a key food, being associated with the most important food uses.

The great importance of wheat flour is due to the ability to form a viscoelastic dough characterized by a polymeric protein network embedded by starch, whose processing leads to the production of a wide range of products (Delcour et al., 2012).

Biscuits, or cookies, is a term used for a variety of primarily flour-based baked food products. They are baked products, generally containing three major ingredients: soft wheat flour, sugar and fat, and have a low water content (1–5%) (Chevallier et al., 2002). Other ingredients, such as leavening agents, emulsifiers and salts can be listed in the formulation, but in minor concentration. On the basis of the formulations, there are several types of biscuits with specific features and formulations (Manley, 2000). As example, in the sugar-snap cookie preparation, all the ingredients can be mixed together in a single step, by forming a non-extensible short dough or, if the fat is first mixed together with the water and sugar, a cream, then flour is added with minimal mixing leading to a non-elastic and non-extensible dough with a minimal gluten network development (Pareyt & Delcour, 2008).

What happens during baking is strictly related to the recipe of the short-dough biscuits. The baking entails the expansion of the dough as a consequence of the vaporization of the water and the gases from the leavening powders. The main features of biscuits, size (Doescher et al., 1987) and bite (Hoeseney & Rogers,1994), are determined by the biscuits structure, which in turn is affected by

the baking events. The way the ingredients are mixed and the dough rheological properties influence the intrinsic properties of the product during the baking and the final quality of the biscuits (Piteira et al., 2006). It has been reported that the short-dough biscuits spread at costant rate during baking, because of the chemical leavening and the apparent viscosity decreasing (Miller & Hoseney, 1997), which in turn is determined by the sugar dissolution, fat melting and the competition for water (Hoseney & Rogers, 1994), until the dough sets.

Several studies reported what may happens during baking and the influence that the main ingredients have in the dough setting mechanism. Doescher et al., (1987) reported the influence of the glass transition of gluten proteins during baking, which cause an increase in the dough viscosity leading the gluten protein to be able to entangle.

By contrast, Slade et al., (1993) and Chevallier et al., (2000) noticed that in the short dough processing the development of the gluten network is limited, as a consequence of the high amount of fat (up to 25%) and sugar (up to 30%) and the low water content. The ability of gluten proteins to cross-link resulted to be affected, especially, by the high amount of sugar, which competing for the water, can prevent or delay the cross-linking reactions, affecting the gluten functionality (Pareyt et al., 2009). In this view, the structure of biscuits can be seen as a complex matrix where protein aggregates, lipids and sugars embedded starch granules. The sugar melting and subsequent glass transition during the cooling stages would guarantee a cohesive structure through the formation of bridges between protein and lipids or through a continuous phase of molten sugars, which embed all the other ingredients (Chevallier et al., 2000), becoming glassy during cooling.

The flour protein characteristics were found to play a role in determining the biscuits dough set, structure and quality (Pareyt et al., 2010), forming large protein aggregates during baking, which affect the spreading rate.

In this context, the polymer size distribution of gluten proteins was studied in biscuits where the inclusion of another flour was provided for the formulation, roasted peanut flour, assessing how this can affect the aggregation event and, eventually, the allergenic properties.

1.2 Allergens

An allergen can be described as an antigen that cause an allergic response (Johansson et al., 2008). Most of them are proteins that can derive from different sources, such as animals or plants or their processed products too.

Food allergy is defined as “an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food” (Sicherer, 2011).

These reactions refer to immune-mediated reactions (that can be divided into IgE- and non-IgE-

mediated reactions) and non immune mediated reactions (Fernández-Rivas & Miles, 2007).

Food allergy (hypersensitivity) is an IgE immune-mediated reaction and exists an underlying immunological response mechanism for them, while *Food intolerances* are non-IgE mediated reactions and refers to difficulty in digesting certain foods.

Another difference between food allergy and food intolerance is in the times of the individual's response. For food allergies, the reaction is quick (from 3-5 minutes to 2 hours); in food intolerances, response times are longer (up to 2-3 days) for the appearance of symptoms.

Non immune mediated reactions, instead, do not cause immune system responses but are linked to enzymatic factors (as in the case of the lack of specific enzymes) or to pharmacological factors.

The exposure to allergens in foods is subject to a number of factors that may be genetic, but which are widely related to the age of the individuals and to their geographical location. In general, the major allergens in foods are milk, egg, peanuts and tree nuts, fish, shellfish, soy, wheat, fruits and legumes.

1.3 Peanut

Peanut (*Arachis hypogaea L.*) is an important crop grown worldwide and belongs to the family of Leguminosae, together with beans and peas. Legumes are edible seeds enclosed in pods. They are one of the most important source of protein and for their characteristics (54% oil, 21-36% protein and 25% carbohydrates) and with 18 amino acids, considered as essential in the diet are used to produce a large variery of products, such as oil, snacks, butter and sweets.

1.4 Peanut allergens

Several researches have been conducted to study the characteristics of peanuts and biochemical and immunological studies demonstrated that the peanuts have different allergens located in the protein of the cotyledon and did not differ significantly between the different varieties of peanuts (Shewry et al., 2007).

There are 11 peanut allergens (Ara h 1-11) that are currently studied (Krause et al., 2010). The three major peanut allergens that are recognized by the vast majority of peanut allergic individuals and that have been studied are Ara h 1, a vicilin-like protein (Dunwell, 1998; Pomes et al. 2005), Ara h 2, a conglutin–homologue protein (Dunwell, 1998; Viquez et al. 2001) and Ara h 3/Ara h 4, glycinin proteins (Rabjohn et al., 1999; Viquez et al. 2002; Piersma et al., 2005). Recently it was shown that Ara h 3 and Ara h 4 are isoallergens and can be designated as the allergen Ara h 3/4 (Kleber-Janke et al., 1999; Piersma et al., 2005; Boldt et al., 2005).

The allergens Ara h1, Ara h3 and Ara h4 belong to the cupin superfamily. The cupin superfamily is

a group of proteins with a similar β-barrel domain (Breiteneder & Radauer, 2004). The allergen Ara h1 is the most abundant (20% of the total protein) followed by Ara h2 (~10%) and Ara h3/4 (Burks et al., 1998; Koppelman et al., 2001). Ara h1 is a 63–68 kDa glycoprotein (Burks et al., 1995) assembled in di- and trimeric complexes (Bushmann et al., 1996; Shin et al. 1998) and two of its genes encoding 626 and 614 amino acids have been well described (Burks et al., 1995; Wichers et al., 2004). The allergenic protein Ara h3/4, recently purified consists of an acidic and a basic sub-unit (Piersma et al., 2005; Koppelman et al., 2003; Restani et al., 2005). The two sub-units remain covalently linked by an intermolecular disulfide bridge and associate into a very stable hexameric structure (Boldt et al., 2005; Jung et al. 1998). The acidic sub-unit has a molecular mass in the range of 40–45 kDa, whereas the basic sub-unit has a mass of ca. 25 kDa. Ara h 3/4 is mainly proteolytically modified (truncation at multiple sites), with possible glycosylation. Proteolytic truncation was observed for the acidic sub-unit, but not for the basic sub-unit, resulting in a series of polypeptides ranging from 13–45 kDa (Piersma et al., 2005).

The allergens Ara h2, Ara h6 and Ara h7 belong to the prolamin superfamily. This superfamily is characterized by a structure composed of eight cysteine residues connected by four α-helices and stabilised by disulphide bonds (Shewry et al., 2007).

Ara h2 (conglutin family, or 2S albumin) belongs to the Prolamins superfamily, it is a glycoprotein and has molecular weight of 17 kDa. This polypeptide is considered one of the major peanut allergens with ten recognised binding sites that contribute to its high allergenicity (Zhuang & Dreskin, 2013). Maleki *et al.* (2003), also, demonstrated that the heat treatment can exalts its allergenicity.

Ara h6 (conglutin family) belongs to the Prolamins superfamily and has a molecular weight of 15 kDa. Both from the structural point of view and for its allergenicity (with seven IgE-binding epitopes) and resistance to heat treatments is very similar to Ara h 2.

1.5 Wheat as allergen

Wheat is one of the ‘big eight’ food allergens which all together account for about 90% of all allergic responses (Shewry, 2009).

Wheat proteins can induce a "gluten-related disorder", a condition related to the consumption of gluten-based products, including celiac disease (CD), dermatitis herpetiformis, gluten ataxia, and non-celiac gluten sensitivity (NCGS) (Sapone et al., 2012).

Wheat-based foods can trigger immune-mediated adverse reactions including both IgE-mediated food allergies and the gluten intolerance syndrome celiac disease (CD), a T-cell mediated condition, which is triggered by the presence of numerous T-cell reactive epitopes found in the repetitive

domain of prolamins (Smith et al., 2015), affecting around 1% of the European population (Mustalahti et al., 2010).

Several studies have been conducted to identify the peptide sequences which are recognized by intestinal T cell lines and it has been defined that gluten proteins and especially gliadin fractions can produce symptoms in sensitive persons (Arentz-Hansen et al., 2002; Ellis et al., 2003). Among these CD-immunodominant gluten peptides, the most immunogenic is the α-gliadin-derived 33-mer (residues 57-89) (Shan et al., 2002).

1.6 The effect of thermal processing on food allergens

Thermal processing can modify food proteins giving rise to unfolding and aggregation events, as well as chemical modifications.

As a consequence, the proteins could remain monomeric and soluble in a diluted solution, but with a high protein concentrations, as in food matrices, they can form large macromolecular aggregates and gel networks (Mills et al., 2009).

There are some factors which are essential to understand how the processing could affect the unfolding and the aggregation events: in the food matrix, especially, the interactions occurring with fats and sugars, the presence of water (protein becomes thermostable in low-water content matrices) and the combination of processing time and temperature. The modifications induced by the thermal processing can influence the digestibility of the protein and so the potential ability to elicit a reaction of the immune system (Verhoeckx et al., 2015).

An allergic reaction can be caused by a simple sequence of few amino acids, along the primary structure or a three dimensional motif of the protein structure, known as linear and conformational epitopes (Sathe et al., 2005).

The allergenic properties of a protein can be modified since most of the foods or food ingredients are subjected to processing, which may destroy epitopes on a protein, generate new forms (neo-allergen formation) (Spies, 1974), modify, mask or unmask an allergenic epitope and increase or decrease or having no effect on allergenicity.

Conformational epitopes are commonly more susceptible to processing than the linear ones, which are more affected by the hydrolysis processes (Sathe et al., 2005).

Both thermal and non thermal processing are implicated in modifying the potential allergenic properties, such as heating, fermentation, including endogenous enzymatic hydrolysis, enzymatic and acid hydrolysis, physical treatments (high pressure processing or extrusion), the use of preservatives, changes in pH or the combination of these (Verhoeckx et al., 2015, Mills and Mackie, 2008).

Food processing can affect the allergenicity, but there are no clear rules. The allergenicity can be enhanced by the thermal processing as well as it can be destroyed.
What is clear is that the structure and the stability of the allergen as well as the structure of the matrices, in which an allergen can be incurred, play a key role.

2. Experimental design

The experimental design provided for the production of three different types of biscuits: biscuits with 100% of wheat flour, biscuits incurring 4% of peanut flour and biscuits incurring 20% of peanut flour. The aim of the work was to assess how the addition of peanut flour affected the protein extractability and the gluten protein polymerization through the determination of the sodium dodecyl sulfate (SDS)-Extractable and Unextractable Protein through monodimensional gel electrophoresis (SDS-PAGE) and size exclusion liquid chromatography (SE-HPLC). Moreover the detection of changes in the immunoreactivity of the main allergenic epitopes of wheat and peanut flours was assessed in the baked products through the application of the immunoblotting.

3. Materials and methods

3.1 Materials

Commercial biscuit flour [protein content 8.3% (db, dry base) and 12% Fat-Ligh roast peanut flour were used as raw materials. Commercial caster sugar, margarine and sodium bicarbonate were purchased at local supermarkets.

3.2 Cooking making

A traditional biscuits dough recipe was used to produce biscuits with flour (50%,), sugar (16 %), fat (15%), water (18%), salt (0.3%), sodium bicarbonate (0.2%, flour base), ammonium bicarbonate (0.1%, flour base) and corn oil (0.1% For the preparation of the two types of biscuits with a different formulation, 4% and 20%, of roasted peanut flour, respectively, replaced,the commercial wheat flour. In the case of 20% peanut-biscuits, more water (22%) was needed to obtain a proper dough.
The dry ingredients were creamed in a Philips Professional mixer HR1565 (Philips, Amsterdam, Netherlands) for 10 min, then oil was added, mixing for 5 min (speed 1) and 5 min (speed 3).
Then, deionized water was added to the dough, mixing for 5 min (speed 3). At the end, the cream was packed in a food grade bag to be baked. The dough was spread with a roller, getting ≈ 5mm thickness and the dough was cut with a circular cookie cutter (inside diameter ≈ 65mm).The dough pieces were weighed (≈ 20g/biscuits) and immediately baked for 18 min at 150 °C in a pre-heated

electric fan oven (Whirpool Corporation, Benton Harbor, Michigan, USA) on a stamped steel baking tray with baking paper. The biscuits were cooled down at ambient temperature for 20 min, and packed in labelled food grade bags until the analysis.

3.3 Sample preparation

All the biscuits samples were finely grinded using pestle and mortar, until reaching a sample flour-like. Protein samples were extracted in duplicate using 1g of flour (i.e Wheat flour and Roasted Peanut Flour) or biscuits in a sample:buffer ratio of 1:10. The sample buffer used was 50 mM Tris-HCl (pH 8.8), 50 mM DTT, 7 M urea, 2 M thiourea, 2 % CHAPS. After ensuring a homogeneous suspension was achieved, samples were incubated at 60 °C in a water bath for 15 min; samples were vortexed and sonicated for 30 sec each 5 min. Finally, samples were centrifuged at 10.000xg for 10 min and the supernatant were collected and freezed (-20°C) until the use.

For each sample (i.e flours and biscuits) protein content was determined using the 2D Quant-Kit ™ from GE Healthcare, according to supplier's instructions.

The protein extract from three indipendent samples was analyzed in triplicate.

3.4 Sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS PAGE) and Immunoblotting

SDS-PAGE and immunoblotting analyses were performed for biscuits samples. For the SDS-PAGE, to 30 µL of the extracted proteins, 15µL 200 mM DTT and 15 µL NuPAGE LDS buffer were added and samples were heated to 80°C for 5 min in a heat block.

Samples (10 µg) were loaded onto 12% NuPAGE Bis-Tris gel together with either Mark 12™ markers for SDS-PAGE and SeeBlue™ prestained markers for immunoblotting.

Electrophoresis was performed for 40 min at 200 V using with MES-SDS (pH 7.3) as the running buffer (Invitrogen, UK).

SDS-PAGE gels were fixed for 1 h in 50% v/v methanol (MeOH), 10% v/v TCA and stained overnight with SimplyBlueTM safestain prior to destaining with distilled water and imaging using a Typhoon Trio scanner (GE Healthcare, Buckinghamshire, UK).

For immunoblotting, SDS-PAGE gels were soaked in 25 mM Tris-HCl pH 8.3 containing 192 mM glycine, 20% v/v Methanol for 30 mins.

Proteins were electroblotted onto nitrocellulose membranes at 15 V for 25 min (Biorad, Hertfordshire, UK). Membranes were washed six times for 5 min in PBS (2mM NaH_2PO_4, 8 mM Na_2HPO_4, 0.58 M NaCl, pH 7.4, containing 0.05% (v/v) Tween 20 (wash buffer)) at ambient temperature. Membranes were blocked for 1h at ambient temperature in blocking buffer containing 0.01 M washing buffer with 5% (w/v) skimmed milk powder. After the blocking step, membranes

were washed six times for 5 min with wash buffer at ambient temperature and incubated overnight at 4°C with specific animal antibodies to peanut; Ara h1, Ara h3, Ara h2 and to gluten; mAb R5, mAb G12 and mAb 0610, diluted in blocking buffer (Table 1). Membranes were washed six times for 5 min with washing buffer at ambient temperature before incubating for 1 h with secondary anti-rabbit HRP conjugate diluted for peanut and egg allergens, and anti-mouse alkaline phosphatase-conjugate allergens diluted in blocking buffer for gluten proteins. Then membranes were washed six times for 5 min with wash buffer at ambient temperature. Peanut and egg blots were developed for 10 min using Super Signal West Dura substrate solutions diluted 1:1 (v/v), gluten blots were developed for 10 min using phosphate substrate solution diluted 1:2 (v/v) in milliQ water. Blots were rinsed with milliQ water and dried prior to imaging (Fujifilm LAS-1000, Fuji, Japan) (Smith et al., 2015).

Table 1. Summary of antibodies and substrate used in Immunoblotting of biscuits

Food allergens	Primary antibody	Dilution of primary antibody (blots) (v/v)	Secondary antibody	Dilution of secondary antibody	Substrate
Peanut	Ara h 1 (7S) Ara h 3 (11S)	1:5000	anti-rabbit HRP conjugate	1:10000	Super Signal West Dura
	Ara h 2 (2S)	1:1000			
Gluten	mAb R5 mAb G12 mAb 0610	1:100	Anti-mouse AP conjugated		Phosphatase substrate solution

3.5 Densitometric analysis

Densitometric analysis was carried out on selected polypeptides, reproducibly resolved as discrete bands on duplicate immunoblots, using ImageJ software (Schneider et al., 2012).

3.6 Size-exclusion liquid chromatography (SE-HPLC)

Polymeric proteins from wheat flour, roasted peanut flour and from all the samples of biscuits (control biscuits, 4% peanut biscuits ,and 20% peanut biscuits); were extracted following the two-step extraction procedure (Gupta, Khan, & MacRitchie, 1993), using two different extraction buffers.

Briefly, polymeric proteins were extracted from samples (0.1 g) with 1 mL of phosphate buffer (pH 6.8), containing 2% SDS, (Buffer A). The suspension was vigorously shaken for 30 min and then centrifuged for (10 min, 10,000 x g, 25°C). The supernatant represented the "SDS-Extractable Polymeric Proteins".

The pellet was re-suspended in 1 mL of the same buffer and sonicated 30s at power (300 W) (Microson Ultrasonic cell distrupter; Misonix Inc., Farmingdale, NY), ensuring that the samples were completely dispersed and then heated at 30°C for 30 min. After centrifugation (10 min, 10,000 x g, 25°C), the supernatant was recovered and contained the SDS-Unextractable Polymeric Proteins,, intending proteins extractable only with sonication".

The same protocol was applied using the same extraction buffer aided by a denaturing and a reducing agent, 2M urea, 3% dithithreitol (DTT) (Buffer B).

SE-HPLC was performed according to Johansson, Prieto-Linde, & Jonsson, (2001), using a liquid chromatograph Shimadzu HPLC (LC-20AP) equipped with a Phenomenex Biosep SEC-S4000 column (300 x 7.8 mm, Phenomenex, Torrence, CA, USA). The SE-HPLC column was calibrated using protein standards with a range of molecular weights (kDa) as follows: Cytochorme c (12.4), Carbonic Anhydrase (29.0), albumin, bovine serum (66.0), alcohol Dehydrogenase (150.0) and β-Amylase (200) (Sigma-Aldrich, Saint Louis, Missouri, USA).

The percentage of unextractable polymeric proteins (UPP) was calculated as described by Kuktaite, Larsson, & Johansson, (2004).

4. Preliminary results

As general features, the biscuits produced replacing wheat flour with increasing percentages of peanut flour were characterized by a darker colour (tendentially orange) and were softer than the control biscuits. Further details will be added to these results to complete the technological properties of these products (i.e heigh and diameter).

4.1 SDS-PAGE and immunoblotting results

In the figure 1 are shown the electrophoretic patterns obtained by SDS-PAGE of wheat flour (WF), roasted peanut flours (RPF) and biscuit control (CB), 4% peanut-biscuits (4PB) and 20% peanut-biscuits (20PB).

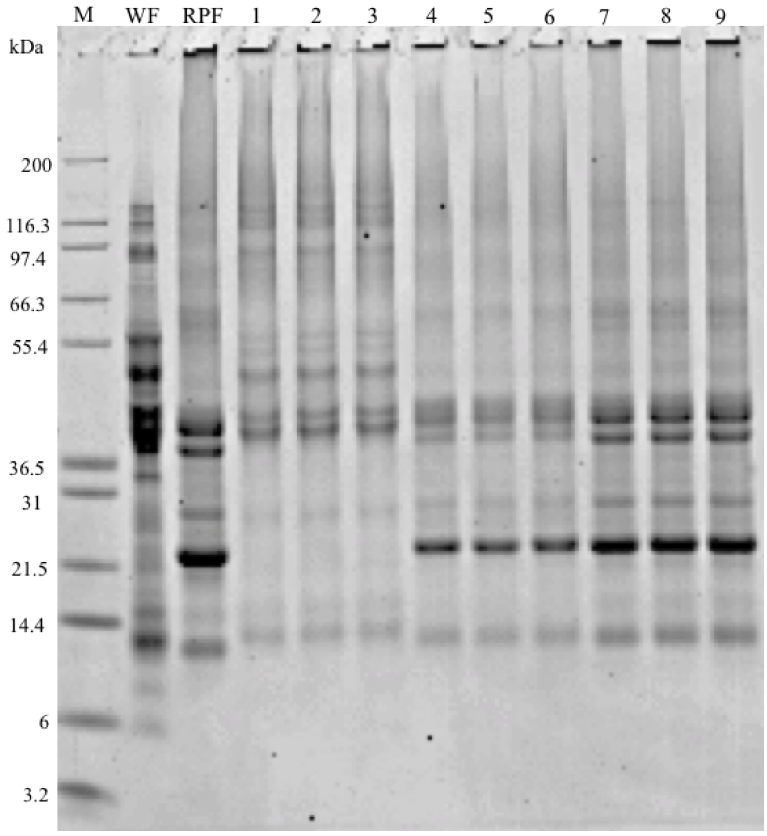

Figura 1. Reducing SDS-PAGE separation of total proteins extracted from wheat flour (WB) and roasted peanut flour (RPF) and biscuits. Lanes 1-2-3 show the total proteins of three indipendent Control Biscuits (CB); lanes 4-5-6: show the total proteins of three indipendent samples of 4%- peanut biscuits (4PB); lanes 7-8-9: show the total proteins of three indipendent samples of 20%- peanut biscuits (20PB); M: marker.

The electrophoretic profiles of wheat flour (WF) showed the expected bands of High Molecular Weight Glutenin Subunits (HMW-GS), included between 116 and 67 kDa, bands between 67 and 10 kDa, contained mainly Low Molecular Weight Glutenin Subunits (LMW-GS), gliadins (α, β e γ) and low molecular weight globulin and albumins (Shewry, 2009). As also expected, roasted peanut flour (RPF) showed the bands related to the main peanut proteins Ara h 1, and Ara h 3 (acid and basic subunits) and a less evident band related to Ara h 2 (Marsh et al., 2008; Johnson et al., 2016). The control biscuit (lanes 1-2-3) showed an electrophoretic profiles quite similar to its respective flour, with the evidence of high molecular weight bands and complexes, which did not enter into the resolving gel, as result of the baking process. Along the electrophoretic pattern of the 4%-peanut biscuit (lanes 4-5-6) the presence of faint protein bands, from both wheat and roasted peanut flours, was observed together with a more intense protein band with an estimated molecular weight of 22 kDa, deriving from the presence of the roasted peanut flour. In the 20%-peanut biscuits profiles, it was singular to observe the higher evidence of the peanut protein bands in contrast to the very faint protein bands related to the wheat flour (Fig. 1).

Changes in the immunoreactive polypeptides in the biscuits, where wheat flour was replaced with with different percentages of roasted peanut flour (4% and 20%) was assessed through the immunoblotting (Figure 2). As concerns the wheat protein, three antibodies were used: R5, which recognized the epitopes QQPFP, QQQFP, LQPFP, and QLPFP (Kahlenberg et al., 2006); G12, which recognized the epitope QPQLPY (Morón et al., 2008) which occurs three times in the 33-mer and IFRN 0610 monoclonal antibody, raised against the total glutenin fraction, which recognizes epitopes QQSF and QPFP, (Brett et al. 1999).

The figure 2 showed that the antibodies reactivity decreased, increasing the amount of peanut incurring in the biscuits. Indeed, the densitometric analysis on selected polypeptides, reproducibly resolved as discrete bands, showed, as general feature, a decreasing trend passing from the control biscuits (lane 1) to the 20%-peanut biscuit (lane 3), using as reference the densitometric values of wheat flour (WF). It was clear also from the blots, the presence of high molecular weight aggregates, whose polypeptides were recognized by the three antibodies, but whose intensity decreased in the case of 20%-peanut biscuits. This result could be explained as the consequence of the replacing of wheat flour with the 20% of peanut flour or as the effect of the interaction between gluten and peanut proteins, which causing a conformational changes, decreased the wheat protein solubility and/or reactivity.

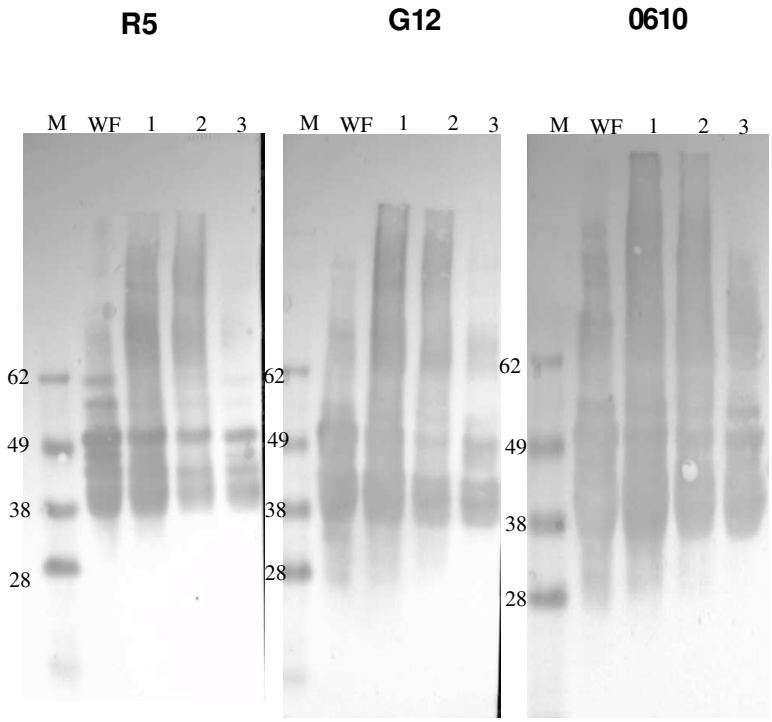

Figura 2. Immunoblotting with R5-G12-0610 antibodies

The image of the blots obtained using the antibodies against the main peanut proteins are shown in the figure 3. The immunoblotting was carried out considering the control biscuit (lane 1) as negative control sample and the peanut flour (PF) as positive control sample.

The Ara h1 protein, whose bands look diffused in the SDS-PAGE, was recognized in the 4%- and 20%-peanut biscuits, according to the amount of roasted peanut flour that was added. As expected, the total absence.of reactivity in the control biscuit lane (lane 1) was also detected. The recognition of the Ara h2 protein was observed only in the 20%-peanut biscuits (lane 3) as a faint band, beyond of a non-specific immunodetection in the control biscuit (lane 1) at the estimated molecular weight between 48 to 55 kDa, highlighting a possible problem of cross-reactivity of the used antibody. Finally, the Ara h3 immunodetection, as in the case of Ara h1, reflected the immunoreactivity of the positive control (PF), recognizing both the acid and basic subunits, according to the amount of peanut flour that was added.

These preliminary results allowed to hypothesize that the baking process and the addition pf peanut flour to the formulation, gave rise to a new gluten arrangement, which affected the protein solubility and immunoreactivity, according to the stochiometric ratio existing among the proteins of different nature involved in the aggregation event.

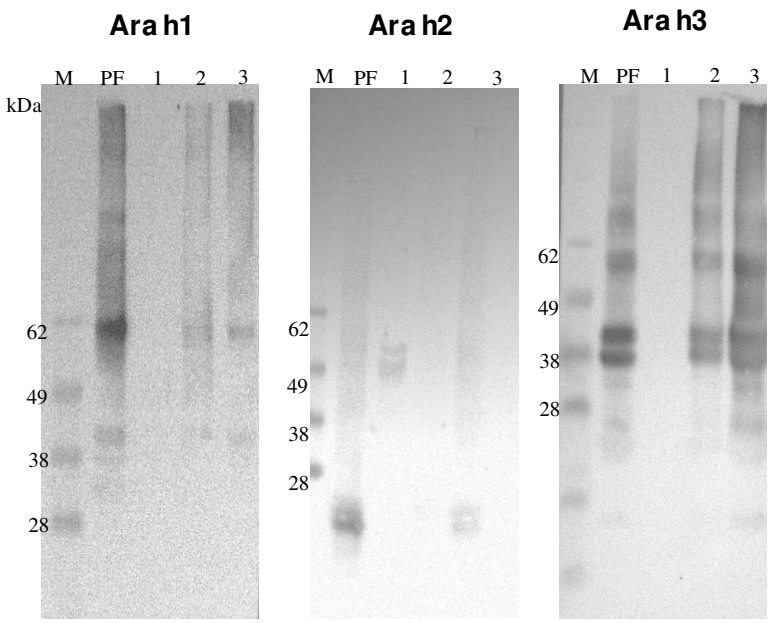

Figura 3. Immunoblotting with antibodies against Ara h1, Ara h2, Ara h3

4.2 SE-HPLC analyses and protein extractability of wheat and roasted peanut flours

The molecular weight distribution of the SDS-Extractable and SDS-Unextractable polymeric proteins was assessed using the two-step extraction procedure developed by Gupta et al., (1993) and evaluating the different protein extractability using two buffers: (1) 2% SDS-sodium phosphate buffer (50 mM; pH 6.9), the buffer described by the procedure, and (2) the same buffer aided with the addition of denaturing (Urea) and reducing (DTT) agents.

The differences in the flour and biscuits protein extractability, using the two extraction buffers, are shown in the Figure 4, Panel A-B and Figure 5, Panel A-B, respectively. The protein extractability was evaluated as the sum of the peaks area obtained from the chromatographic fractionation of the SDS-Extractable (SDS-EP) and SDS-Unextractable proteins (SDS-UP).

As concerns flour samples, the extractability of wheat flour remained almost the same with the two buffers (Fig. 4A and Fig. 5A), with slight modifications occurred in the chromatographic profiles, with the disappearance of the small monomeric protein peak (SMP, Peak 4 of the chromatographic profile, data not shown) (data not shown). As expected, a higher SDS-extractable fraction than SDS-unextractable was detected, being the formation of large unextractable protein aggregates an event promoted by the processing (Bruneel et al., 2010; la Gatta et al., 2017a, la Gatta et al., 2017b) and demonstrating that changing the buffer, just hydrophobic interactions and low energy bonds were disrupted. In the case of roasted peanut flour (RPF), the addition of the denaturing and reducing agents increased the protein extractability (Fig. 4A and Fig. 5A), changing the proportion

between the SDS-Extractable and SDS-Unextractable fractions, therefore, highlighting the involvement of disulfide and hydrophobic bonds in the protein polymers of this flour.

The extraction efficiency of the two buffers on bakery products, added other informations. For the three types of biscuits (i.e control, 4%-peanut biscuits and 20%-peanut biscuits) the use of the denaturing and reducing agents increased the protein extractability, with a more remarkable increase in the case of 20%-peanut biscuits samples. The extractability of control biscuits increased with the buffer (2), exihibitig a SDS-Extractable protein fraction higher than the SDS-Unextractable fraction. The 4%-peanut biscuits extractability, indeed, seemed to be less modified by the supplementation of the denaturing and reducing agents with respect of 20%-peanut biscuits, suggesting that the addition of the peanut flour affected the protein network arrangement, and, therefore, the protein extractability, as related to the stochiometric proportions.

As general features, the higher protein solubility with the buffer supplemented with denaturing and reducing agents confirmed the presence of hydrogen bonds and disulphide bridges and the greater efficiency of this buffer on biscuits also demonstrated that the formation of these types of crosslinks occurred during the bakery processing.

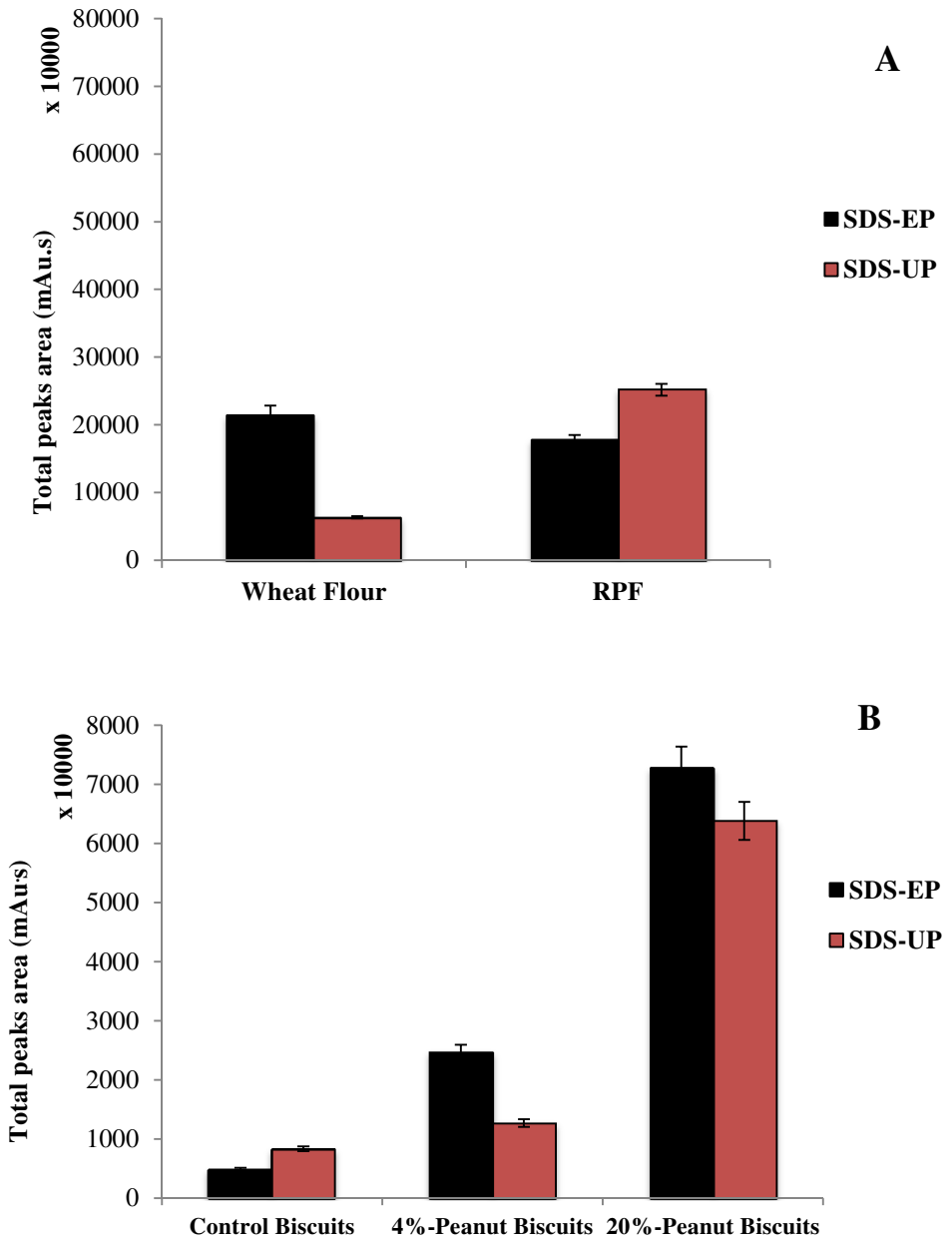

Figura 4. Protein solubility with 2%SDS-Sodium phosphate buffer of SDS-Extractable (SDS-EP) and SDS-Unextractable (SDS-UP) fractions of flour samples (Panel A) and baked products (Panel B)

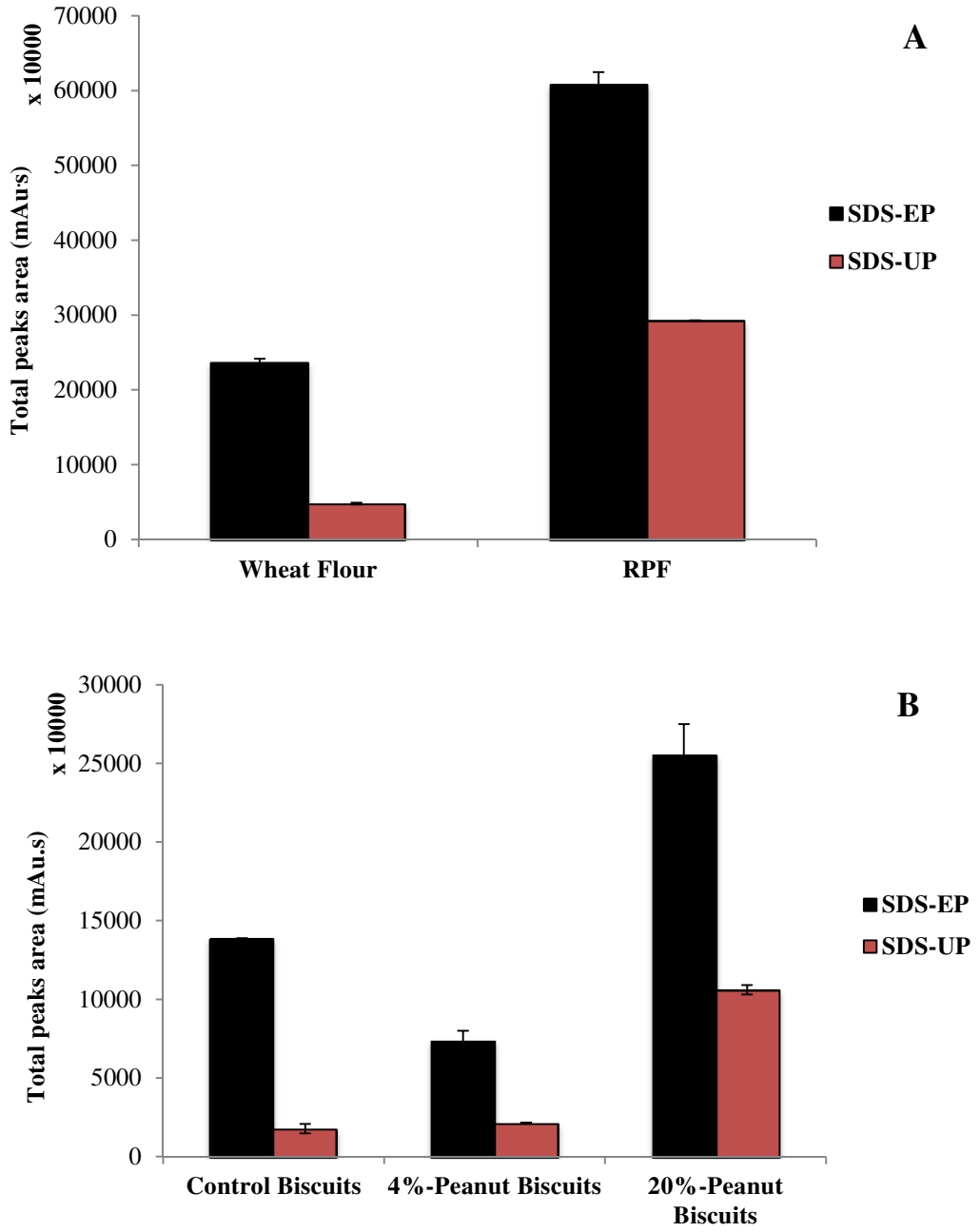

Figura 5. Protein solubility with 2%SDS-Sodium phosphate buffer aided with 2M Urea and 1.5% DTT of SDS-Extractable (SDS-EP) and SDS-Unextractable (SDS-UP) fractions of flour samples (Panel A) and baked products (Panel B)

To better understand the formation of large protein aggregates occurred during the bakery processing in the three different types of biscuits, we evaluated the amount of total unextractable polymeric proteins (tUPP) and large unextractable polymeric proteins (lUPP), which were expressed as percentages values and were shown in the Figure 6. The first index takes into account the first two peaks eluted in the chromatographic separation, evaluating the incidence of the high molecular weight unextractable peaks (peak 1 + peak 2) on the total extracted peaks (SDS-extractable + SDS-unextractable), as also indicated by the formula. It was clear, from our results, that the addition of peanut flour led to the formation of high molecular weight protein aggregates, as related to the percentage of the added peanut flour, being the value obtained from the 20%-peanut biscuits higher than that of 4%-peanut biscuits, which were, moreover, higher than control biscuits.

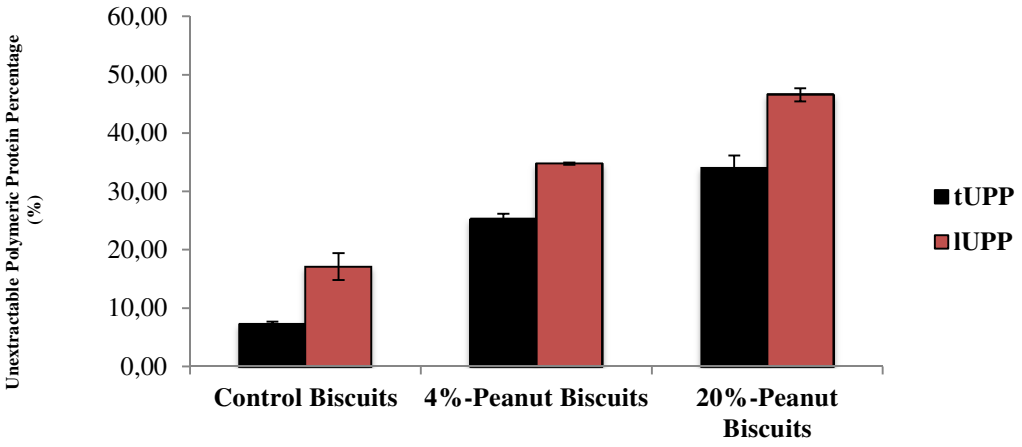

Figura 6. Total and Large Unextractable Polymeric Proteins (tUPP, lUPP) of the biscuits

These results were confirmed by the calculation of lUPP, which accounted for the formation of very large protein aggregates, considering just the area of the peak 1 (high molecular weigh peak) of the elution profiles. The different percentages of total (tUPP) and large unextractable polymeric proteins (lUPP), demonstrated as the proportion of gluten and non-gluten proteins can give rise to aggregates with different structures. The possibility that the protein aggregates arrangement in supramolecular structures can depend on the proportion and the nature of proteins allowed us to speculate on a hierarchical self-assembling of the aggregates, as also found by la Gatta et al., (2017) for gluten protein from wheat and einkorn flours mixturex in the pasta production.

5. Draft of the Discussion

The efficiency in the protein extraction is related to the changes in the protein structure due to the denaturation and/or chemical complexation/modifications phenomena, occurring within the food matrix, that can affect protein solubility (Poms & Anklam, 2004; van Hengel, 2007).

During the baking, protein denaturation takes place and, since the solubility of denatured proteins decreases with the increase of heating, the total protein extractability is expected to be affected by the physical–chemical characteristics of the extraction solution (Poms et al., 2004; Westphal et al., 2004).

Electrophoresis analyses showed a higher efficiency of extractability of roasted peanut flour proteins, since already with the addition of 4% of this flour, the wheat proteins seemed represented by a negligible fraction with respect to the more extracted peanut proteins. This results allowed us to suggest a structure made by a compact gluten protein network surrounded by peanut proteins linked through non covalent interactions in addition to the covalent bonds generated by the mixing and baking processes. Actually, the recognition of gluten protein epitopes decreasing from control to 20%-peanut biscuits, both for the decreasing amount and for baking process. This latter, producing a conformational changing, probably led to the high exposition of the amino acid sequences for the epitopes recognition in the control biscuits, whereas the incurring of peanut flour and the aggregation with gluten proteins should mask some of them. Observing the immunodetection of peanut allergens, Ara h1, Ara h2 and Ara h3, their recognition occurred in concomitance of peanut flour addition in the mixtures. Namely the 20%-peanut biscuits showed the highest band intensity for the three tested peanut allergens.

It was noteworthy that the most allergenic antigen, Ara h2, presented the lowest band intensity, also in the densitometric analysis of the immunoblots, despite of its major allergenic potentiality and even with the highest percentage of peanut flour added (20%).

On the basis of these considerations, our results could speculate that Ara h2, being characterized by eight cysteine residues, stabilised by disulphide bond and probably owing to its molecular weight, was not visible in the electrophoretic patterns of peanut biscuits mixtures, taking part to the formation of supramolecular gluten network and/or being embedded in it, with a possible decrease of immunoreactivity.

Equally interesting were the highest reactivity of the Ara h3 antibody and the intermediate behavior of Ara h1, which allowed us to image an architectural structure made by a central core comprised the supramolecular gluten proteins aggregates enveloped by the peanut proteins.

From the chromatographic results and by the percentages of the unextractable polymeric proteins, a different protein aggregation machinery, occurred in the baked products can be inferred, which was

related to the bakery processing, as main cause, and by the amount of the peanut flour that was added. Actually, Pareyt et al., (2010) already found that the baking process decreased the level of SDS-Extractable proteins, influencing also the characteristics of the biscuits, such as the diameter. The gluten protein properties determine the formation of a proper gluten network, which in turn may be affected by the introduction of a new element, as the roasted peanut flour.

Therefore, the assessment of the composition through mass spectrometry analysis of the protein aggregates could be able to provide for the nutritional properties of the products, through the detection of the involved proteins. This assessment on the eluted peaks is ongoing and further informations will be achieved.

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Session 2

Meat Products

New Details in Meat Protein Organization in Cooked Pork Products

In this session, the two shown papers deal with the meat protein organization in cooked pork products.

In a previous paper Di Luccia et al., (2015) assessed the protein modifications in cooked pork products evaluating, the completely different protein organization in whole anatomicul cuts (Cooked hams) and comminuted products (Mortadella and Wurstel) through a proteomic approach, with the detection of a great amount of insoluble residue in the emulsioned products. Some of the results of this work were reported in a chapter of the book *Proteomics in Food Science – From Farm to Fork*, where more informations were developed.

A deepening on the protein organization in cooked meat products was carried out, evaluating the molecular weight distribution of protein aggregates through size-exclusion liquid chromatography (SE-HPLC) and the composition of these aggregates with tandem mass-spectrometry (LC-MS-MS) and monodimensional gel electrophoresis (SDS-PAGE).

This approach allowed to better understanding the differences in the protein arrangement occurring in cooked pork products with respect of the raw material, as a consequence of the heat treatment and to ponder how the formation of heat-induced supramolecular structures can influence for the texture properties of cooked pork products.

Publications

- Di Luccia, A., la Gatta, B., Rutigliano, M., Rusco, G., Gagliardi, R., & Picariello, G. Protein Modifications in Cooked Pork Products. Michelle L. Colgrave (Ed.*). Proteomics in Food Science – From Farm to Fork*, 2017, (pp. 199–214). Elsevier Inc.
- Rutigliano, M., Picariello, G., Trani, A., Di Luccia, A., la Gatta, B. (2019). Protein aggregation in cooked pork products: new details on the supramolecular organization. Food Chemistry, 294, 238 – 247.

Paper 1: Protein modifications in cooked pork products

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Abstract

Heat treatment induces protein modifications in both cooked whole anatomical cuts and in emulsioned sausages. Heat treatment results in shrinkage and water loss, protein rearrangements, oxidation of amino acid residues and polymeric aggregation. The proteomes of whole-cut meats (*e.g.* Parma and ‘‘Praga’’ cooked hams) and comminuted pork (*e.g.* mortadella and würstel) products have been compared to raw pork using traditional proteomics approaches employing two-dimensional gel electrophoresis (2-DE) coupled to image analysis and mass spectrometry (MS). These studies revealed that besides the heat-induced breakdown of a part of the myosin heavy chains, the 2-DE pattern of cooked ham was highly similar to that of raw pork. However, MS-based analysis showed protein modifications, including extensive oxidation of methionines. By contrast, likely due to emulsification, comminuted sausages were characterized by an abundant insoluble protein fraction. Interestingly, in comminuted sausages, tropomyosin and myosin light chains were exclusively found in the insoluble protein fraction. Experimental evidence showed that the protein aggregation systems of cooked hams and emulsion sausages reflected the processing conditions and were definitely different, the former being characterized mainly by disulfide bridges and the latter by additional covalent inter-protein links.

1. Introduction

Muscle proteins (~20%) represent the primary constituents of meat and are classified on the basis of their solubility. The myofibrillar proteins are salt-soluble (~45–55%), the sarcoplasmic fraction is water-soluble and comprises the main metabolic enzymes (~30–35%) and the connective tissue (or stroma) proteins are soluble in dilute acidic solutions (~10–15%). Their physiological role is summarized in Table 1.

In raw meat muscle proteins are organized into supramolecular structures via self-assembling and self-organization machinery that acts to build a soft scaffold to protect muscle cells and to permit the physiological mechanisms that convert chemical energy from nutrients into mechanical energy. Such architectonic supramolecular structures are necessary for the contractile properties of the monomeric unit, known as the sarcomere (Figure 1).

Table 1. Physiological role of the main meat proteins

Proteins		Characteristics	Function
Myofibrillar	Myosin	Two large polypeptide chains, "heavy chains" (200 kDa) and three small chains "light chains"	Contractile action
	Myosin Light Chain I	Light chain isolated with alkali treatment (20.7-25 kDa)	
	Myosin Light Chain II	Light chain isolated with 5.5'-dithiobis(2-nitrobenzoic acid) (19-20 kDa)	
	Myosin Light Chain III	Light chain isolated with alkali treatment (16-16.5 kDa)	
	Actin	G-actin:globular actin polymerizes to form F-actin:fibrous actin	
	Tropomyosin	Regulatory protein (66-68 kDa) composed of two polypeptide: α- chain (34 kDa), β-chain (36 kDa)	
	Troponins C, I, T	Protein involved for the regulation of contraction-relaxation processes. TnC binds Ca^{++}, TnI inhibits actomyosin ATPase; TnT binds tropomyosin.	
	α- and β-actinin	α-actinin (95 kDa) and β-actinin (34-37 kDa) bind actin	
	M-protein	Binds to myosin (165 kDa)	
	C-protein	(140 kDa)	
	Titin	Massive Protein. Molecular weight of around 1 million Dalton.	Scaffold proteins
	Desmin Nebulin Vimentin Synemin	Intermediate filaments. These proteins represent longitudinal intrafibrillar and trasverse interfibrillar bridges.	
Sarcoplasmic	Glyceraldehyde phosphate dehydrogenase Aldolase Creatine kinase	Enzymes of the glycolytic pathway	Energetic and other globular cytoplasmic proteins
	Myoglobin	Myoglobin (17 kDa) stores oxygen in muscle cells, it is a pigmented protein.	
Connective tissue	Collagen	Type I (epimysium) Type II and III (perimysium)	Fibre enveloping

	Type III, IV and V (endomysium)
Reticulin	Collagen Type III
Elastin	Fibrillin (Glycoprotein)

The conversion of muscle to meat is due firstly to the enzymatic action of cytoplasmic enzymes, m- and μ-calpains, and then, with pH diminution, to the action of lysosomal enzymes, the cathepsins. These enzymes act to break the supramolecular structure of sarcomere weakening the Z-discs and destroying the anchoring of the actomyosin complex and myofibrillar proteins (Figure 1). Biochemical and structural changes increase the tenderness of meat in turn affecting quality attributes of meat palatability such as flavour and juiciness traits (Maltin et al., 2003; Calkins and Hodgen, 2007). Consumer behaviour has had implications in terms of both food safety and the development of better sensory attributes (Mottram, 1998). The first attempts at food processing by cooking meat is thought to have originated with primitive humans that savoured roast meat accidentally produced by forest fires. Cooked meat was found to be more palatable and easier to chew and digest. The consumption of cooked meat thus began with the ability to generate and control fire thus contributing to human evolution. Successive animal domestication and breeding allowed humans not only to consume the meat but to store the meat by producing both dry cured meats and cooked meat (Civitello, 2007).

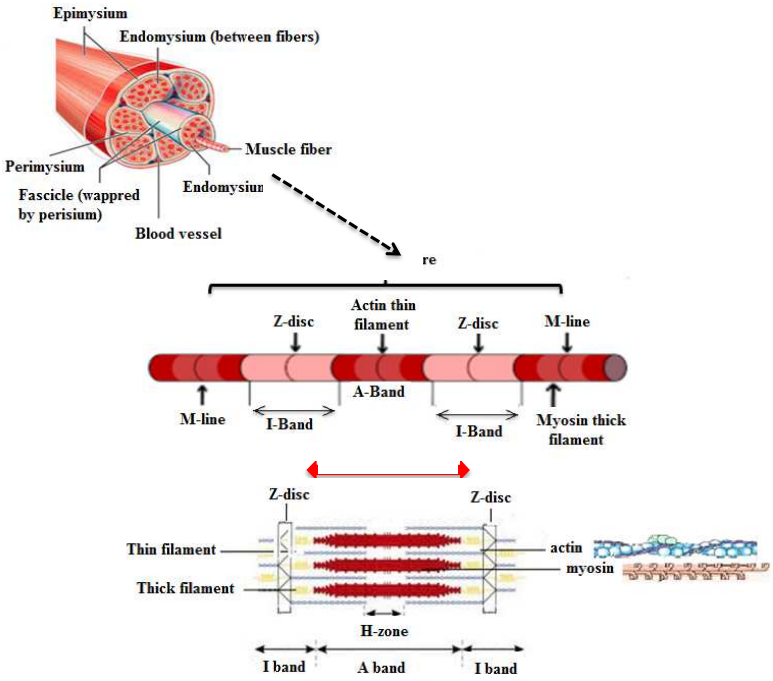

Figure 1. From supramolecular architecture of myofibril to the proteins of contractile monomeric unit, sarcomere.

Heat treatment induces unavoidable modifications to the structural arrangement of the main meat constituents (Barbera and Tassone, 2006; Tornberg, 2005), especially proteins as a direct consequence of degradation, denaturation, oxidation and polymerization events (Barbieri and Rivaldi, 2008; Gatellier et al., 2009). When heat is applied to meat, two general changes occur: the muscle fibres become tougher and the connective tissue becomes tenderer. Specifically, actin, myosin and other muscle fibre proteins undergo significant changes. The polypeptide chains composed of amino acids (the basic components of proteins) unfold, in a process termed denaturation, and may undergo structural rearrangements such as coagulation. The result of this process is shrinkage, moisture and fat loss, and toughening of the muscle fibre. The tenderizing effect of moist heat on connective tissue results from the conversion of collagen to gelatin. The extent to which these changes occur in a piece of meat depends on both the time and temperature of cooking.

The denaturation of the muscle proteins influences the structural characteristics (Tornberg, 2005) and the water distribution of the meat (Bertram et al., 2006). Such structural changes lead to substantial loss of water (cooking loss) in the range of 15 to 35%. However, the amount of cooking loss is highly dependent on the cooking method, the cooking time/temperature and the end-point temperature (Aaslyng et al., 2003). Thermographic studies of meat have shown that protein denaturation generally takes place in three steps: (1) myosin (rod and light chain) denaturation occurs at ~40–60°C; (2) denaturation of sarcoplasmic protein and collagen occurs at ~60–70°C; and finally (3) denaturation of actin occurs around 80°C (Deng et al., 2002; Stabursvik, et al., 1984; Stabursvik and Martens, 1980; Wright et al., 1977; Wright and Wilding, 1984).

Bertram et al., (2006) found that denaturation of specific muscle proteins resembled the change in water characteristics during cooking. Thus, the greater changes in water characteristics took place between 40°C and 50°C, which suggested that the denaturation of myosin heads was important for subsequent cooking loss. Moreover, the denaturation of myosin rods and light chains at ~53–58°C also affected the water distribution, whilst the denaturation of actin at ~80–82°C was correlated directly to expulsion of water from the meat (cooking loss). Straadt et al. (2007) demonstrated pronounced shrinkage of fibres upon cooking giving rise to large gaps between the cooked muscle fibres, as well as at the level of the individual myofibrils.

During shrinkage the triple-stranded helix is destroyed to a great extent and the decreased dimensions of the fibres and retractable strength is directly related to the number of crosslinks formed (e.g. hydroxylysinorleucine, dihydroxylysinorleucine, histidinohydroxymerodesmosine, pyridinoline). Concomitantly, water expulsion from the myofibrillar matrix takes place and this was postulated to be one of the mechanisms responsible for cooking loss given by Bertram et al. (2006)

and Micklander et al. (2002). Shaarani et al. (2006) identified three distinct water populations in raw meat: free water located in the sarcoplasmic area (Honikel, 1988) that could be easily mobilised e.g. through minor physical forces formed upon shrinking of myofibrils at the time of rigour mortis; immobilized water that was bound either by steric effects of attraction between the filaments or hydrogen bound with muscle proteins or other macromolecules; and water that was closely associated with macromolecules.

In cooking meat or its products the reorganization of protein structures and water holding properties have, unquestionably, a greater importance for their commercial value and consumer acceptance. In this chapter we explore the disruption of the supramolecular structure of muscle proteins and the rebuilding of a different supramolecular architecture by self-assembling and self-organization in a hierarchical fashion, as a consequence of the heat treatment.

2.1 The history of common pork products.

Two different models of cooked meat products are generally manufactured, from a whole anatomical pork cut (*e.g*. leg or shoulder) and comminuted pork pieces from different anatomical cuts. Cooked hams from leg or shoulder preserve the structural organisation of anatomical cut, whilst mortadella and würstel lose the muscular filamentous network due to extensive grinding resulting in particles of size less than 0.9 mm.

The origin of cooked ham dates back to the mid-fifteenth century, as quoted noted in Libro de coquinaria written by Martino of Como (circa 1465), where it was obtained from the hind limb of a pig, from which the fat was removed, cut, boned, massaged, processed and, finally, steamed. ‘‘Praga’’ ham is a related specialty from the Trieste area of central Europe, which formerly belonged to the Austro-Hungarian Empire. For this style of ham, the cooking stage is carried out in special hot-air ovens rather than by exposure to wet steam. Following this, the smoking stage is entirely natural and is based exclusively on beech wood.

Cooked hams are produced by heating in an oven to a core temperature of 70°C, following brine injection and tumbling. Generally, a cooking time of 1 h per kg of meat product is required, which means an overall time of 10–12 h. One of the most important quality attributes of cooked ham is the juiciness, therefore, the cooked ham production starts with the choice of raw ham piece with higher pH values and ionic strength which are associated with better water-holding capacity (Puolanne et al., 2001; Puolanne and Halonen, 2010). The water-holding capacity is linked to the pH of the ham; pH values within the range 5.8 to 6.2 may assure good water retention.

Comminuted meat products include mortadella di Bologna, a typical Italian cooked sausage, and würstel, a sausage manufactured worldwide. The origin of the word ‘‘mortadella’’ is somewhat

controversial, but one of the most reliable hypotheses is that it derives from the late Latin ‘‘mortarium’’, which described the mortar and pestle, in which the friars in Bologna (Italy) prepared the mixture of pounded meat mixed with fat and spices. Nowadays, comminution is accomplished by grinders that reduce the granulometry of the meat to less than 0.9 mm and the cooking is performed in stages (drying, pre-cooking, firing and second firing) with temperatures as high as 80°C for 19–20 h overall (Barbieri et al., 2013). The würstel is linked to the butcher Johann Georg Lahner, who in 1807, invented the frankfurter sausage that gradually spread to the entire Austro-Hungarian Empire (Lahner, 1969). Würstels are cooked at variable times and temperatures, typically for about two hours or until the whole product reaches a temperature of 70°C. Following this, the sausages are traditionally smoked with beech wood, to give them a characteristic flavour. Therefore, the heat-induced effects on the structural and conformational arrangement of the muscle proteins within these products are expected to be different.

2.2 Effect of heat treatment on meat proteins in cooked pork products.

An undesirable effect of heat on the cooked meat processing is the shortening of fibres. The cooking process increases the intramuscular connective tissue contribution to toughness, in the range 20–50°C and only above 60°C is there a prominent myofibrillar contributions (Bendall and Restall, 1983). The transverse shrinkage of the fibre starts at 35–40°C and then increases almost linearly as a function of temperature and at 65–80 °C shrinkage of meat is visible through a volume reduction in the muscle fibres, increasing their toughness (Lepetit et al., 2000). Therefore, the shrinkage of the connective tissue per se starts at 60°C and around 65°C it contracts more intensely. However, the amount of shrinkage varies substantially from about 7% area up to 19% area. The discrepancy in results could, among other things, be due to large biological variation within a muscle and among different muscles. The shrinkage phenomenon is strongly reduced by applying pressure to the entire meat cut in a mould or to finely minced meat pieces. Through application of pressure a linear structure similar to that of native collagen (triple-stranded helix) is maintained thus avoiding the shortening phenomenon that is due to denaturation and the consequent restructuring of the random coils in the collagen network. In highly comminuted meat products, collagen fibres are already broken such that the shrinkage is not perceived.

In addition, heat treatments and processing result in decreased solubility of the sarcoplasmic proteins in cooked ham and comminuted products, which are attributed to the increased hydrophobicity that occurs as a consequence of heat denaturation and coagulation (Promeyrat et al., 2010). This is caused by destruction of the hydrogen-bonding and/or electrostatic interactions at high temperatures wherein the orientation of the dipole of water is disturbed, destabilizing the

protein–water solvation system that exists in the ‘‘native’’ state. The conformational transitions and the exposure of hydrophobic amino acids, which, in normal conditions, are hidden within the inner core of the globular proteins (Chelh et al., 2007; Santé-Lhoutellier et al., 2008), promote the aggregation of proteins due to hydrophobic interactions. Davey and Gilbert (1974) found that heat aggregation of sarcoplasmic proteins could occur up to 90°C, and they suggested that during cooking, sarcoplasmic proteins might contribute to the consistency of the meat by forming a gel that links together several structural elements.

3. Proteomic studies of cooked meat products

Most of the proteomic investigations and related studies have examined the change in protein pattern during the transformation of fresh pork meat either to cooked or dry cured ham (Di Luccia et al., 2005; Di Luccia et al., 2015; Barbieri and Rivaldi 2008; Pioselli et al., 2011; Paredi et al., 2012).

Cooked hams and emulsion sausages represent two different models of cooked meat products. As noted previously, the former are generally manufactured from a whole anatomical cut (leg), that preserves the structural arrangement of the muscle and the latter are produced with comminuted pork meat pieces from different anatomical cuts. Therefore, the heat-induced effects on the structural and conformational arrangement of the muscle proteins are expected to be different. A more recent study confirmed this expectation and showed different patterns of protein modifications as a result of heat treatments and processing (Di Luccia et al., 2015). One of the main observations in this work was the almost complete insolubility of sarcoplasmic proteins in cooked ham and comminuted products, which was attributed to the increased hydrophobicity, as a consequence of heat denaturation and coagulation (Promeyrat et al., 2010).

The two-dimensional electrophoresis (2-DE) map of the muscle proteins from raw pork and cooked samples is shown in Figure 2.

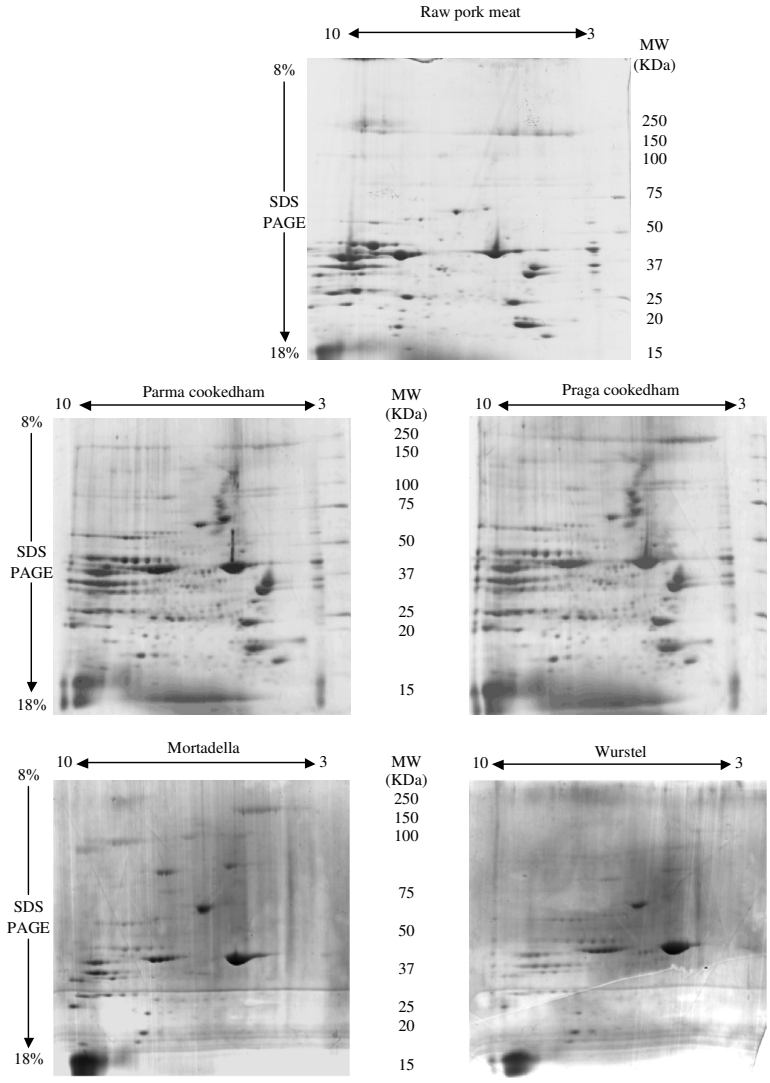

Figure 2. 2-DE analysis of meat proteins extracted with denaturing and reducing buffer (DRB) from raw meat, cooked hams and emulsion sausages (I extraction). (From Di Luccia et al., Food Chemistry 172 (2015) 447–455).

As expected, myofibrillar proteins migrated within the acidic side of the 2-DE map, whereas sarcoplasmic proteins tended to be spread towards the alkaline side, above pH 7.0. Despite the tumbling and heat-treatments that cooked hams undergo, the main myofibrillar and sarcoplasmic proteins are characterized by a higher total number of detectable protein spots (211 and 242 respectively) compared to raw pork (156). In the case of emulsion sausages protein extract is characterized by a very limited number of detectable spots (70 and 68 respectively) and surprisingly, myofibrillar proteins such as TPMs, myosin light chains (MLCs), and other minor proteins involved in muscle contraction were almost completely missing. Table 2 shows the total number of spots (*ns*) for the various cooked products, the percentage of variation compared to raw pork, and the total volume of the spots. It is noteworthy as the values of *ns* for Parma and ‘‘Praga’’ cooked hams represent 135% and 156%, respectively, of the raw pork *ns*; the values of *ns* of mortadella and würstel were 45% and 44% respectively, compared to the raw pork. The total

volumes increased as the number of spots increased. These finding were defined by the variation of spot density as the ratio (D_{ns}/|DV|), where D_{ns} is the variation in spots and |DV| is the absolute value of the variation in the volume, derived from the image analysis. The variation in spot density determines the degree to which the protein changes in the cooked products with respect to raw pork. The variation of spot density was negative for emulsified sausages and positive for cooked hams. Spot matching, as assessed by image analysis, showed the similarities and differences between the cooked products and raw pork. Parma and ‘‘Praga’’ cooked hams matched raw pork for 58% and 53% of the spots respectively, while mortadella and würstel matched for 22% and 21% respectively. The sarcoplasmic proteins of emulsion sausages also occurred at lower spot numbers and intensities than in cooked hams (Figure 2). These qualitative and quantitative differences in the 2-DE protein patterns depended on a series of factors, including: (i) decrement of the stain intensity of sarcoplasmic spots that made it easier to single out individual proteoforms within a contiguous train of spots; (ii) the appearance of newly-formed spots around pI 7.0 in cooked hams, which probably arose due to the heat-induced breakdown of large protein chains (*e.g.* myosin); and (iii) the detection of additional spots in the 50–100 kDa MW range, probably due to partial protein aggregation.

Table 2. Spot identified from the 2-DE gel electrophoresis of total proteins obtained from first extraction by Image Master analysis. (From Di Luccia et al., Food Chemistry 172 (2015) 447–455).

Samples	**Number of total spot. (n_s)**		**Total volume (V_t)**	**n_s/*V_{trp}·10^{-3}**
Raw pork	156	100	87383.47	0,00
Parma cooked ham	211	135	116648.36	1,85
Praga cooked ham	242	156	116752.10	2,90
Mortadella	70	45	35920.16	-1,67
Würstel	68	44	34399.42	-1,66

*V_{trp}= total volume of raw fresh pork sample

The missed detection of several expected major myofibrillar and sarcoplasmic proteins in the 2-DE of emulsion sausages (Figure 2) suggested that they probably constituted an insoluble protein fraction (IPF). Indeed, the 2-DE analysis, aided by ultrasonication, of cooked pork products that were extracted by a denaturing and reducing buffer (DRB), revealed several additional proteins for mortadella and würstel (Di Luccia et al., 2015) and, to a lesser extent, for cooked hams. In particular, the analysis of the DRB fraction of proteins extracted from emulsion sausages showed the presence of intense spots of both sarcoplasmic and myofibrillar proteins, primarily actin. Sequential extraction by DRB (extraction I) and ultrasonication-aided DRB (extraction II)

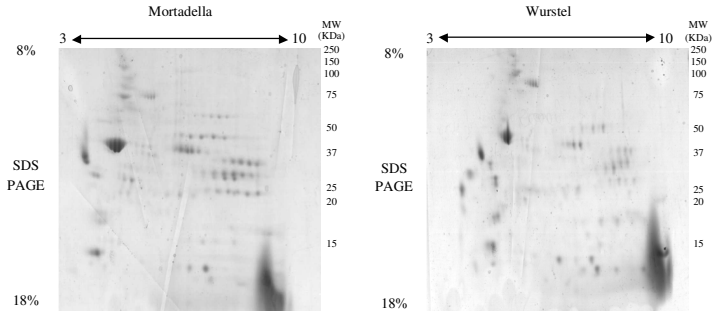

Figura 3. 2-DE analysis of proteins obtained from sonication-aided denaturing and reducing buffer (DRB) of mortadella and würstel (II extraction). (From Di Luccia et al., Food Chemistry 172 (2015) 447–455).

showed that ultrasonication increased the ratio of myofibrillar-to-sarcoplasmic *ns* values (M/S) from 0.23 to 0.93 and from 0.17 to 0.88 for mortadella and würstel, respectively. However, the total spot volumes were stunningly different between the two products, the values for mortadella being much higher (Figure 3). The percentage contribution of actin to the total volumes also increased more than twice with ultrasonication (extraction II), indicating that it has an important role in the formation of soluble and insoluble protein aggregates in emulsion sausages. The different ratios and compositions of the two protein fractions reported by Di Luccia et al. (2015) suggested that insoluble protein fraction aggregates were formed by non-reducing covalent cross-linking and differed both qualitatively and quantitatively between the two emulsion sausages. These findings indicated that the meat preparation and heat treatment of the production processes induced the formation of peculiar gel networks due to protein aggregation, and this was mainly through the mechanisms of coagulation, denaturation, oxidation, and inter-chain cross-linking of polymers.

Other proteomic studies performed to define the quality and optimize the processing parameters through the identification and monitoring of molecular markers, Barbieri and Rivaldi (2008) and Pioselli et al., (2011) analysed the exudate during the manufacturing of cooked ham. The exudate is formed due to the injection of brine, extensive tumbling and increased temperature. Basically, three steps are crucial to this process (Barbieri and Rivaldi, 2008): (i) formation of an exudate rich in myofibrillar proteins; (ii) the formation of a network at a temperature near 62°C; and (iii) the loss of exudate in cooking subsequent to the formation of a myofibrillar matrix. These authors identified desmin in the exudate as a marker of the heat-induced modifications. Desmin steadily increased in any sample of the exudate of the cooked ham sample, but it decreased or even disappeared once the meat structure reached a steady state, and the cooking process could be stopped. On the other hand, the lack of desmin in the exudate indicates that the meat is not sufficiently cooked, or that the meat structure is too strong for a good network to be formed. In the cooking phases of production, raw

ham and raw pork undergo changes. Muscle meat proteins denature which causes deep structural changes, such as the destruction of cell membranes (Rowe, 1989), the transversal and longitudinal shrinkage of meat fibres, the aggregation and gel formation of sarcoplasmic proteins, and the gelation and solubilization of connective tissue (Tornberg, 2005). To avoid shrinkage and fibre separation, and to obtain homogenous products, the application of pressure on the pork leg is necessary during cooking in order to ensure that the muscle fibres cohere by gel formation, due to the denaturation of proteins and the solubilization of connective tissue. In a system in which the fibres are forced to remain in a firm structure, an increase in temperature first causes myosin heavy chain (MHC) denaturation, followed by an increase in emulsification, sarcoplasmic coagulation and successive incorporation within the myofibrillar network. Finally, the fibres adhere to each other due to a collagen gelation that acts like glue. The result is a compact and homogenous product in which the fibres are no longer distinguishable. In this case, protein networks are generated mainly through disulfide links and hydrophobic interactions, as demonstrated by a very small insoluble residue obtained from total protein extraction in the DRB. This also explains the progressive decrease of myofibrillar proteins in the exudate from ham during the cooking process.

Emulsion sausages (*e.g.* mortadella and würstel) lack the fibre structure that serves to increase the solubilizing effect on the muscle proteins. Moreover, the process of comminution results in incorporation of air into a system that is composed primarily by fat, proteins and water. In such a system, the conditions for generating both metal-catalysed and myoglobin/H_2O_2 oxidative phenomena, that underlie the formation of reducible and non-reducible link formations, exist (Bhoite-Solomon et al., 1992).

The dissolution of the myofibrillar protein into the water-phase during comminution and blending represents the most important factor for judging the quality of the protein network and the structure of the emulsion sausage. Solubilization and heat denaturation of the myofibrillar and soluble sarcoplasmic proteins leads to a protein solution with emulsifying properties that acts to conglomerate the exposed hydrophobic residues with fats, and is also prone to oxidation. These events underlie the formation of aggregates and, likely, the polymeric protein networks. With extraction using a denaturing and reducing buffer, we obtained larger amounts of protein residue from the emulsion sausages than from the cooked hams. Moreover, the complete solubilisation of the aggregate by breaking the disulfide bonds (Singh et al., 1990) and, probably, other covalent bonds was achieved by combining the use of sodium dodecylsulfate (SDS) with sonication. This explains the different 2-DE patterns of proteins obtained from the total protein extract and from the IPF supporting the existence of two types of aggregation in the gel matrix residue, one

characterized by the presence of actin alone and the other with interactions between actin and the remaining myofibrillar proteins and some of the associated sarcoplasmic proteins.

4. Spot identification by MALDI-TOF MS

Protein identification has been also accomplished in previous 2-DE analysis (pI range 3–10) for muscle proteins from different mammals (Bendixen, 2005). We note the occurrence in cooked pork products of a neo-formed series of spots in the range 65-110 kDa; this is particularly evident in the 2-DE maps of cooked hams. They were identified as α-actinin, MHC and BSA. The MHC spots migrated at similar pIs, but with lower MW than the parent protein, which indicated an extensive cooking-induced breakdown of the chain during the cooking process. Interestingly, almost all of the tryptic peptides of the MHC fragments mapped to the central and C-terminal protein region, indicating that the 2-DE spot arose from the fibrous (C-terminal) region of myosin. On the other hand, the N-terminal heads (up to the actin-binding domains) of MHC are for the most part engaged in the formation of the coalesced heat-induced gel networks, and this could be why they are resistant to extraction (Sharp and Offer, 1992; Tornberg, 2005). The majority of the other identified spots corresponded to intact protein chains. This was also established by comparing the theoretical and experimental 2-DE coordinates (MW/pI), which demonstrated that heat-induced proteolysis is a rather uncommon event and affects MHC almost exclusively. Many of the methionine residues were partially oxidized, as demonstrated by MALDI-TOF MS mapping (Figure 4) of the tryptic peptides of actin (Di Luccia et al., 2015).

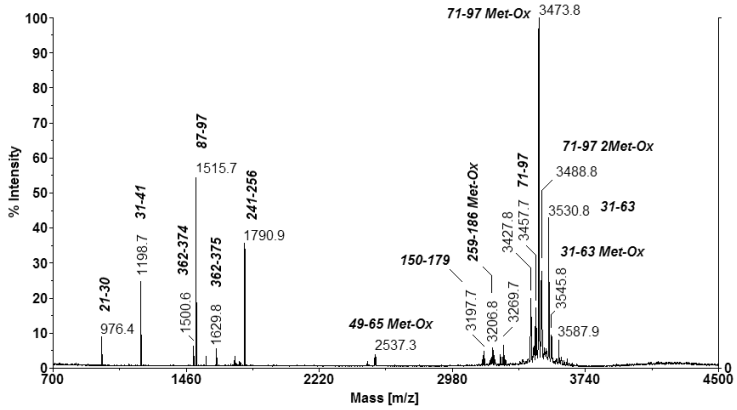

Figure 4. MALDI-TOF spectrum of Actin. Met-Ox indicates peptides containing oxidized methionine.

5. Supramolecular rebuilding of meat proteins in cooked pork products

During cooking, muscle proteins undergo conformational changes and alterations to their aggregation state determined by the cooking method. The transition from a native conformation to an unfolded one in the two models (whole anatomical cuts and comminuted emulsified sausages) involves two different self-assembling mechanisms resulting in a new architectural network. Molecular self-assembly is a powerful approach to build up novel supramolecular architectures. Supramolecular self-assembly is mediated by weak non-covalent bonds, notably hydrogen bonds, ionic bonds (electrostatic interactions), hydrophobic interactions, π-π stacking, van der Waals interactions and water-mediated hydrogen bonds. Proteins interact and self-organize to form well defined structures that are associated to their intrinsic ability to respond to environmental conditions.

The supramolecular assemblies determine a greater network with an architecture that includes meat proteins. This example also illustrates that different quaternary structures can be generated from the same biopolymer through small changes linked to the environmental conditions. Understanding the changes to the induced-structure in meat proteins is important for the production of meat products. On heating, a filamentous network is formed where each strand is composed of myosin filaments, however, recently Di Luccia et al. (2015) found that actin plays an important role in this supramolecular filamentous network.

Protein aggregation from the denatured proteins involves the formation of higher molecular weight complexes as supramolecular structures (Schmidt, 1981) that are stabilized by cross-links formed at specific sites on the protein chains or by non-specific bonding occurring along the protein chains. Protein-protein hydrophobic interactions are usually the main cause of subsequent aggregation (Cheftel et al., 1985) providing that the protein concentration, thermodynamic conditions, and other conditions favour the formation of a new tertiary architecture (Schmidt, 1981).

Cross-linking of protein aggregates, following denaturation, usually involves oxidative chemical reactions of proteins resulting from the covalent interaction of their functional groups. In fact, oxidative protein cross-linking can involve different amino acids: basic and aromatic amino acids and cysteine are particularly prone to reacting with free radicals during the cooking process (Petruk et al., 2012; Santé-Lhoutellier et al., 2008). Basic amino acids are oxidized to generate carbonyl products (Stadtman and Levine, 2003; Uchida, 2003). The thiol groups of cysteine can be oxidized with the formation of disulfide bridges (Lund et al., 2011). Tyrosine in myofibrillar proteins can generate dityrosine cross-links (Gerrard, 2002; Lund et al., 2011) and the combination of thiol radicals can lead to thiol-tyrosine bonds (Lund et al., 2008; Martinie et al., 2012; Petruk et al., 2012).. Finally, the interaction of reactive amino acids, such as lysine and cysteine, with

(bifunctional) aldehyde products arising from lipid oxidation can also induce protein networking through the formation of Schiff bases (Gardner, 1979; Refsgaard et al., 2000). In cooked meat products such as comminuted cooked sausages and cooked ham (Estévez et al., 2005; Estévez and Cava, 2004, 2006; Estévez et al., 2007; Sun et al., 2010) the extent of protein carbonylation was also investigated. Carbonylation is an irreversible and non-enzymatic modification of proteins that involves the formation of carbonyl moieties induced by oxidative stress and other mechanisms (Berlett and Stadtman, 1997). These modifications may be directly derived from oxidative damage to proteins and/or by inducing conformational changes leading to denaturation (Xiong, 2000).

The presence of these various links results in the formation of larger aggregates or/and insoluble polymeric protein networks. In the light of these results, it is possible to hypothesize that the cooking of a whole anatomical cut or of comminuted pork pieces generates different aggregates and/or insoluble polymeric protein networks.

Cooked ham, from whole anatomical cuts, maintains the supramolecular architecture as raw meat but the protein denaturation, caused by heat treatment, rearranges meat proteins during cooked meat processing (Di Luccia et al., 2015). In emulsion sausages, the comminution causes mechanical disruption of the native supramolecular organization, reducing fibre dimensions before protein denaturation through heat treatment can reorganize the large aggregates as supramolecular structures (Figure 5). As matter of fact, in both cases, of whole and comminuted cuts, the disrupted structure of stroma and myofibrillar proteins existing as random coils, which are soluble in water, is called gelatin. However, the response to heat treatment is dependent on the chemical and physical properties of the meat protein fraction.

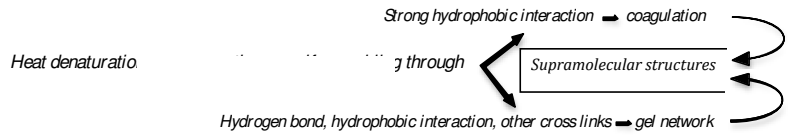

Figure 5. Schematic representation of the mechanisms involved in supramolecular structures formation by heat treatment.

6. Conclusions

Proteomic approaches have proved that emulsion sausages contain larger amounts of insoluble residue, such as gelled collagen, than cooked hams. The presence of an insoluble protein fraction with a high myofibrillar-to-sarcoplasmic protein ratio can be recovered from the insoluble residue of emulsion sausages. Actin is the main protein in emulsion sausages, and it forms aggregates with tropomyosins and myosin light chains that occur almost exclusively in the insoluble protein fraction. Proteins in cooked pork products are subjected to an oxidative environment, the effects of

which are evidenced by the MS identification of a high proportion of oxidized methionine in the muscle proteins. The differences observed between the protein composition of cooked ham and emulsion sausages were ascribed to the degree of solubilization of the myofibrillar proteins, their distribution in the network gel of cooked products, the temperature cycle utilized, the presence of fats, and the status of the pork cut (whole cut or comminuted). The gel properties of cooked pork products determined the nature of the myofibrillar network and their inclusion within the coagulated sarcoplasmic protein matrices, giving rise to a homogenous gel matter bonded by covalent and non-covalent links. Denatured MHCs, and probably solubilized actin, contributed to the homogeneity of these products by emulsifying the fat matter within the myofibrillar and sarcoplasmic gel. Finally, proteomic studies have lead to a deeper understanding of the rebuilding of denatured meat proteins into novel supramolecular structures determined by self-assembling and self-organisation machinery.

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Paper 2: Protein aggregation on cooked pork products: new details on the supramolecular organization

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Food Chemistry, 294, 238 – 247.

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Abstract

The molecular weight distribution of protein aggregates from raw meat and cooked pork products was assessed by size exclusion-high performance liquid chromatography (SE-HPLC). Electrophoretic analysis under reducing conditions showed that the high molecular weight SE-HPLC peak (peak 1) of the cooked products contained protein aggregates in addition to high molecular weight muscle proteins, while the second peak (peak 2) still contained aggregates and <50 kDa proteins. The protein aggregates composition was investigated by HPLC-tandem mass spectrometry. Different classes of proteins were identified and the cooked products showed a more complex composition and organization, according to the muscle structure and the technological procedures, respectively. The key role of actin in the building of the protein networks was also confirmed. The different multi-protein systems found in the cooked products suggest the protein re-organization in heat-induced supramolecular structures, which might be responsible for the texture and the structural properties of the final products.

1. Introduction

In the course of human culinary history, men have developed mild or severe thermal technological processes to preserve raw meat. Besides increasing the storage time, heat treatments improve meat sensory properties (Mottram, 1998). However, several meat structural modifications occur at variable degrees, depending on the time-temperature regimen and on the technological processes. In particular, heat treatment induces drastic modifications to the native arrangement of the main protein constituents (Barbera & Tassone, 2006; Tornberg, 2005), as a consequence of protein denaturation (Barbieri & Rivaldi, 2008), amino acid oxidation (Lund, Lametsch, Hviid, Jensen, & Skibsted, 2007; Di Luccia, la Gatta, Nicastro, Petrella, Lamacchia, & Picariello, 2015) and aggregation events (Promeyrat, Gatellier, Lebret, Kajak-Siemaszko, Aubry, & Santé-Lhoutellier, 2010; Di Luccia et al., 2015). As concerns protein denaturation, the unfolding events change the orientation of the water dipole, destabilizing the protein–water solvation system of the ‘‘native’’ state (Kinoshita & Yoshidome, 2009). Therefore, due to conformational transitions, proteins expose those hydrophobic amino acids normally buried within the inner core of the globular structure (Chelh, Gatellier, & Santé-Lhoutellier, 2007; Santé-Lhoutellier, Astruc, Marinova, Grève & Gatellier, 2008), promoting protein – protein hydrophobic interactions (Davis & Williams, 1998; Yu, Morton, Clerens, & Dyer, 2016). Additionally, heat treatments determine the formation of intermolecular disulfide, dityrosine and thiol-tyrosine bonds, as a consequence of the oxidation of sulphur containing amino acids (cysteine and methionine) as well as tyrosine oxidation and the formation of other types of not reducible covalent bonds (Gerrard, 2002; Lund et al., 2011). The alteration of the levels of amino acid residues along with the protein structure modifies the native three-dimensional meat protein network (Yu et al. 2016), leading to a new heat-induced protein architecture. Changes of pH and ionic strength of the medium represent further chemical-physical factors contributing to determine protein re-organization, thereby leading to induced “irreversible” assembly of denatured proteins (Bouhallab & Croguennec, 2013).

In the light of the vast variability in the protein structure (stability, net charge, presence of hydrophobic and hydrophilic residues) and the chemical-physical characteristics of the medium, there are endless possibilities for the re-organization of proteins in these types of supramolecular architectures, which also affect the functional and structural properties of the protein networks (Bouhallab & Croguennec, 2013). The formation of protein aggregates in cooked meat has a primary impact on the technological and nutritional properties of the final products (Promeyrat et al., 2010; He et al., 2018), which are expected to significantly vary between whole anatomical cut products and emulsioned sausages.

Di Luccia et al., (2015) and Di Luccia, la Gatta, Rutigliano, Rusco, Gagliardi, & Picariello (2017), found out that the emulsioned sausages and cooked ham were characterized by a different protein arrangement as a consequence of the different technological processes and heat treatment. Emulsioned sausages contained a larger amount of insoluble protein residues, which also included collagen, compared to cooked ham. The presence of this insoluble protein residue denoted the presence of high molecular weight (MW) protein aggregates in the cooked meat products, which became somehow soluble only after sonication. These aggregates were characterized by a high myofibrillar-to-sarcoplasmic ratio, highlighting the involvement of myofibrillar proteins, especially actin, in the building of a heat-induced supramolecular protein assembling.

However, the co-occurrence of several proteins in the meat native architecture may influence the final protein aggregation machinery and, consequently, the physico-chemical properties of the cooked meat.

The aim of this study was a specific investigation of the effect of heat treatment on the protein organization in cooked products compared to raw matter through the assessment of the MW distribution of protein aggregates by size exclusion liquid chromatography (SE-HPLC) and evaluation of the composition of fractionated protein aggregates by proteomics.

2. Materials and Methods

2.1 Samples

Traditional meat products were selected for this study. Six samples each of cooked ham, “mortadella di Bologna”, würstel and raw pork were purchased at the local markets.

2.2 Two-Step Extraction Procedure for the analysis of meat protein aggregates

To study meat protein aggregates from raw and cooked samples, we adapted the conditions previously used to separate polymeric protein aggregates from cereal-based products (Gupta, Khan, & MacRitchie, 1993), with some modifications. The buffer containing Na_2HPO_4/NaH_2PO_4 50 mM pH 6.8; 2% SDS was supplemented with denaturing (2M Urea) and (3% DTT) reducing agents and it is thereinafter designated as denaturing and reducing buffer (DRB). We applied a two-steps extraction procedure, which yielded two protein fractions: a free protein fraction (SDS-extractable) and a linked protein fraction (SDS-unextractable or extractable with sonication).

Briefly, proteins were extracted from the ground samples of raw and cooked meat products (1 g) with 10 mL of phosphate buffer (pH 6.4), containing 2% SDS, 2M urea, 3% dithithreitol (DTT) and 1 mM of Pefabloc® (Sigma) which worked as a protease inhibitor. The suspension was vigorously shaken for 30 min and centrifuged for 10 minutes at 10,000 x g at 25°C, using an IEC CL31R

Multispeed centrifuge (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The supernatant contained the "SDS-extractable proteins" or the "free protein fraction".

The resulting pellet was re-suspended in 10 mL of the same buffer and sonicated 30s at 300 W (Microson Ultrasonic cell distruptor; Misonix Inc., Farmingdale, NY), to ensure the samples were completely dispersed, these steps were repeated for three times. After centrifugation (10 min, 10,000 x g, 25°C), the supernatants were recovered and comprised "Proteins extractable with the aid of sonication" or "linked Protein fractions".

For mass spectrometry analysis, "Total" proteins were extracted from ground meat samples (1 g) in 10 mL of the same buffer as described above, shaken, vortexed and sonicated for 30 sec and then centrifuged (10 min, 10,000 x g, 25°C).

2.3 SE-HPLC analysis

SE-HPLC was performed using a liquid chromatograph Agilent 1100 Series system (Santa Clara, CA, USA) equipped with a Phenomenex Biosep SEC-S 4000 column (300 x 7.8mm, Phenomenex, Torrence, CA, USA). Each sample (20 μL) was injected onto the column and the separation was monitored at 214 nm. The mobile phase was 50% acetonitrile (v/v in water) containing 0.1% trifluoroacetic acid with constant flow rate 0.7 mL/min. The SE-HPLC column was calibrated using protein standards with a range of molecular weights (KDa) as follows: Vitamin B12 (1.35), Myoglobin (17.0), ovalbumin (44.0), γ- globulin (158.0) and thyroglobulin (670).

In the SE-HPLC chromatograms, high MW protein peaks (peak 1, peak 2 and peak 3) of SDS-extractable and SDS-extractable with sonication fractions were considered as the free (FPA) and the linked protein aggregates (LPA), respectively.

Two additional peaks at lower MW (peak 4 and peak 5) were detected for both the extracts.

The total extracted protein aggregates in raw and cooked meat products were calculated as the sum of the FPA and LPA total peaks area, giving also an idea of the total protein extractability for each sample. The contribution of each protein fraction (free and linked) on the total extracted aggregates was given as percentage by:

$$\% \text{ FPA} = [\text{FPA total peaks area}/(\text{total FPA area} + \text{total LPA area})] \times 100$$

$$\% \text{ LPA} = [\text{LPA total peaks area}/(\text{total FPA area} + \text{total LPA area})] \times 100$$

Furthermore, the contribution of individual high MW peak on both FPA and LPA total area (peak 1 + 2 + 3) was also evaluated and calculated, as follows:

$$FPA_i = [peak_i \text{ area} / FPA \text{ total area}] \times 100$$

and

$$LPA_i = [peak_i \text{ area} / LPA \text{ total area}] \times 100$$

2.4 Determination of protein content

Nitrogen content of pellets obtained after the extraction of the LPA was estimated through the application of the Kjeldahl method and it was converted into protein content using a factor of 6.25. The analyses were carried out by an automatic digestion unit and through an automatic distillation and titration system (VELP Scientifica Srl, Usmate, Monza-Brianza e Italy). Three measurements for each sample were performed.

2.5 Collection of SE-HPLC fractions

SE-HPLC fractions of protein aggregates were manually collected, concentrated in a Speed-vac (Savant SPD 1010, Thermo Fisher Life Technologies, Carlsbad, CA, USA), lyophilized and stored at -20 °C. An aliquot of each polymeric protein fraction was re-suspended in 6M guanidine HCl, 50 mM Tris, 1 mM EDTA (pH 8.0) containing 10 mM dithiothreitol (DTT). After reduction of disulfide bonds (55°C, 1h), cysteins were alkylated with 55 mM iodoacetamide (final concentration) for 40 min in the dark. Proteins were desalted using prepacked Econopak (Biorad, Milan Italy) G25 size exclusion columns, quantified with the Bradford assay and digested overnight at 37°C with proteomic grade modified trypsin (Sigma), using a 1:50 enzyme to substrate (w/w) ratio.

2.6 Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) of collected fractions

SE-HPLC fractions were diluted (1:1 v/v) with Laemmli solution 2x (0.0625 M Tris-HCl pH 6.8, 2% SDS, 10% glycerol (v/v)) and 0.01% bromophenol blue (BPB) and boiled for 2 minutes at 100°C. To separate the extracted proteins under reducing conditions, 5% DTT was added and SDS-PAGE was performed on a 12% gel, using horizontal electrophoresis system Hoefer SE 600 (GE-Healthcare Bio-Sciences, Little Chalfont, U.K). The electrophoresis separation was performed at 30 mA for gel at 10°C. Image analysis was carried out using Quantity One software (Bio-Rad, Hercules, CA); the band percentage was calculated from the trace quantity (Intensity x mm) of each band related to the total trace quantity of each electrophoretic pattern.

2.7 LC MS-MS

HPLC-electrospray ionization (ESI) - high resolution tandem mass spectrometry (MS/MS) analysis was performed using an Ultimate 3000 high performance liquid chromatography instrument (Dionex/Thermo Scientific, San Jose, CA, USA) coupled with a Q-Exactive Orbitrap mass spectrometer (Thermo Scientific). Tryptic peptides from SE-purified protein fractions were diluted in 0.1% (v/v) formic acid solution. Approximately 0.5 ug of peptides were loaded onto a 5mm long, 300 µm i.d. pre-column (LC Packings, USA) using the HPLC autosampler and separated by an EASY-Spray™ PepMap C18 column (2 µm, 15 cm x 75 µm) 3 µm particles, 100 Å pore size (Thermo Scientific™). Eluent A was 0.1% formic acid (v/v) in HPLC-MS grade water; eluent B was 0.1% formic acid (v/v) in acetonitrile. The column was equilibrated at 5% B. Peptides were separated applying a 4–40% gradient of B over 60 min. The flow rate was 300 nL/min. The mass spectrometer operated in data-dependent mode and all MS1 spectra were acquired in the positive ionization mode with an *m/*z scan range of 350 to 1600. Up to 10 most intense ions in MS1 were selected for fragmentation in MS/MS mode. A resolving power of 70,000 full width at half maximum (FWHM), an automatic gain control (AGC) target of $1x10^6$ ions and a maximum ion injection time (IT) of 256 ms were set to generate precursor spectra. MS/MS fragmentation spectra were obtained at a resolving power of 17,500 FWHM. In order to prevent repeated fragmentation of the most abundant ions, a dynamic exclusion of 10 s was applied. Ions with one or more than six charges were excluded. Spectra were elaborated using the Xcalibur! Software 3.1 version (Thermo Scientific).

Mass spectra were elaborated using the Proteome Discoverer 2.1 software (Thermo Fisher), restricting the search to the Mammalia protein database extracted from the Uniprot. The search was then refined restricting it to the *Sus* species.

Searching parameters included Met-oxidation and N-terminus Gln pyroglutamic as variable peptide modifications; carbamidomethylation as a constant modification of Cys; a mass tolerance value of 5 ppm for precursor ion and 10 Da for MS/MS fragments; trypsin as the proteolytic enzyme; missed cleavage: up to 2. The false discovery rate and protein probabilities were calculated by Target Decoy PSM Validator working between 0.01 and 0.05 for strict and relaxed searches, respectively. The identification was validated through the Protein Percolator tool of the Proteome Discoverer.

2.8 Statistical analysis

The results from six independent samples for each product were compared by one-way variance analysis (ANOVA). A Duncan's multiple range test, with the option of homogeneous groups

($p<0.05$), this was used to determine the significance between samples. The statistical analyses were performed using IBM SPSS Statistics v.21 for Windows and XLSTAT Base 2018 Addinsoft**.**

3. Results

3.1 Assessment of protein aggregates of raw meat and cooked pork products

Meat protein aggregates were assessed substantially according to the conventional procedure used for the study of wheat polymeric protein (Gupta et al., 1993; Kuktaite, Larsson & Johansson, 2004). However, to improve the effectiveness and efficiency of the extraction methodology, the extraction buffer was supplemented with denaturing and reducing agents (Di Luccia et. al., 2015). In this manner the proteins of meat products were fractionated into a free protein fraction (SDS-extractable), which included proteins and protein aggregates that were merely dissolved in DRB and a linked protein fraction (SDS-unextractable), i.e. the fraction still embedded in the cooked muscle structure, which became soluble after sonication.

For each sample, the chromatographic profiles obtained according to the two-step extraction procedure were shown in Figure 1. In all the cases, the chromatographic profiles consisted of three main peaks, which were labelled as peak 1, peak 2 and peak 3, as a shoulder of peak 2 (more evident in the case of the two emulsioned products). The elution was obtained between 6 and 10 minutes, corresponding to an estimated MW in the 500 - 180 kDa range. Two additional minor peaks, eluting between 10 and 12 minutes, were labelled as peak 4 and peak 5 and were more prominent in the free protein fraction than in the linked one. Most likely, they were soluble monomeric proteins, expectedly less represented in the linked protein fraction (i.e SDS-unextractable fraction). In Table 1 is reported the area of each peak at high MW (peak 1, peak 2 and peak 3) which were labelled as free protein aggregates (FPA) and linked protein aggregates (LPA), according to the extraction procedure, and the sum of the total area of peaks of FPA and LPA, this latter defined as estimated extractability (EE) value.

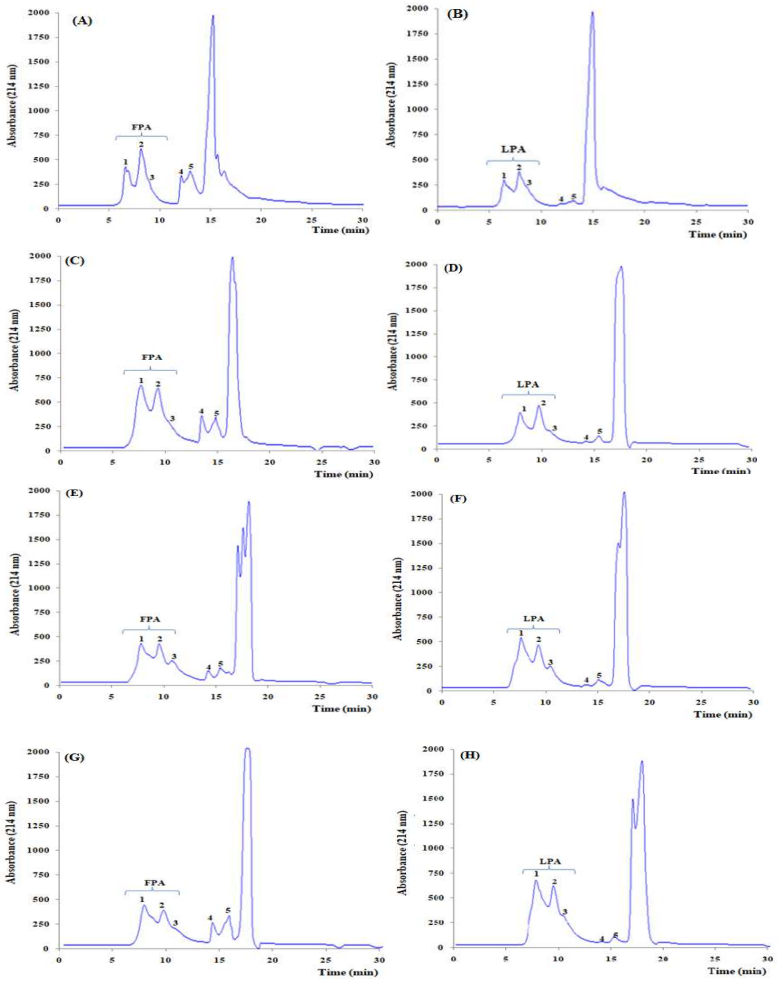


Figure 1 - SE-HPLC profiles of Free and Linked protein Fraction. Free protein fraction of Raw Pork (A), Cooked ham (C), Würstel (E) and Mortadella (G); Linked Protein Fraction of Raw Pork (B), Cooked ham (D), Würstel (F) and Mortadella (H).

Raw pork exhibited more abundant FPA than LPA, because of the expected presence of unaggregated proteins, which were easily released from the native supramolecular muscle network. Regarding the cooked products, cooked ham showed the highest value for the FPA, whereas mortadella had the highest value for the LPA. To have a clearer framework of the incidence of the FPA and LPA on the total extracted protein aggregates (FPA+LPA), we reported the values of the peaks area, considering the EE values as the 100% of the extractability and the relative percentage of each aggregates fraction (FPA and LPA) for each sample, as shown in Figure 2A. The FPA percentages followed the order: cooked ham (65,0%) > würstel (49,8%) > mortadella (38,3%), demonstrating the greater extractability of cooked ham than the emulsioned products. Thus, LPA followed the inverse trend with mortadella (61,7%) > würstel (50,2%) > cooked ham (35,0%) highlighting how the formation of an emulsion of minced meat with fat, air and water, could be at the origin of the reduced extractability of denatured and associated muscle proteins from comminuted products.

Table 1. High molecular weight peaks area in the raw meat and cooked products, labelled as Free Protein Aggregates (FPA) and the Linked Protein Aggregates (LPA). Total peak area is given by the sum of the three peaks detected. The EE, estimated extractability values, represent the average of the sum from total peaks area of the Free Protein Aggregates (FPA) and Linked Protein Aggregates (LPA).

	Free Protein aggregates (FPA)				**Linked Protein Aggregates (LPA)**				
	Peak 1 (mAu•s)	***Peak 2 (mAu•s)***	***Peak 3 (mAu•s)***	***Total Peaks Area (mAu•s)***	***Peak 1 (mAu•s)***	***Peak 2 (mAu•s)***	***Peak 3 (mAu•s)***	***Total Peaks Area (mAu•s)***	***EE (mAu•s)***
Raw Pork	19365,47±785,12	29556,10±477,99	15555,40±320,45	64476,97±679,72	17298,97±382,52	18090,60±190,79	15942,40±636,76	51331,97±385,32	115808,93±820,66
Cooked Ham	52292,30±423,70	53442,33±484,11	29900,83±427,37	135635,47±1129,80	30469,50±468,85	30023,77±265,29	12538,57±235,03	73031,83±478,28	208667,30±781,45
Mortadella	34817,37±385,33	23328,07±385,47	21074,23±439,59	79219,67±369,91	62209,51±849,25	34365,44±810,91	30998,68±689,74	127573,6±1666,86	206793,3±1341,05
Wurstel	40440,50±454,13	29675,30±610,31	23719,03±832,93	93834,83±126,15	45891,43±315,19	30110,90±126,96	18593,50±540,06	94595,83±265,36	188430,66±253,58

However, sonication improved the extractability also in these cases, suggesting the substantially non-covalent nature of the aggregation. All the differences among the FPA and LPA area values were significant ($P < 0.05$) except for the case of würstel, which had no significant difference between the FPA and LPA peak area values.

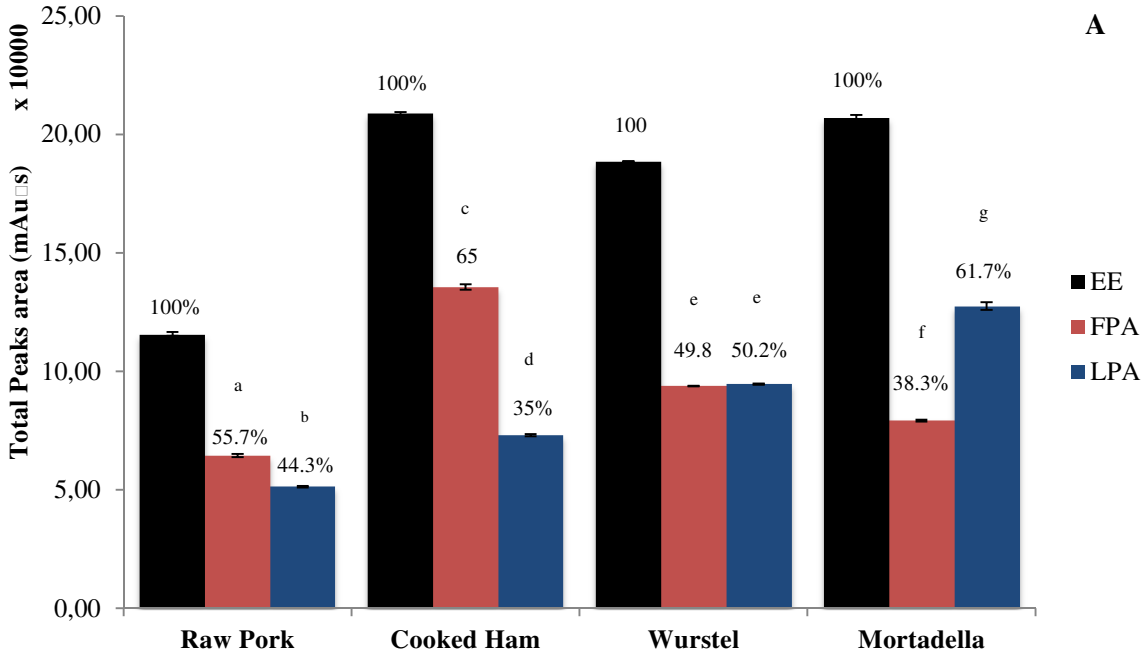
The distribution of the protein aggregates in the raw and cooked products was more precisely evaluated by determining the contribution of each peak to the total area of FPA and LPA (Fig. 2B-2C), expressed as percentage normalized values. In the FPA (Fig. 2B), the peak 1 of the comminuted products (i.e. mortadella and würstel) had the greatest influence on the total area, while in the case of cooked ham there was a negligible difference between the percentages of peaks 1 and 2. As expected, in the case of raw pork, peak 2 (≈200 kDa) had the greatest incidence on total area, since the formation of larger aggregates is an event promoted by the heat treatment (Promeyrat et al., 2010).

The introduction of the sonication step to extract proteins from the pellets after the first extraction step, in order to obtain the linked protein fraction (Fig. 2C) did not affect the extractability of cooked ham, whose peaks percentages only slightly increased. On the contrary, mortadella and würstel showed a significant increase of the percentages for peak 1, probably due to a significant solubilization of protein aggregates linked to the matrix, induced by DRB as well as by the sonication (Di Luccia et al., 2015). Raw pork showed the same proportion among the three peaks, with a consistent decrease of the percentage of peak 2 and the increase of the low MW fraction (peak 3), which in the other cases, always decreased.

Possible residual proteins in the pellets after the linked protein fraction extraction were determined by the Kjeldahl method. Indeed, a protein residue was found and quantified, as reported in the Table 2. It was interesting to note that, despite a higher weight of the dry residue for raw pork and cooked ham than the emulsioned products, the pellets resulting from these latter products were higher in the crude proteins percentages.

Table 2. Dry weight and percentage of crude proteins in the residue resultant after the extraction of the Linked Protein Fraction (LPA).

	Raw Pork	Cooked Ham	Mortadella	Wurstel
Dry weight residue (g)	6.14	6.53	3.75	4.19
Crude Protein (%)	2.78	2.96	5.02	4.59



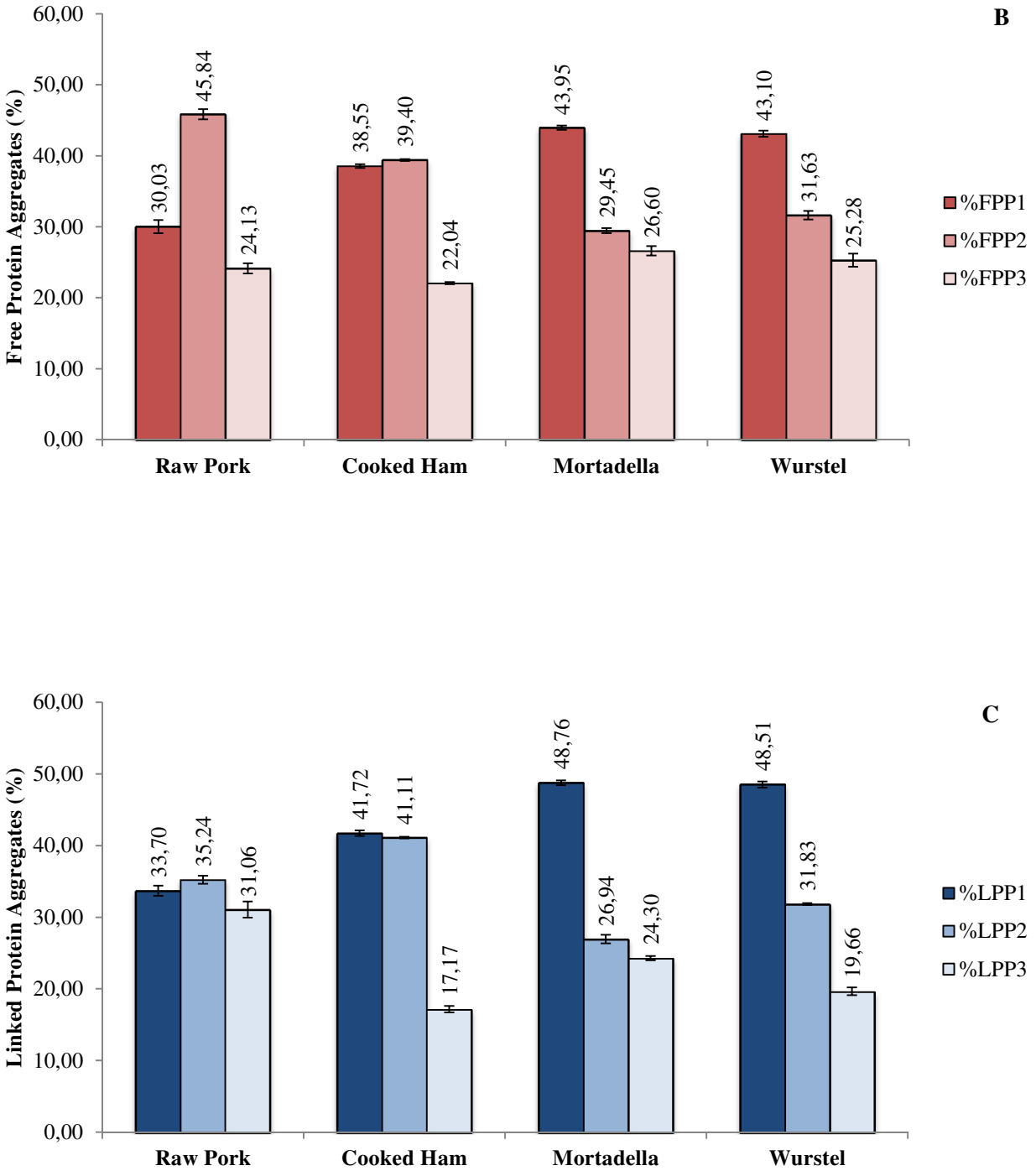


Figure 2A – Free and Linked Protein fractions percentages evaluated as related to the Estimated Extractabilty (EE) values. **Figure 2B** – Influence of each peak accounting for the Free Protein Aggregates (FPA) on the total peak area in the raw and cooked products. **Figure 2C** – Influence of each peak accounting for the Linked Protein Aggregates (LPA) on the total peak area in the raw and cooked products.

We used the principal component analysis (PCA) (XLSTAT Base 2018 Addinsoft) to investigate on the relationships among the high and low MW protein fractions and the technology of production. Two factors were extracted, named F1 and F2, explaining 45 and 37% of the total variance respectively, with a cumulated explained variance of 83% (Figure 3). Considering the loading factors, it could be observed that F1 showed a positive correlation with all the peaks areas of the linked protein fraction (i.e insoluble fraction) and a negative correlation with all peak areas of the free one (i.e soluble fraction). In other words, higher was the score of a sample for F1, higher was the proportion of its proteins present in an insoluble form. The principal component F2 showed positive correlation with almost all the considered peaks area with the exception of peak 4 of both free and linked fractions. The score plot reported in figure 3 showed a great separation of sample types. Raw meat samples that, obviously, are not processed and therefore they are characterized by no protein denaturation fall in the negative part of the diagram showing negative correlation with both F1 and F2. The area of peak 4 of the free (i.e soluble) and linked (i.e insoluble) fractions showed a particularly high correlation with this group of samples. In the light of these findings, F1 could be thought as thermal denaturation effect, whereas F2 could be considered a factor linked to the production processing.

Moreover, cooked ham samples were placed on the upper part of the diagram, having a high positive correlation with component F2 and a negative correlation with F1. This suggested that the production processing affected cooked ham more than protein denaturation, as actually in this quarter almost all the peaks were from the free protein fraction and, especially, we noted the aggregates at high MW (peak 2) and low MW monomers (peak 5). This finding for the cooked ham confirmed the partial denaturation of the muscle protein because of the intact structure of the muscular fibre (i.e. sarcomere). Therefore, only the soluble fraction (i.e. the sarcoplasmic, soluble part of myofibrillar and connective proteins) aggregated. Würstel samples were placed in the positive quadrant of F1 and F2, owing to the combined effects of production processing and thermal treatment. Finally, mortadella samples showed a positive correlation with F1 and an almost zero correlation with F2.

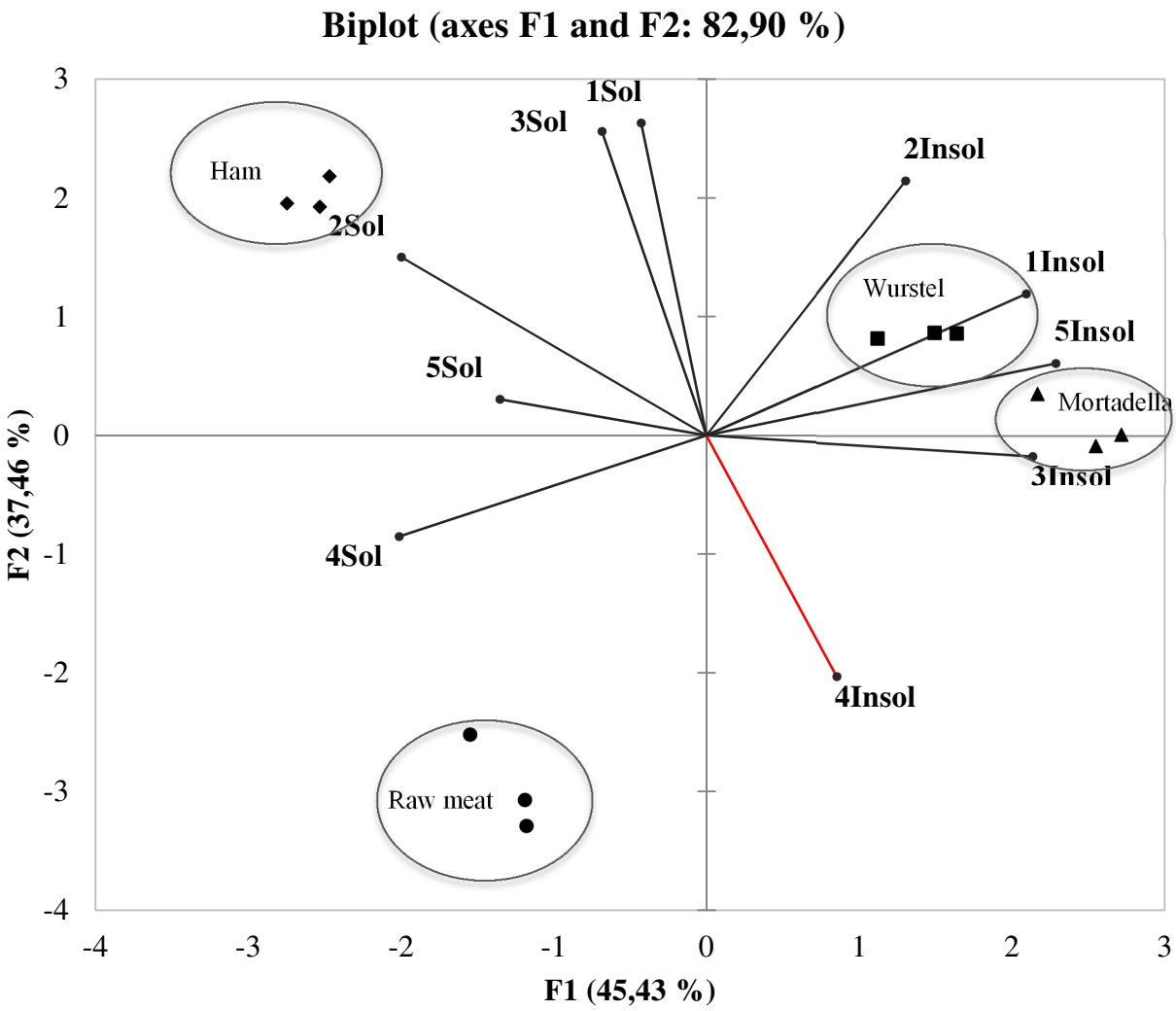


Figure 3. Principal component analysis of peaks area of raw meat and cooked products samples.

3.2 SDS-PAGE of total proteins and SE-HPLC peaks

The SE-HPLC fractions were individually analysed by SDS-PAGE under reducing conditions. The electrophoretic patterns of cooked products were compared to the sarcoplasmic and myofibrillar proteins of raw pork (lane 1 and lane 2), as shown in figure 4. The electrophoretic patterns of unfractionated cooked products (lane 3, cooked ham; lane 6, mortadella; lane 9, würstel) were very complex and showed the main bands belonging to sarcoplasmic and myofibrillar proteins, with a protein smear at high molecular weight. Interestingly, a thick band (AgR) at the top of the resolving gel was observed for both total proteins and peak 1, confirming the presence of large protein aggregates, which were not able to enter the resolving gel. The presence of high MW protein aggregates, persisting under reducing conditions, suggested the existence of not-reducible inter-chains cross-links. The electrophoretic lanes relevant to peak 1 of the cooked products (lane 4, cooked ham; lane 7, mortadella; lane 10, würstel) mainly showed high MW protein bands,

including AgR and the heavy chain myosin (210 kDa), which was missing in peak 2. The electrophoretic patterns relevant to peak 2 (lane 5, cooked ham; lane 8, mortadella; lane 11, würstel) showed protein bands with MW lower than peak 1, including the actin band (42 kDa). The results of the image analysis of the electrophoretic patterns of the peaks are reported in Supplementary Table 2. The band percentage was calculated from the trace quantity (Intensity x mm) of each band related to the total trace quantity of the respective electrophoretic pattern, considering those with values higher than 5%. In Supplementary Table 2, we divided the MW range into three sections: the range 400-240 kDa, which was that of the protein aggregates; the range 100- 42 kDa, in which we detected most of the protein bands related to the electrophoretic patterns of peak 1; the range 38-23 kDa, in which most of the protein bands related to the electrophoretic patterns of peak 2 were detected. The band percentages related to the protein aggregates (range 400-240 kDa), as expected, were higher in peak 1 than in peak 2, for all the cooked products, with AgR scoring as the highest. In the range 100-60 kDa, we detected protein bands only in the lanes of peak 1 of each product, while the band of actin (42 kDa), shown in bold in the Supplementary Table 2, appeared as a boundary band, being present in both the peaks. Its relative intensity was higher in the emulsioned products than in cooked ham, with a band percentage higher in peak 2 than in peak 1. The protein bands of cooked ham and mortadella were clearly split in peak 1 and 2. In both the cases, peak 1 patterns (lane 4 and lane 8, respectively) showed protein bands up to 42 kDa, while peak 2 lanes revealed protein bands with a lower MW. In contrast, the electrophoretic patterns of würstel samples showed a higher number of bands than the other two cooked products and a balance in the protein composition of the two peaks. Actually, several bands below actin up to 25 kDa were revealed also in peak 1 and bands at lower MW than actin band were detected in peak 2, but with lower band intensity compared to the other two products.

Table 3. Band percentages of the high molecular weight peaks (peak 1 and peak 2) obtained from the electrophoretic patterns (SDS-PAGE).

MW	Band Percentage (%) Peak 1			Band Percentage (%) Peak 2		
	Cooked ham	**Mortadella**	**Wurstel**	**Cooked ham**	**Mortadella**	**Wurstel**
400-380	22,37	23,58	17,39	4,84	5,02	8,52
220-240	18,26	10,34	10,98	5,45	4,84	5,47
100-98	11,89	10,03	7,75			
92-95	12,12	10,04	7,62			
57-60	11,7	9,28	7,09			
45-47	10,96	8,87	5,95	6,46	9,63	6,12
42	**12,7**	**19,12**	**16,81**	**17,72**	**20,65**	**20,40**
38				8,64	10,14	7,59
35		8,74	7,26	9,47	14,68	13,06
32-30			6,55	14,46	14,17	7,44
28			6,33			7,99
25			6,27	19,63	9,01	16,44
23				13,33	11,87	6,97

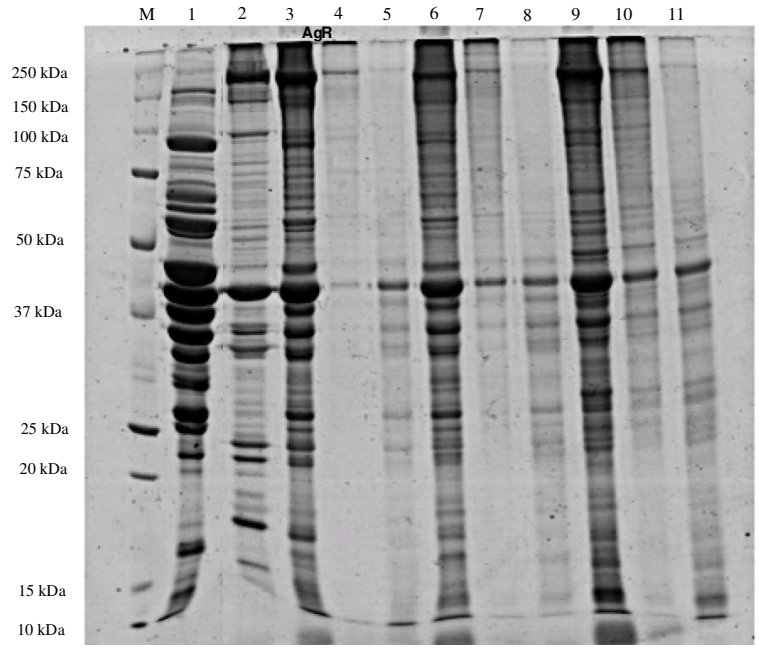


Figure 4 - SDS-PAGE of total protein samples and high molecular weight peaks (peak 1 and 2) under reducing conditions. M: marker; Lane 1: raw pork sarcoplasmic proteins; lane 2: raw pork myofibrillar proteins; lane 3: Cooked ham total polymers; lane 4: Cooked ham (peak 1); lane 5: Cooked ham (peak 2); lane 6: Mortadella total polymers; lane 7: Mortadella (peak 1); lane 8: Mortadella (peak 2); lane 9: Würstel total polymers; lane 10: Würstel (peak 1); lane 11: Würstel (peak 2).

3.3 Mass spectrometry analysis

The identification of the total proteins, which eluted in the collected peaks after chromatographic separation, was assessed using a shotgun proteomic approach. In Table 4, we reported the number of identified proteins, grouped according to their role in the muscle cell. It was noteworthy that in all samples, sarcoplasmic proteins were the main component ranging from 58 to 69%. Significant differences in the percentage of sarcoplasmic proteins were detected between peak 1 and peak 2 of raw meat and cooked ham, while the emulsioned sausages showed similar percentages. In the computation of the sarcoplasmic fraction, we identified the main intracellular enzymes and proteins with functional properties, while in the myofibrillar fraction, we found the main structural and scaffold proteins, along with their isoforms (es. myosin, actin, troponin, tropomyosin, desmin).

The number of the identified proteins for mortadella and würstel was higher than raw pork and cooked ham, denoting higher protein variability in the composition of the emulsioned sausages. This hypothesis was supported by the presence of a discrete number of other proteins in these products, including collagen, proteoglycans (es. lumican, decorin, tuftelin, fibromodulin) and proteins with typical functions. In addition, two milk proteins, α_{s1}- and β-casein were identified in mortadella samples, most likely due to the use of milk proteins as emulsifier additives (Carmona, Ruiz-Capillas, Jimenez-Colmenero, Pintado, & Herrero, 2011).

Table 4 . Composition of protein peaks by shotgun proteomic approach

		Intracellular			Extracellular		Detected Proteins	Total proteins
		Sarcoplasmic	Myofibrillar	Sarcoplasmic proteins (%)	Collagen	Others		
Raw Pork	Peak 1	11	8	57,9	-	-	19	**62**
	Peak 2	29	14	67,7	-	-	43	
Cooked Ham	Peak 1	18	10	62,1	-	1	29	**74**
	Peak 2	31	14	68,9	-	-	45	
Mortadella	Peak 1	40	17	66,7	1	2	60	**229**
	Peak 2	46	17	68,7	-	4	67	
	Peak 3	70	23	68,6	-	9	102	
Wurstel	Peak 1	34	18	62,1	-	2	54	**121**
	Peak 2	19	11	61,3	-	1	31	
	Peak 3	34	17	60,7	-	5	56	

4. Discussion

Generally, food is constituted by multi-protein systems, whose native supramolecular organization depends on the conditions of the medium and on the interaction with other constituents. The architecture of the food protein systems can be significantly affected by the technology.

Muscle fibres are organized in supramolecular assemblies arranged in aligned myofibrils in order to accomplish their physiological role (Epstein & Fischmxan 1991; Ohlendieck, 2011). The basic unit of such architectonic supramolecular structure is the sarcomere, in which the macro-protein, titin, works as a scaffold for the ordered assembly, also contributing to the muscular mechanics (Gregorio, Granzier, Sorimachi, & Labeit, 1999). Upon heating, meat proteins denature, re-arranging and consequently inducing a re-organization of the native supramolecular structure into a heat-induced one (Shao, Zou, Xu, Wu, & Zhou, 2011; Bouhallab & Croguennec, 2013). The machinery of this re-arrangement is based on a new recognition amongst the unfolded protein structures, which is driven by non-covalent interactions and is stabilized by the formation of disulfides and other covalent bonds (Gerrard 2002). Some of these modifications can be ascribed to the Maillard reaction (Mohammed, Hill & Mitchell 2000), such as the formation of dehydroprotein (lysinoalanine, lanthionine, deydroalanine) (Friedman, 1999), dityrosine bonds (Lund, Heinonen, Baron, & Estevez, 2011), while others stem from oxidation events (Lund et al., 2007).

The supramolecular organization in raw pork and in cooked pork products was different and was assessed by evaluating the MW distribution of protein aggregates and identifying the proteins involved in their formation, using proteomics. The application of a two-steps extraction procedure

allowed us to obtain a more efficient extractability and the sonication provided a supplementary energy to release those proteins and/or aggregates strongly associated to the food matrix. The evaluation of the peaks area of the high MW aggregates (FPA and LPA) pointed out the differences between the raw matter and the transformed products. The overall chromatographic results of the raw pork, showing a minor presence of protein aggregates and the lowest values for both FPA and LPA, were due to the native muscle protein organization in an insoluble and stable supramolecular architecture (sarcomere) (Gregorio et al., 1999). Actually, the formation of large protein aggregates is an event promoted by heat treatments and, therefore, the higher values detected in the LPA fraction of comminuted products confirmed the findings of Di Luccia et al., (2015), where the proteomic approach revealed the presence of a great insoluble protein residue, whose extractability increased only after the extraction aided with sonication. Although, the presence of large protein aggregates was evident in all the cooked products, their different proportions (Figure 2B-2C) was likely the result of a different organization, according to status of the raw matter (whole anatomical cut or comminuted), chemical and physical conditions of the medium (pH, water holding and tenderness degree) and processing conditions.

The production of cooked ham involves that the whole anatomical cut is put in the mould, pressed and cooked. With this step all soluble meat proteins, including the structural proteins from the tenderization process, are trapped into the myofibrillar network, a well-ordered pre-defined structure, that is a more accessible system, mainly characterized by disulfide bonds and hydrophobic interactions (Di Luccia et al., 2015; Di Luccia et al., 2017). The emulsioned products, obtained by finely chopped meat, with a destroyed native muscular fibre structure, showed a more complex protein arrangement, with LPA prevailing over FPA, which also induced the formation of an insoluble protein residue. The different dry weight of the insoluble residue following the two-step extraction procedure of the raw pork and cooked ham samples compared to those of the comminuted products was due to the differences in the native and denatured structures. The firm structure of cooked ham made an insoluble residue with a weight higher than the comminuted products, as a consequence of the dimension of the particles, the lacking of the muscle structure and the presence of the air in the meat emulsion. In a system such as cooked ham, the two-step extraction procedure allowed great protein extractability, leaving an insoluble part, mainly formed by gelled connective tissue, represented by the low amount of crude proteins resulting in the residue. The higher amount of crude proteins in the insoluble residues of mortadella and würstel samples highlighted the complexity of protein arrangement in the comminuted products, where the two-step extraction procedure was effective only after the sonication step (LPA fraction > FPA fraction, Table 1), therefore suggesting the presence of large aggregates and a remarkable insoluble

protein fraction (Supplementary Table 1). In addition, it was interesting to note that the FPA and LPA were both the 50% of the total extracted protein aggregates (EE value) in the würstel samples, probably due to the different structure of this product with respect to the mortadella. Indeed, the structure of the würstel is completely homogenous, while mortadella shows a structural discontinuity, represented by the small cubes of lard, which are enclosed in the emulsion.

The PCA analysis also confirmed that two main factors could explain the differences in the cooked meat products protein aggregates: thermal denaturation and processing conditions. In the comminuted products, the linked protein aggregates (LPA) were more represented, while in the cooked ham samples the free protein aggregates (FPA) were predominant, confirming only a partial protein denaturation. In addition, raw meat contained only a negligible part of protein aggregates, deriving from the native supramolecular muscle organization. Further information derived from the influence of the technological processes, being the raw meat and the cooked products located in different quarter of the diagram. Amongst the cooked products, the comminuted were located in the same quarter, which was different from that of cooked ham, highlighting the role that the comminution combined with heat treatment had in the formation of completely different heat-induced protein architecture.

The composition of the protein aggregates of cooked products was deduced observing both number and nature of the involved proteins. As demonstrated by SDS, the actin band, detected in both the high MW peaks, seemed to have a crucial role in the building of the heat-induced supramolecular structure of the cooked products, confirming the findings of Di Luccia et al. (2015; 2017). Notably, the comminuted cooked products, especially würstel, exhibited proteins with MW < 42 kDa even in peak 1. This finding suggested that in this case small monomeric proteins might be tightly bound to the protein aggregates by non-covalent and strong hydrophobic interactions, possibly also mediated by the presence of the emulsioned lipids, giving rise to a more complex architecture or a “supra-structure”. The protein identification in the peaks by HPLC-tandem mass spectrometry revealed that all the main sarcoplasmic and myofibrillar proteins were found and the sarcoplasmic proteins were the most abundant in all the cooked products.

Cooked ham showed a protein pattern quite similar to that of raw pork, according to the ordered structure of the product, with a higher number of proteins in peak 2 rather than in peak 1, as could also inferred from the chromatographic (Figure 1) and electrophoretic patterns (Figure 4). In particular, the peak 2 of cooked ham contained myozenin-1. This protein is part of the complex Z-line structure, identified as a biomarker of tenderness by Beldarrain et al., (2018) for the first time. Interacting with troponin and myotilin at the Z-line of the muscle, myozenin-1 increases the cohesion of the sarcomere (D’Alessandro et al. 2012), reducing the activity of proteases during the

tenderization process, thus favouring the formation of a tough structure. Morzel et al., (2004) supposed that this protein was also a target for proteolysis, decreasing in the post-mortem phases of pork meat. Overall, from the results obtained for cooked ham samples and according to the findings of Di Luccia et al., (2015) and Di Luccia et al., (2017), the arrangement of the protein structure was mainly characterized by disulfide bonds and hydrophobic interactions, as confirmed by the high extractability under denaturing and reducing conditions and the lower percentages of crude protein found in the residues. The compact structure of a whole anatomical cut product is the result of the denaturation and aggregation events, which lead to the formation of supramolecular heat induced assembling, involving the main sarcoplasmic, myofibrillar and scaffold proteins, forming an ordered network, held together by the collagen. The protein composition of the three peaks detected in comminuted products (i.e mortadella and würstel), in which peak 3 was clearly evident, revealed a great variability, with a higher number of detected protein than in cooked ham and raw pork. Despite the evidence for a very low extractability of sarcoplasmic protein in the cooked products (Di Luccia et al., 2015), MS analysis revealed their involvement in the formation of protein aggregates. In addition to the sarcoplasmic, muscular and scaffold proteins, other functional proteins were detected, according to the different preparation and status of the raw material. Collagen was detected only in peak 1 of mortadella samples, while proteoglycans were the most represented class of proteins identified in peaks 2 and 3, also in the case of würstel. Proteoglycans are macromolecules with a protein core covalently linked to glycosaminoglycan chains (Lepetit, 2008). The role of proteoglycans is to link collagen fibrils and stabilize the intramuscular connective tissue. They are degraded during post-mortem aging, leading to the weakening of collagen fibrils (Nishimura, 2010). In products, such as mortadella and würstel, the aggregates arising from protein re-organization are characterized by disulfide and hydrogen bonds, as demonstrated by the high extractability with DRB, but the detection of large aggregates even after the sonication step (LPA, peak 1), could be due to the presence of an insoluble protein associated through bonds other than covalent ones. In the case of comminuted products, the presence of a multi-phase system led to a complex protein arrangement, where also the fat plays an important role. Actually, in the emulsioned products, the properties of fat and the protein matrix are closely interrelated, because the physical entrapment of the fat within the dense meat protein matrix (Andersson et al., 2000) and the possible interaction with the monomeric proteins at the protein-fat interface can allow the building of a ‘supra-structure’, in which, as shown, different classes of proteins are involved.

5. Conclusion

Heat treatment induces the formation of large protein aggregates organized in multi-protein systems, as assessed using proteomics. The combined use of SE-HPLC, electrophoresis and proteomics provided us information about the nature of the aggregates formed in different cooked pork products as a function of the state of the raw matter and processing.

The structural organization resulting from our findings consists of coagulated sarcoplasmic proteins enclosed into the inner part of the myofibrillar network, through covalent and/or non-covalent or tight hydrophobic bonds. A more complex protein arrangement machinery occurs in comminuted products, as a consequence of the involvement of different classes of proteins in an emulsified matrix. The supramolecular protein organization in a tight structure can account for the texture properties of cooked comminuted products. Further investigation could be undertaken to assess how these supramolecular structures could entrap or release nutritionally interesting or bioactive components.

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Session 3

Milk Products

The supramolecular organization within the milk system

Milk is the secretion of the mammary gland and has distinctive features according to the composition and the structure of its costituents (Jensen et al., 1995).

The native casein micelles organization within the milk system is a clear example of a supramolecular structure, where the molecular entities are held together through non-covalent intermolecular interactions (McMahon & Oommen, 2008).

The model for casein micelles organization within the milk system has been discussed for many years (McMahon & Brown, 1984, McMahon & McManus, 1998; de Kruif & Holt, 2003; Dalgleish et al., 2004). According to the results of the analyses carried out to explain their internal structure (Karlsson et al., 2007; Marchin et al., 2007), the casein micelles were described as mostly arranged in a complex network of globular and linear aggregates of proteins, where the phosposerine residues of α_{s1}-, α_{s2}- and β-CN stabilize the calcium phosphate, as amorphous nanoclusters.

Among the main costituents, the fat is another key element in the milk system, exsisting as small droplets, i.e the milk fat globules, which are enveloped in a biological membrane, comprising proteins, glycoproteins, enzymes, phospholipids, cholesterol and other component (McPherson & Kitchen, 1983), i.e the milk fat globule membrane (MFGM). The role of MFGM is to protect the milk fat from lipolysis (Walstra & Jennes, 1984) and avoid the aggregation and the fat coalescensce events. The supramolecular structure of the fat globule in the raw milk (≈ 4μm) is destabilized as a consequence of the application of several possible dairy processes, with the consequent removal or severe damage to the MFGM and the resulting interactions with the protein matrix, which may affect the sensory, functional and nutritional properties of the dairy products (Lopez, 2005).

Therefore, during the processing of milk, the applications of some treatments and in this specific context, the application of the thermal treatments, lead to the disruption of the native supramolecular organization of the milk costituents, implying a new rearrangement.

As a matter of the fact, the UHT (Ultra High Temperature) treatment on milk provides for the application of temperatures between 135 and 150° C for 3-5 seconds, in order to preserve the microbiological quality and prolong its shel-life. This type of treatment involves significant structural changes in proteins, whose native supramolecular architecture (casein micelle) is modified with consequences on milk stability. Following protein denaturation, UHT milk is characterized by two main phenomena occurring during storage, gelification and sedimentation. The causes of gelification are not clear yet, but the explanations of this phenomenon are attributable both to the activity of proteases and the chemical-physical effects of protein rearrangement due to

non-enzymatic actions (reaction of Maillard) (Datta and Deeth, 2001), with the consequent formation of a 'gel' that involves the whole matrix.

The sedimentation process, on the other hand, is strictly related to the characteristics of the raw material, to the intensity and type of the applied heat treatment (direct or indirect system), to pH variations and ionic calcium concentration (Deeth & Lewis, 2016; Gaur, Schalk & Anema, 2018), representing a problem for the consumers and, consequently, for the food industry.

In the light of these considerations, in this session the protein composition of the heat induced supramolecular aggregates of commercial UHT milk was assessed by a proteomic approach. Moreover the dynamic arrangement of milk proteins in UHT skimmed milk samples, obtained from local dairy industries, along the storage for 1, 3 and 5 months was studied. This approach allowed to assess the protein rearrangement within the UHT milk system, which shifts across three forms: soluble aggregates, suspended insoluble supramolecular aggregates, leading to the formation of the sediment.

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Draft Article

- Rutigliano, M., Rusco, G., Picariello, G., Bulgari, O., Spadaccino, G., Gagliardi, R., Di Luccia, A., Addeo, F., la Gatta, B. Protein aggregation machinery in UHT milk: supramolecular insights.

Draft Article. Protein aggregation machinery in UHT milk: supramolecular insights.

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Abstract

Two investigations were performed on UHT milk samples. The former provided for the assessment of protein composition in four UHT milk supernatants stored at ambient temperature for 1, 3 and 5 months. The amount of the insoluble residue decreased during the first month and then, slightly changed, until the fifth month of storage. The protein composition of the supernatants showed a greater variability than the insoluble residue, suggesting the presence of a dynamic arrangement within the protein system of UHT milk, which moves towards the formation of the sediment. The latter study provided for a proteomic study on six commercial UHT milks by reducing and non-reducing SDS-PAGE and by non-reducing alkaline Urea-PAGE / reducing SDS-PAGE two-dimensional gel electrophoresis coupled to tandem mass spectrometry. Whey proteins, mainly β-lactoglobulin, interact with the casein units, essentially α_{s1}-, α_{s2}- and κ-CN, forming the main heat-induced supramolecular protein aggregates in the supernatant, while the insoluble residue showed only the casein fractions.

1. Introduction

The milk intended for human consumption is subject to thermal treatment to guarantee microbiological safety and to extend the commercial shelf life. Ultrahigh-temperature (UHT) milk undergoes heating at 135-150° C for 3-5 s, in order to produce a commercially sterile product and to enable the storage at room temperature for up to nine months (Holland, Gupta, Deeth, & Alewood, 2011; Grewal, Chandrapala, Donkor, Apostolopoulos, & Vasiljevic, 2016). Depending on the severity of the treatment, heat induces deep nutritional modifications and physicochemical changes, (Sakkas, Moutafi, Moschopoulous, & Moatsou, 2014) which involves, particularly, the milk protein fraction. Heat treatment alters the native supramolecular organization of milk casein micelles (McMahon & Oommen, 2008), leading to the formation of heat-induced protein assemblies that, through a new protein recognition build up induced supramolecular structures (Bouhallab & Croguennec, 2013). Therefore, milk protein heat denaturation leads to protein-protein cross-links, which affect milk stability, resulting in age gelation and sedimentation events, during the storage of UHT milk. In particular, the sedimentation is an evident phenomenon that it is observed at the bottom of the container with the formation of a layer of material, composed mainly of protein. The sediment formation is influenced by several factors, including raw milk quality, homogenizer location in the heating process, type and severity of heat treatment and variation of pH and ionic calcium concentration (Deeth & Lewis, 2016; Gaur, Schalk & Anema, 2018).

Protein aggregation in the sedimentation phenomenon is driven by the unfolded whey protein chains, which are able to interact with each other, or with micelles or κ-CN and α_{s2}-CN, through the formation of disulfide-bonds and hydrophobic interactions, as described by Patel, Singh, Anema & Creamer, 2006; Chevalier & Kelly, 2010). Furthermore, Friedman (1999) reported that additional cross-links were involved in the formation of protein aggregates after heat processing, while Holland, Gupta, Deeth & Alewood, (2011) evaluated the extent of these cross-links along the UHT milk storage, depending on temperature.

Several authors reported also the formation of soluble aggregates formed during the heat treatment of skim milk, involving the main whey proteins linked with κ-casein through hydrophobic interactions and thiol/disulfide interchanges, with a size ranging from 30 to 100 nm (Guyomarc'h, Law & Dalgleish, 2003; Li, Dalgleish & Corredig, 2015). The involvement of β-lg and/or α-la in the soluble aggregates (i.e non sedimentable material), was found to be strictly related to the heat temperature (Corredig & Dalgleish, 1999; Oldfield, Singh, & Taylor, 1998), while β-casein and/or α_{s1}-casein were not found (Donato & Dalgleish, 2006). The complexity of the mechanisms involved in the milk protein modification as a result of heat treatment has stimulated several authors to face

this issue through high-resolution proteomic approaches (Patel et al., 2006; Chevalier & Kelly, 2010; Holland et al., 2011).

Therefore, in the light of all these considerations, the aim of our work was dual:

- to evaluate the dynamic arrangement of proteins in UHT milk skimmed samples between the liquid phase or supernatant, containing any serum phase casein and whey proteins and the insoluble forms (insoluble residue and sediment) along the storage;
- to define the presence and the composition of the heat induced protein aggregates in commercial UHT milk samples and related insoluble residues using a mondimensional electrophoresis (SDS-PAGE^{R} and SDS-PAGE^{NR}) and assessing the composition of the larger heat induced protein aggregates applying 2D electrophoresis (alkaline urea PAGE^{NR}/ SDS-PAGE^{R}) coupled to tandem mass spectrometry (LC-MS-MS).

2. Materials and methods

2.1 Milk samples for the proteomic study (PS)

Two investigations were performed on UHT milk in this work: a *proteomic study* (PS) of UHT milk and *the assessment of the protein dynamic arrangement* (PDA) of UHT milk.

For the proteomic study UHT milk samples were purchased at local supermarkets: two semi skimmed milks (indicated as sS^{1} and sS^{2}), two whole milks (indicated as W^{1} and W^{2}), a semi skimmed milk with reduced lactose (sS^{rl}), a skimmed milk (S) and whole pasteurized milk (P), which were centrifuged to recover the liquid phase or supernatant, containing any serum phase casein and whey proteins (PSsup) and the insoluble residue (PSres).

In this work, we used the term ‘insoluble residue’ to refer to the sediment obtained by centrifugation, while the term ‘sediment’ was used to refer to the natural sediment found at the bottom of the container.

The protein composition of PSsup and PSres was studied by a proteomic approach. All the commercial samples were stored from their purchase for about 6 weeks at ambient temperature and they were analysed about 6 weeks before their expiration date.

2.2 Milk samples for the assessment of protein dynamic arrangement (PDA)

For the assessment of the protein dynamic arrangement, four skimmed UHT milks, produced with a direct system, were obtained from local dairy industries. For each milk four batches were provided from the industries, in order to be analysed immediately (time 0) and after 1, 3 and 5 months of storage at ambient temperature. The natural sediment of UHT milk samples was obtained according to the gravimetric procedure of Ramsey & Swartzel, (1984) and weighted at each stage. The

resultant milk samples were processed, at each time of storage, to obtain the insoluble residue following the same centrifugation procedure described above, obtaining the UHT supernatant samples (PDAsup) and the insoluble residue samples (PDAres).
An overview of the two investigation procedures is illustrated in the Scheme 1.

2.3 Sample preparation for the electrophoretic analyses

UHT milk samples were shaken in their containers and a 50 mL aliquot was centrifuged (3000 *g*, 15 min, 4° C) using an IEC CL31R Multispeed centrifuge (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The upper solid fat was manually removed; the supernatants (PSsup) were recovered and stored at -80° C until electrophoretic analyses. The resultant insoluble residues (PSres), were washed three times with -20° C cold acetone (1:5 w/v), centrifuged and dried overnight under vacuum. Protein residues (0.01 g) were solubilized in 1.0 mL of 9 M Urea for electrophoretic analyses. All samples were analysed in triplicate.
Image analysis was carried out using Quantity One software (Bio-Rad, Hercules, CA); the protein variations were expressed from the trace quantity (Intensity x mm) of each band related to the total trace quantity of each electrophoretic pattern.

2.4 SDS-PAGE analysis

Superantants and insoluble residues aliquots (100 µL) were diluted (1:1 v/v) in a 2x Laemmli buffer (0.0625 M Tris–HCl, pH 6.8, 2% SDS, 10% (v/v) glycerol). All samples were analyzed under reducing (5% (w/v) dithiotreitol, DTT) and non-reducing (no DTT included) conditions.
Electrophoresis was carried out on large pore 12% polyacrylamide gels (16 x 18 cm) using a horizontal electrophoresis device Hoefer SE 600 (GE Healthcare, Milan, Italy) or on 15% polyacrylamide gels (8,6 x 6,7 cm) using a Mini-PROTEAN tetra cell (Bio-Rad, Hercules, CA). The gels were stained with 0.25% w/v Coomassie Brilliant Blue G-250 (CBB) overnight and destained with water. Prestained SDS-PAGE standards (Bio-Rad, Richmond, CA, USA) were used as protein molecular weight markers.

2.5 Two-dimensional gel electrophoresis: Alkaline Urea^{NR} / SDS^{R}- PAGE (2-D AU^{NR}/SDS^{R}-PAGE)

Two-dimensional gel electrophoresis was performed using, as the first dimension, an alkaline urea gel electrophoresis under non-reducing conditions and, orthogonal to this, as the second dimension, a SDS-PAGE electrophoresis under reducing conditions (AU$^{\boldsymbol{NR}}$ / SDS^{R}-PAGE). The 2-D AU$^{\boldsymbol{NR}}$ / SDS^{R}-PAGE was used to identify the components of the protein aggregates of both PSsup and PSres. In the AU first dimension, the PSsup and PSres samples were analysed under non-reducing conditions. The AU electrophoretic step was carried out using 7.5% polyacrylamide gels. After

separation, each lane, which contained all the protein bands, including the protein aggregates that had not migrated and still resided in the stacking gel, was cut. Proteins were reduced by soaking gel strips in 10% (w/v) SDS supplemented with 1.5% (w/v) DTT for 5 min. The excess of SDS-containing buffer was removed with deionized water. The strips were loaded on 15% SDS-PAGE gels (16 x 18 cm) and sealed using 0.5% (w/v) low melting point agarose (GE healthcare) to ensure contact between AU gel strips and the SDS-PAGE gel. Proteins were separated at 30 mA for 3 hours. Proteins were stained with Coomassie Brilliant Blue G-250.

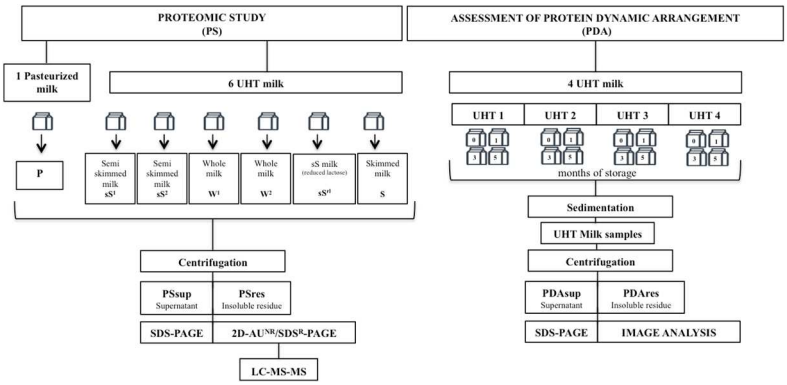
2.6 In-gel tryptic digestion of protein spots

Protein spots were in-gel digested according to Petrella et al., (2015). Briefly, gel spots were manually excised and destained by repeated washing with 50% acetonitrile (v/v) in 25 mM NH_4HCO_3. The proteins were then reduced by incubation with 10 mM DTT (1 h, 56 C) and alkylated with 55 mM iodoacetamide (45 min at room temperature in the dark). The gel plugs were thoroughly washed with NH_4HCO_3, dehydrated with 100 µl of 100% acetonitrile and dried in a SPD1010 speed-vac (ThermoFisher Waltham, Massachusetts, USA). The gel pieces were rehydrated with 10–15 µL proteomic grade modified trypsin (Promega, Madison, WI, USA, 12.5 ng ml^{-1} in 25 mM NH_4HCO_3) and proteins were digested overnight at 37° C. The resulting peptides were extracted three times by 40 µl of 50% acetonitrile (v/v)/5% formic acid (v/v). Extracts were combined, dried in a vacuum centrifuge, and re-dissolved in 50 µL 0.1% formic acid (v/v) and submitted to nanoflow high performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry (nano-HPLC-ESI-MS/MS).

2.7 nano-HPLC-ESI-MS/MS analysis

NanoHPLC-MS/MS identification of protein spot was performed using an Ultimate 3000 nano-flow ultra-high performance liquid chromatography (Dionex/Thermo Scientific, San Jose, CA, USA) coupled to a Q-Exactive Orbitrap mass spectrometer (Thermo Scientific). Tryptic peptide digests were loaded through a 5 mm long, 300 µm i.d. pre-column (LC Packings, USA) using a Famos autosampler (Thermo) and separated by an EASY-Spray™ PepMap C18 column (2 µm, 15 cm x 75 µm) 3 µm particles, 100 Å pore size (Thermo Scientific). Eluent A was 0.1% formic acid (v/v) in Milli-Q water; eluent B was 0.1% formic acid (v/v) in acetonitrile. The column was equilibrated at 4% B. Peptides were separated applying a 4–40% gradient of B over 45 min at a 300 nL min^{-1} constant flow rate, injecting variable amount ranging between 1/5 and 1/10 (based of the apparent intensity of the spot) of the peptide extract. MS1 precursor spectra were acquired in the positive ionization mode scanning the 1600-300 m/z range, with resolving power of 70.000 full width at half

maximum (FWHM), automatic gain control (AGC) target of $1x10^6$ ions and maximum ion injection time (IT) of 256 ms. The spectrometer operated in data-dependent acquisition, selecting up to 10 most intense ions for MS/MS fragmentation, applying a 10 s dynamic exclusion. Fragmentation spectra were obtained at a resolving power of 17.500 FWHM. Ions, with one or more than six charges, were excluded from MS/MS selection. Spectra were elaborated using the Xcalibur Software 3.1 version (Thermo Scientific). Raw files of the LC-MS runs were used to identifying the proteins using the Proteome Discoverer 2.1 software (Thermo Scientific) with Sequest search engine. Searches in the Uniprot database were taxonomically restricted to Bos Taurus (downloaded on October 2017). Database searching parameters were the following: carboxymethylcysteine as a constant modification; Met oxidation, N-terminus Gln as pyroglutamic acid and Ser/Thr phosphorylation as variable modifications; mass tolerance value of 5 ppm for precursor ion and 8 ppm for MS/MS fragments; trypsin as the proteolytic enzyme; missed cleavage up to 2. False discovery rate (FDR) set at 1% and protein identification scores were calculated by Target Decoy PSM Validator.



Scheme 1 - Schematic representation of the two paper sections

3. Results

3.1 Study of the dynamic arrangement of milk proteins (PDA samples)

At the beginning of this study we compared the protein composition of four UHT skimmed milk samples: as general features, UHT milk 1 and UHT milk 3 and related supernatans (sup1, sup3), insoluble residues (res1, res3) and sediments (sed1, sed3) (time 0) electrophoretic patterns under non reducing and reducing conditions are shown in Figure 1. Under non-reducing condition (Fig. 4A), milk (lane 1 and 2) and supernatant (lane 3 and 4) samples showed α_s-, β-CN fractions, a negligible band related to κ-CN and a smear of heat-induced aggregates, which did not entry into the resolving gel (AgW and AgR). The protein composition of the sediment (lane 5 and 6) and the

insoluble residue (lane 7 and 8) samples was not different and showed, mainly, the casein fraction and a smaller amount of aggregates.

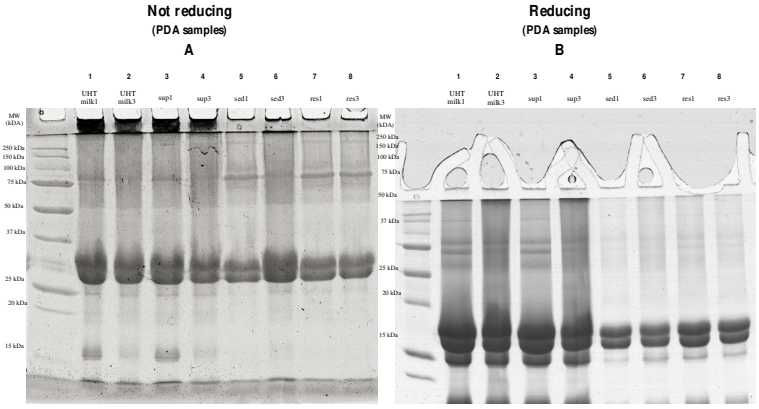


Figure 1. SDS-PAGE (T=15%; C=2,5%) of the UHT milk 1 and UHT milk 3 and related supernatants, sediments and insoluble residues for the study of the assessment of protein dynamic arrangement (PDA) under reducing (A) and not-reducing (B) conditions. In the two panels, the lanes 1 and 2 show the electrophoretic pattern of UHT milk (UHT milk1 and UHT milk3); lanes 3 and 4, the supernatant (Sup1 and Sup3); lanes 5 and 6, the sediments (Sed1 and Sed3); lanes 7 and 8, the insoluble residue (Res1 and Res3).

Under reducing conditions (Fig. 4B), the electrophoretic patterns of milks (lane 1 and 2) and supernatant (lane 3 and 4) samples showed the disappearance of the high molecular weight aggregates (AgW and AgR) and the concomitant showing of more bands, included in the range of 75 – 150 kDa. The presence of casein fractions, including the κ-CN, and whey proteins (β-Lg and α-La) bands was detected, as well. In the electrophoretic patterns of the sediments (lane 5 and 6) and insoluble residues (lane 7 and 8) the casein bands (α_s-, β-CN and κ-CN) were much more intense than the whey proteins, which were clearly present in a greater extent than under non-reducing conditions. Therefore, the first observation was that the casein and whey proteins were distributed in the supernatant and in the dispersed and sedimented forms with a different casein/whey protein ratio.

The dynamic shift of milk proteins was investigated on four skimmed UHT milk supernatant (Sup1, Sup2, Sup3 and Sup4) and related dispersed insoluble residues (Res1, Res2, Res3 and Res4) over 5 months of storage. The amount of the insoluble residue and sediment, the pH trend and the supernatant and insoluble residue protein trend variations throughout the storage, are shown in Figure 2.

The highest amount of insoluble residue was obtained at the beginning of the storage period (Fig. 2 Panel A) for all the samples (Res1, Res2, Res3 and Res4), whereas the lowest one occurred after 1 month. After the first month, the insoluble residue formation increased up to the third month, to undergo a subsequent decrease until the fifth month, except for the Res1 sample. Concomitantly, a linear decrease of pH (Fig. 2 Panel B), from 6.68 to 6.53, was observed throughout the storage

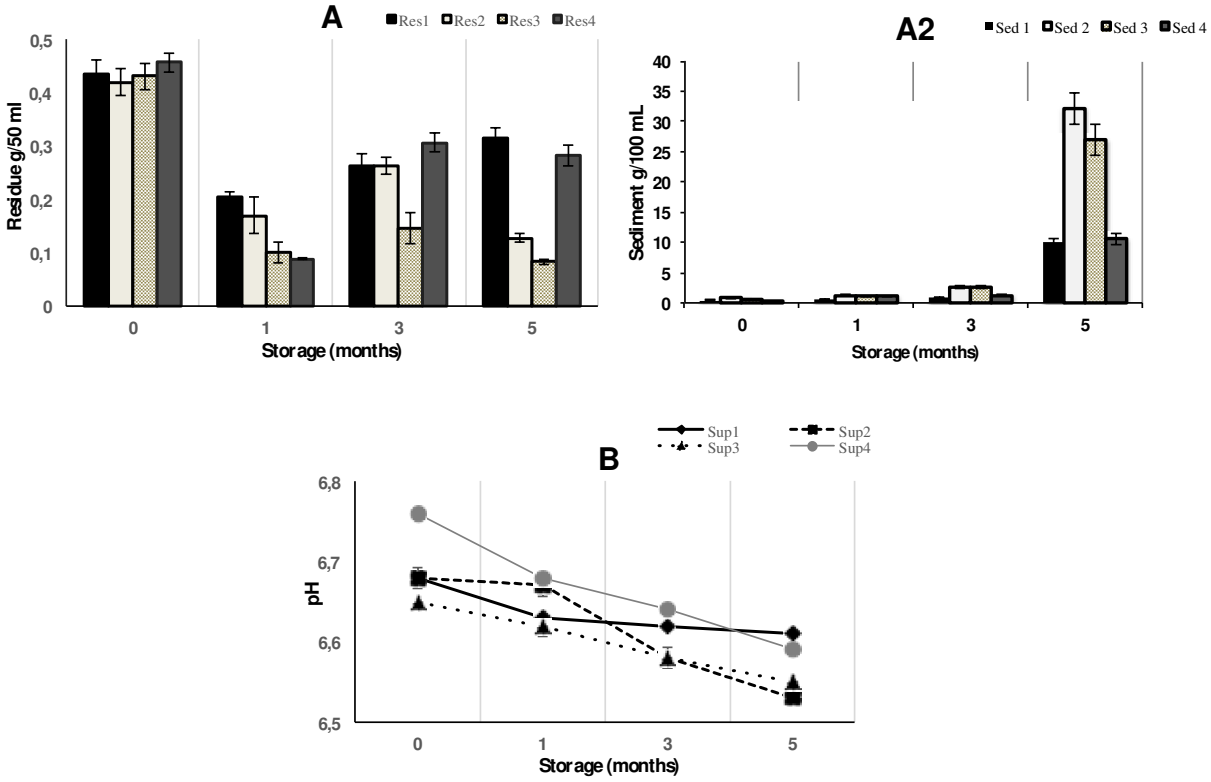
period, with a variation of 0.15 ± 0.04 pH units, recorded for the supernatants of UHT milk 2 (Sup2) and 3 (Sup3).

The supernatant of UHT milk 1 (Sup1) showed the slowest decrease, resulting in a negligible variation of pH (0.07 unit), and, consequently, the highest pH value (i.e. 6.61) at the end of the storage. The UHT milk 4 (Sup 4) showed a pronounced variation of pH (0.14 unit), but it recorded the highest pH value (6.76) at the beginning of the storage and the same final value of UHT milk 1.

From the panel related to the amount of natural sediment recorded at each stage of storage, (Fig. 2 Panel A2), it was clearly as the formation of a sediment became considerable only after the third month, with the highest values reached after five months of storage. At the end of storage, the highest amount of sediment was recorded in UHT milk 2 and UHT milk 3 samples, where the concomitant lowest value of the insoluble residue (Fig. 2 Panel A) and pH (Fig. 2 Panel B) were detected. In this regard, it was noteworthy that at the end of the storage (fifth month) the UHT milks 1 and 4 with a pH value higher than 6.55 showed more dispersed phase and less sediment than the UHT milks 2 and 3, which reached a pH value lower than 6.55. This could suggest that the UHT milk protein aggregation event is over time influenced by the pH variation and by pH value higher than 6.55; these results were consistent with that obtained by Gaur et al., (2018).

The trend of casein and whey proteins variation (expressed as trace quantity) was monitored in the supernatants and insoluble residues samples throughout the storage time, as results by SDS-PAGE^{R} coupled to image analysis and shown in Figure 2 (panel C-F). The casein and whey proteins, included in high molecular weigh aggregates, built by non-reducing covalent bonds included in the range of 75 – 150 kDa, were not computed in the calculation of the shown trends. In the supernatants (Fig 2C), the trace quantity of the casein fractions showed an opposite trend with respect to the insoluble residue formation (Fig. 2A). The casein fractions of the supernatant of UHT milk 3 (Sup3) and UHT milk 4 (Sup4) increased from the beginning of storage to the first month and a linear decrease was observed throughout the remaining period. The supernatant of UHT milk 2 (Sup2) showed a not significant decrease between the third and fifth month, whereas the supernatant of UHT milk 1 (Sup1) showed, in the same period, a not significant increase. The trends of the whey proteins (α-LA and β-LG) trace quantity of the Sup1 and Sup3 exhibited an increase up to the first month and then a decrease throughout the remaining storage period. Differently, the whey proteins trace quantity of Sup4 increased up to the third month and decreased only during the last period, while in the case of Sup2, the whey proteins trace quantity showed an oscillating trend, fitting a cubic function. In the case of the insoluble residues, both caseins and whey proteins showed different trends with respect to the related supernatants, exhibiting a general not significant decrease throughout the storage period. Differently, the insoluble residue of UHT

milk 4 (Res4) had the highest band intensity for casein and whey proteins all along the five months of storage. As a general feature, the variation of protein composition of the supernatant samples was more evident, probably, depending on intrinsic factors of the milks and from their response to the heat treatment.



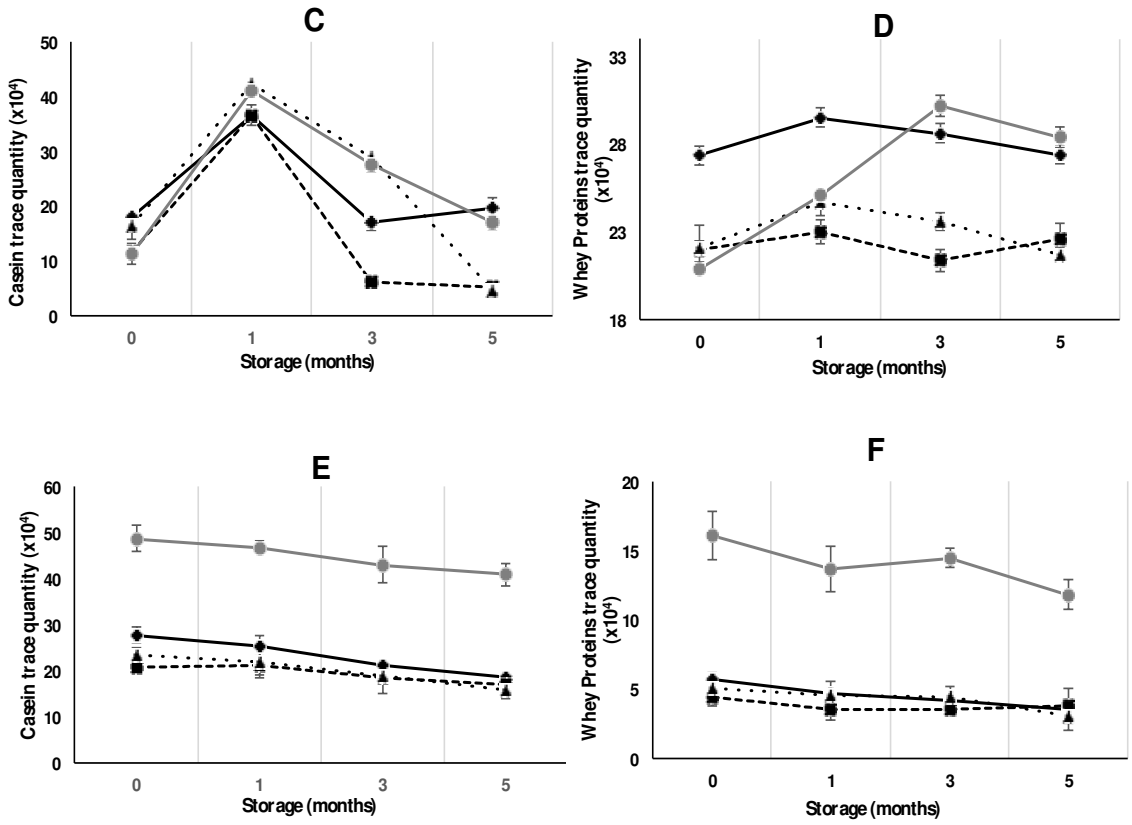


Figure 2. Study of the dynamic arrangement (PDA) of the supernatant (Sup1 (◆□□, Sup2 (■□□, Sup3 (▲) , Sup4 (•· · and insoluble residues (Res1 (◆· · , Res2 (■· · , Res3 (▲), Res4 (•· · samples during the storage (0, 1, 3 and 5 months). **A**: amount of the insoluble residue; **A2**: amount of the sediment; **B**: variation of pH; **C**: trend for the casein fraction in the supernatant; **D**: trend for the whey proteins in the supernatant; **E**: trend for the casein fraction in the insoluble residue; **F**: trend for the whey proteins in the insoluble residue.

All these results supported the shift of soluble and insoluble protein forms within the UHT milk system, as shown in Figure 3. In this figure, the casein and whey proteins percentages obtained from the bands trace quantity values in the supernatants (Sup) and in the insoluble residue (Res) for the four UHT milk samples (UHT milk 1, 2, 3 and 4) are reported, according to the time of storage. It is noteworthy that the proportion of whey proteins (WP) in the supernatant samples was higher than the casein fractions (CN), with exception of the first month of storage, where a decrease in the insoluble residue formation was detected, probably affected by the re-arrangement of the casein fractions into soluble forms (Fig. 2 A and C). In the supernatant samples we found that the average of the percentages of the intact forms of whey proteins was 62% ± 3.4 at the beginning of the storage, 39.3% ± 2.7 after the first month, 59.4% ± 12.3 at third month and 71.1% ± 10.8 at fifth month. Therefore, the decrease of the percentages and the marked increase of the variability of the casein fraction after the first month could indicate a different and dynamic arrangement of this protein fraction in the liquid phase during the storage. Differently, the proportion of casein in the insoluble residue samples was always higher than the whey proteins and the casein-to-whey protein

ratio (80/20) was quite constant during the five months of storage, probably due to the hierarchical aggregation system of casein and whey proteins in the sedimentation process.

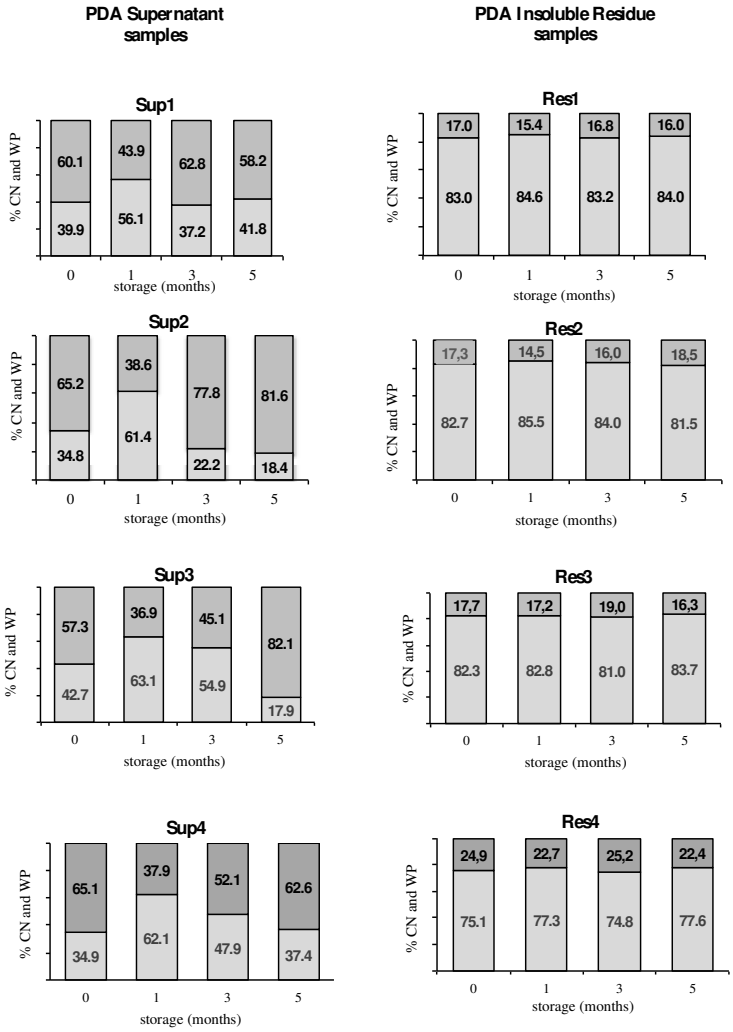


Figure 3. Proportions of the casein fraction and whey proteins obtained from the bands trace quantity values in the supernatant (Sup1, Sup2, Sup3 and Sup4) and insoluble residue (Res1, Res 2, Res3 and Res4) samples for PDA study throughout the storage (0, 1, 3 and 5 months).

In order to focus the contribution of each main milk protein to the variation observed in the supernatants and insoluble residues, we considered the average of the trace quantity of the protein bands detected under reducing conditions in the four UHT milk samples (Fig. 4A-4B).

The values recorded for the three main caseins (α_s-, β- and κ-CN) in the supernatant protein patterns were the same at the beginning of the storage (time 0). However, while the trace quantity of κ-CN band remained constant up to the fifth month, being not significant the observed variations, the trace quantity of α_s, and β-CN bands showed a relevant increase after the first month of storage

and then a decrease until the end. As concerns the whey proteins, the trace quantity of β-Lg band was much higher than that of α-La and while the former showed a slight increase along the storage, the latter remained constant.

The trace quantity variations of the caseins in the insoluble residues highlighted as the most represented proteins were α_s, and β-CN, with values higher than κ-CN, which remained constant along the storage and with values similar to those detected in the supernatants. It was interesting to note as the κ-CN trend in the supernatants was complementary to that detected in the insoluble residues and the contribution of α_s-CN fraction was higher than β-CN in both supernatants and insoluble residues.

Finally, the trace quantity of the considered whey proteins in the electrophoretic patterns of the insoluble residues were lower than those recorded in the supernatants, with the values of β-Lg analogous to those of κ-CN. The constant trend of the main whey proteins and of κ-CN along the storage, confirmed their involvement in the formation of non-sedimentable material in the supernatant (i.e soluble aggregates) linked through hydrophobic interactions and thiol/disulfide interchanges (Guyomarc'h et al., 2003; Li et al., 2015).

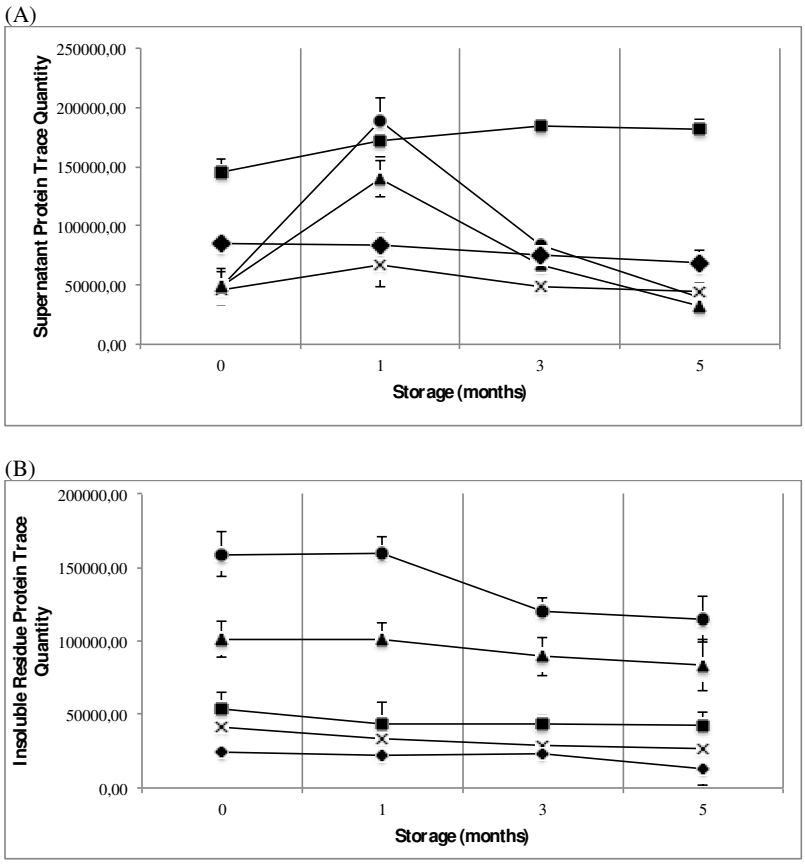


Figure 4. Average of the trace quantity of α_{s1}/α_{s2} casein (•□□□ β-casein (▲), κ-casein (×), β-lactoglobulin (■· · , α-lactalbumin (◆· · of the protein bands separated under reducing conditions in the supernatants (Panel A) and in the insoluble residues (Panel B) in the four UHT milk samples.

3.2 Proteomic study of UHT milk (PS samples)

All UHT commercial milk samples analysed in the proteomic study (Scheme 1) were separated by centrifugation into two fractions, supernatants (PSsup) and insoluble residues (PSres). The amount of the dried insoluble residues for each milk sample is reported in the Table 1. The most abundant amount was observed for the whole fat UHT milk samples (W^1 and W^2), probably due to the fat contribution, since the heat treatment causes changes in the milk fat globule membrane increasing interactions with milk proteins. The milk fat globule surface that is covered by milk proteins after processing of commercial milks is higher for full fat and skimmed UHT milks than the semi-skimmed (Lopez, 2005) and this, possibly, can produce a higher amount of insoluble residue when UHT milk undergoes centrifugation too.

Table 1. Amount of insoluble residue recovered from the centrifugation of the UHT milk

UHT milk	Insoluble residue (g 50 ml^{-1})
Semi skimmed (sS^1)	0,41±0,06
Semi skimmed (sS^2)	0,22±0,05
Whole (W^1)	2,07±0,45
Whole (W^2)	3,62±0,43
Semi skimmed with reduced lactose (sS^{rl})	0,91±0,10
Skimmed (S)	0,86±0,11

The role of disulphide bonds in the heat-induced aggregates of the UHT milk proteins was investigated by comparing the large pore SDS-PAGE under reducing and non-reducing conditions. The electrophoretic patterns of UHT supernatants (PSsup) and insoluble residues (PSres) are shown in the figure 5. Under non-reducing conditions (Fig. 5A), a series of intense bands were detected at MW >250 kDa and at about 76 kDa along with protein aggregates detected in the loading wells (AgW) and at the entrance of the resolving gel (AgR). The pastourized sample (P) showed also an additional band at 60 kDa. Under reducing condition (Fig. 5B), the bands detected between 250 kDa and 60 kDa disappeared or decreased their intensity, denoting that disulphide bonds were involved in the formation of high molecular weight protein aggregates. As concerns the casein (CN) fractions, it was noteworthy the presence of κ-CN band and the increase of smear at level of α_{s2}-CN under reducing condition. The electrophoretic patterns remained substantially unmodified below 25

kDa, in both the conditions, even though the bands related to β-Lg were more intense under reducing conditions.

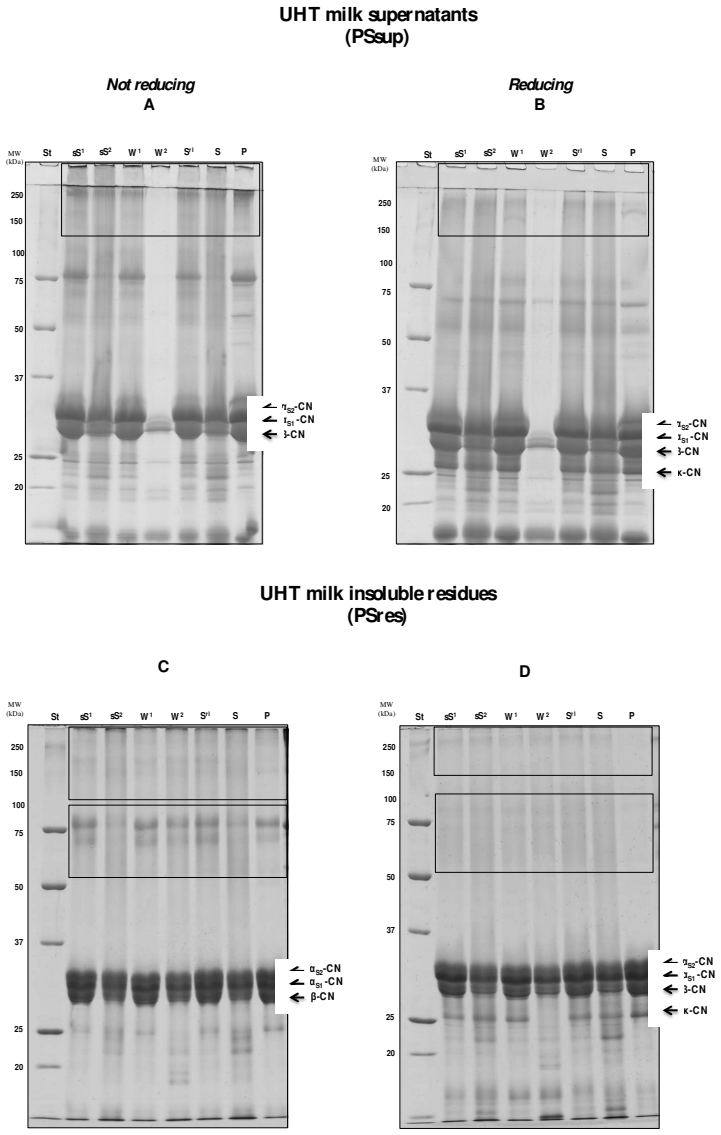


Figure 5. Large pore SDS-PAGE (12%) of UHT milk supernatants (PSsup) and insoluble residues (PSres) under reducing (Panel A and C) and non-reducing (Panel B and D) conditions. Lanes: sS^1 and sS^2, semi skimmed milks; W^1 and W^2, whole milks; sS^{ri}, semi skimmed milk with reduced lactose; S, skimmed milk; P, pasteurized milk; St, protein standard.

Interestingly, the W^2 sample showed only the β-CN band in the supernatant (PSsup; Fig. 5A and 5B), unlike α_s-and β-CN bands were detected both under non-reducing and reducing conditions in the insoluble residue (PSres; Fig. 5C and 5D); κ-CN band was missing in the PSres, under both the conditions (Fig. 5C and D). Mostly, under non-reducing conditions, the insoluble residues (Fig. 5C) contained bands at high molecular weight (mainly around 70 and 75 kDa), which disappeared after

reduction (Fig. 5D). Therefore, it was clear that disulphide bridges were accountable for the presence of heat-induced aggregates also in the insoluble residue.

Since polypeptides with MW<18 kDa escape the detection in large pore electrophoresis, the involvement of the whey proteins in the insoluble residue was explored using 15% polyacrylamide SDS-PAGE under reducing and non-reducing conditions (Fig. 6). The bands related to whey proteins were more evident under reducing conditions than non-reducing conditions (Fig. 6A, 6B). The main whey proteins, β-Lg and α-La, were clearly detected in all the samples. In the case of W^2 sample, the lack of κ-CN, even under reducing conditions, was sustained by the evidence of a band corresponding to para-κ-CN.

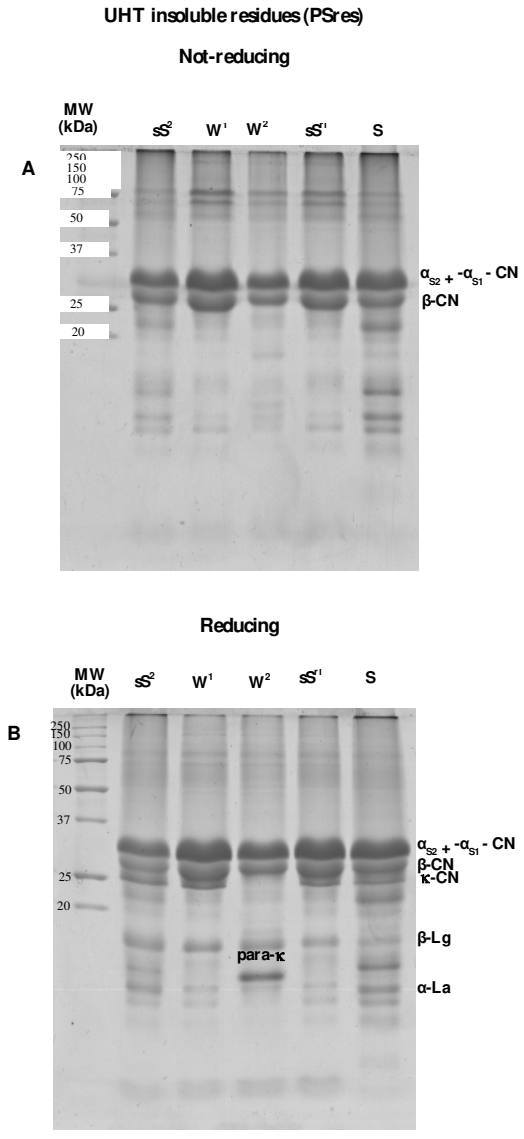


Figure 6. Small pore SDS-PAGE (15%) of the insoluble residues (PSres) under reducing (A) and not-reducing (B) conditions. Lanes: sS^2, semi skimmed milk; W^1 and W^2, whole milks; sS^{r1}, semi skimmed milk with reduced lactose; S, skimmed milk.

3.3 Two-dimensional gel electrophoresis (2-DE) UREA^{NR}/SDS^{R}-PAGE and mass spectrometry analysis

The composition of larger heat-induced protein aggregates in UHT milk (AgW and AgR) samples was investigated by alkaline 2-DE AU^{NR} / SDS^{R}-PAGE (Fig 7). In this part of the study, the commercial UHT skimmed milk sample (S) was shown, since it appeared as proteolyzed and we intented to study also if and how the protein fragments could be involved in the formation of heat-induced protein aggregates. The 2-DE map of the supernatant of the UHT skimmed milk sample (PSsup; Fig. 7A) showed a higher number of protein spots than the corresponding insoluble residue (PSres; Fig. 7B). The high molecular weight aggregates, AgW and AgR, were the hetero-protein aggregates with molecular weight higher than 250 kDa (Fig. 5A), which were the smears in the first dimension (AU-PAGE^{NR}). They were assemblies of whey proteins, caseins and casein fragments, as assessed by LC-MS/MS analysis of the protein spots isolated by 2-DE map.

The identification of the proteins involved in the heat-induced supramolecular structures (AgW and AgR) and of those protein being present in soluble forms (as intact protein or as small aggregate) in the supernatant is reported in the table 2A. In the 2-DE map (Fig. 7A) the protein spots 1 - 11 are referred to the AgW, the smear at the top of the stacking gel, while the spots 12 - 22 to the AgR, the smear that did not enter into the resolving gel. These supramolecular aggregates, which were separated under reducing conditions in the second dimension, seemed to be composed by a scaffold of whey proteins, mainly β-Lg, linking α_{s1}- and κ-CN, lactoferrin, PIGR (polymeric IgG receptor), lactadherin and lipoprotein lipase. AgW supramolecular aggregates also contained α_{s1}-CN (spot 7). The protein spots from 31 to 33 and from 39 to 41 of PSsup were respectively β-LG (MW 18.1 kDa), occurring with A and B variants, and α-LA (14.4 kDa) and its lactosylated forms. According to the molecular weight, spots 25, 26, 29 were β-CN fragments, while spots 34, 35, 36 were α_{s1}-CN fragments. The spots 23, 26, 28, 30, 33, 38 were represented by hetero-protein aggregates of protein fragments, which included different mixtures of α_{s1}-CN, κ-CN, β-CN and β-Lg. Co-migration of proteins in hetero-protein aggregates could be due to the association through covalent or tight hydrophobic binding. The detection of multiple protein chains in 2-DE isolated spots is a rather frequent event when spots are analyzed with high sensitivity MS instruments (Caira et al., 2017).

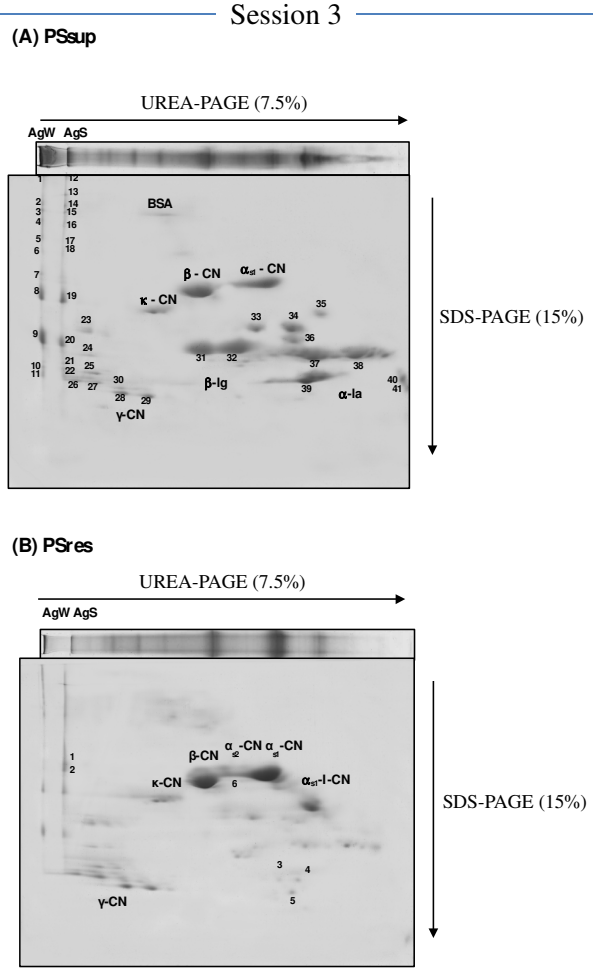


Figure 7. Two-dimensional gel electrophoresis of sample S (UHT skimmed milk): UHT milk supernatant, PSsup (panel A) and UHT milk insoluble residue, PSres (panel B). The first dimension was Alkaline Urea-PAGE under non-reducing conditions and the orthogonal second dimension was SDS-PAGE under reducing condition. The identification of numbered spots was reported in supplementary table 1.

The 2-DE map of PSres (Fig. 7B) was qualitatively similar to that of the supernatant and all the detected spots belonged to the casein fractions. The identification of those spots that were not detected in the 2-DE map of PSsup (Fig. 7A), were also reported in the supplementary table 2B.

The intensity of the larger aggregates in the insoluble residue (Fig. 7B) was lower than that of the supernatant (Fig. 7A) and the spot related to α_{s1}-casein was not found in the AgW, as in the case of the supernatant pattern (Table 2A, spot 7), but it was detected in AgR as aggregate with β-CN (Table 2B, spot 1). A number of protein spots at lower molecular weight than the caseins, assigned to α-La, β-Lg and casein fragments, including γ-CNs, were detected as well.

Table 2. LC-MS/MS based assignment of the protein spots isolated by 2-DE (Fig. 3A-3B)

(A) UHT milk supernatant (PSsup)

spot	Protein	Uniprot accession	No. unique peptides	Coverage (%)
1	lactoferrin	P24627	7	11
	BSA	P02769	4	6
	β-Lg	P02754	4	22
2	lactoferrin	P24627	7	11
	PIGR	P81265	4	7
3	BSA	P02769	11	16
4	β-Lg	P02754	4	25
	lactadherin	Q95114	5	13
	lipoprotein lipase	P11151	4	14
5	β-Lg	P02754	6	38
6	β-Lg	P02754	5	23
7	α_{s1}-casein	P02662	4	26
8	β-Lg	P02754	4	24
	κ-casein	P02668	3	19
9	β-Lg	P02754	9	65
10	β-Lg	P02754	3	22
11	α-La	P00711	3	27
	β-Lg	P02754	3	22
	fatty acid binding protein	C0LSL0	3	18
12	Lactoferrin	P24627	3	7
13	β-Lg	P02754	4	31
	BSA	P02769	10	21
	lactoferrin	P24627	13	20
	actin	P60712	5	22
14	lactoferrin	P24627	9	16
	β-Lg	P02754	3	19
	PIGR	P81265	3	6
15	BSA	P02769	16	32
	β-Lg	P02754	3	22
	α_{s2}-casein	P02663	3	12
16	β-Lg	P02754	3	18
17	β-Lg	P02754	3	25
	κ-casein	P02668	3	20
18	β-Lg	P02754	5	31
	κ-casein	P02668	3	18
19	κ-casein	P02668	3	18
	apolipoprotein A-I	P15497	5	22
	β-Lg	P02754	3	17
20	β-Lg	P02754	8	65
21	β-Lg	P02754	3	22
	α_{s1}-casein	P02662	3	19
	κ-casein	P02668	3	18
22	Not identified			
23	α_{s1}-casein	P02662	7	53
	β-casein	P02666	3	19
	α_{s2}-casein	P02663	3	22
	κ-casein	P02668	3	19
	β-Lg	P02754	3	38
24	α_{s1}-casein	P02662	3	18
25	β-casein	P02666	3	20
26	β-casein	P02666	3	22
	α-La	P00711	4	25
27	α_{s1}-casein	P02662	5	46
28	α_{s1}-casein	P02662	5	39
	β-casein	P02666	3	21
29	β-casein	P02666	4	29
30	α_{s1}-casein	P02662	7	52
	β-Lg	P02754	3	19
	κ-casein	P02668	3	21
31	β-Lg	P02754	15	81

32	β-Lg	P02754	11	73
33	α_{s1}-casein	P02662	5	28
	β-Lg	P02754	4	37
34	α_{s1}-casein	P02662	5	32
35	α_{s1}-casein	P02662	7	53
36	α_{s1}-casein	P02662	6	31
37	β-casein	P02666	5	31
38	α_{s1}-casein	P02662	4	19
	β-casein	P02666	3	25
39	α-La	P00711	9	75
40	α-La	P00711	5	30
41	α-La	P00711	4	26

α-La = α-Lactalbumin, β-Lg = β-Lactoglobulin; BSA = Bovine serum albumin; PIGR = Polymeric IgG receptor

(B) UHT milk insoluble residue (PSres)

spot	Protein	Uniprot accession	No. unique peptides	Coverage (%)
1	α_{s1}-casein	P02662	7	49
	β-casein	P02666	3	19
2	β-casein	P02666	5	31
3	β-casein	P02666	4	22
4	β-casein	P02666	4	22
5	β-casein	P02666	5	27
6	α_{s1}-casein	P02662	6	33
	α_{s1}-casein	P02663	3	21

4. Discussion

In the study of protein arrangement during the UHT milk storage, the first observed phenomenon was the different trend of the pH variation and the insoluble residue and sediment amount recorded. The supernatant protein trends throughout the storage highlighted that casein fraction varied much more than whey proteins. A greater solubilisation of caseins during the first month, with the concomitant lowest value of the insoluble residue recorded for all the samples, should have suggested a re-arrangement of the milk proteins by a self-assembling mechanism. The differences in the amount of the insoluble residue were probably related to the pH variation along the storage, with an average of 0.15 pH units, that affected the protein arrangement in the supernatant, since the trace quantity of the soluble and not linked casein fraction always decreased. Lewis, Grandison, Lin, & Tsioulpas, (2011) found that a very small change in the ionic calcium and/or pH could shift a stable system to an unstable system, thus the lowering of the pH and/or the high ionic calcium levels can promote sedimentation event.

Gaur et al., 2018 confirmed these results and asserted that as the pH decreases, the concentration of ionic Ca^{2+} increases, thus favouring casein micelles association, as a consequence of the κ-CN depletion and the sentivity of phosphocaseins (mainly α_s fractions) to the Ca^{2+} ions. Considering the

single protein variation in the supernatants and in the insoluble residues, the consistent decrease of α_s- and β- CN fractions, as soluble forms, after the first month of storage, confirmed the role that these proteins could have in the formation of non-reducible aggregates, which occurred along the storage of UHT milk (Holland et al., 2011). The concomitant high value found also in the insoluble residue at the beginning of storage, suggested a multifaceted protein organization within the milk system, where the κ-CN depletion and/or κ-CN/β-Lg complex formation kept off the ability to protect micelles from calcium ions (Anema, 2009), leading to the arrangement of a spontaneous supramolecular insoluble protein network (i.e insoluble residue), which involved, mainly, the casein fraction (80%). These events guide the milk protein in a dynamic shift among soluble, insoluble dispersed forms, supramolecular aggregates and, finally, towards the sediment formation, as the casein aggregation event lasts. The electrophoretic investigation on the six commercial UHT milk was carried out to show the protein composition of UHT milk supernatant and the insoluble residue. As expected, milk protein aggregates with high molecular weight were detected, which disappeared under reducing conditions, increasing the number of the bands with a lower molecular weight, with the evidence of κ-CN band and an increase of the intensity of β-Lg band (Fig. 5B). It is known that the formation of disulphide bonds between β-Lg and κ-CN in heated milk occurs as the result of the denaturation process of β-Lg that, exposing free sulfhydryl group, leads to both intramolecular and intermolecular thiol-disulphide exchange reactions (β–Lg: Cys 66, 119, 121 and 160; κ-CN: 11 and 88) (Anema, 2009). An additional information was obtained by the electrophoretic analysis of W^2 sample, that revelead a 'limit situation', showing the β-CN as a faint band in the supernatant and a more intense β-Lg band in both the conditions. Surprisingly, its insoluble residue, under reducing condition, clearly exhibited the α_s- and β-CN bands to a greater extent and β-Lg band to a lower level (Figure 5C - 5D). In addition, W^2 insoluble residue, under reducing conditions, showed the para-κ-CN band, instead of the whole κ-CN (Fig. 5B). The presence of para-κ-CN in the insoluble residue of W^2 sample was due to the proteolytic action of endogenous enzymes (cathepsin from somatic cells) and/or bacterial proteinases occurred on the κ-CN. This was ascribed as a consequence of the status of the raw milk before the heat treatment, determined by an inadequate storage of fresh milk. At this point two considerations were inferred, the first was that part of β-CN shifted in the insoluble residue remaining intact, whatever was the history of UHT milk processing and storage, the second was that the insoluble residue is made essentially of the casein fractions and, to a lower extent, of whey proteins.

As concerns the high molecular protein fraction, the native milk protein polymers in raw milk and the changes at the level of each polymer in heated milk at 90°C were already assessed using non-reducing first dimension and reducing second dimension (SDS^{NR}/ SDS^{R}) technique (Chevalier &

Kelly, 2010), but by this procedure the large aggregates were not visualized, since they were not able to enter into the second dimension. The application of 2D-electrophoresis (AU-PAGE-SDS-PAGE) was used by Patel et al., (2006) to assess the aggregates disulfide-bonding composition in treated milk samples with high casein concentrations.

The aggregation among caseins and between casein and whey proteins is a complex phenomenon, being influenced by the process and the storage conditions and involves several covalent cross-links besides disulphide bridges (Friedman, 1999; Al-Saadi & Deeth, 2008; Holland et al. 2011). These large aggregates, induced throughout processing, were also defined as irreversible supramolecular structures (Bouhallab & Croguennec, 2013).

In this part of the study we assessed the composition of the heat-induced supramolecular protein aggregates in the UHT skimmed milk sample (S sample), because it showed the more complex protein map in the 2-DE AU^{NR} / $SDS\text{-}PAGE^{R}$ (Figure 7A and B), being proteolyzed. The S sample gave us the possibility to understand if and how the proteolysis products can be involved in the constitution of the high molecular weigh aggregates.

The composition of the AgW and AgR supramolecular aggregates was assessed through the application of a proteomic approach (2-DE AU / SDS-PAGE) developed by Patel et al., (2006), but performing the second dimension under reducing conditions (2-DE AU^{NR} / $SDS\text{-}PAGE^{R}$), aiming to assess also of the protein insoluble residue composition. The protein components with different molecular weight, which migrated along the line of AgW and AgR in the second dimension, represented the small aggregates or the intact proteins which formed the above cited supramolecular aggregates. With the second dimension separation only the disulfide-bonded aggregates were detached, while those involving other types of covalent links, persisted. The identification of the spots from Alkaline $Urea^{NR}$ / SDS^{R}- PAGE maps revealed aggregates containing (according to the estimated molecular weight) casein fractions, casein fragments and whey proteins. We found that the two main heat-induced supramolecular aggregates (AgW and AgR) comprised caseins linked with whey proteins and/or their fragments with both reducible –S–S– bridges and non-reducible links. Moreover, we found also casein fragments into the aggregates detectable at lower molecular weight. The spots detected at higher molecular weight (spot from 1 to 6) were, mainly, related to whey proteins, while those at lower molecular weight (spot from 7 to 11) included α_{s1}, κ-CN and whey proteins, mainly, with β-LG, as single protein or as aggregate. Since they were identified under denaturing and reducing conditions, their presence can be due only to non-reducible covalent links. It was noteworthy that α_{s2}-CN was identified in two spots, 15 and 23, combined respectively with BSA and β-Lg, as intact protein and with α_{s1}-, β- κ-CN and β-Lg, as protein fragments, given the estimated molecular weight of this latter aggregate of about 22 kDa. Despite the reducing

conditions of the second dimension, α_{s2}-CN was found to be linked to the other proteins, probably, with the same interaction of α_{s1}-CN, confirming the complexity of the protein aggregates architecture and that the α_{s1}-CN is the most involved protein in the formation of the non reducible aggregates, according to the findings of Holland et al., (2011).

These results demonstrated that the Cys-containing milk protein, namely α_{s2}-CN, κ-CN and the whey proteins, can be engaged in disulphide bridges, generating both co- and hetero-protein aggregates with high molecular weight (Guyomarc'h et al., 2003; Chevalier & Kelly, 2010). Moreover, these aggregates could have a supramolecular architecture built up by monomers of casein fractions and/or casein fragments with whey proteins not only through–S–S– link, but involving also other non-reducible covalent bonds and tight hydrophobic bindings, as demonstrated by the persistence of some protein aggregates on the 2-DE map of UHT milk supernatant.

These findings supported the hypothesis that the proteolysis products, occurring during the UHT milk storage, became part of the supramolecular aggregates, moving the milk proteins towards the formation of soluble/dispersed and then sedimented forms.

The map of the insoluble residue highlighted that this comprised, mainly, the casein fraction and the presence of the β-CN protein fragments (spot 3, 4 and 5, Table 2B) supported a possible proteolytic action of endogenous and bacterial enzymes (Lopez-Fandino, Olano, Corzo, & Ramos, 1993) or even the breakdown of peptide bonds, as a consequence of amino acid oxidation (Wust & Pischetsrieder, 2016). Furthermore, covalent cross-linking of proteins, through Maillard and dehydroalanine products (Sunds, Rauh, Sørensen & Larsen, 2018) contributes to form insoluble particles and sediment (Dalgleish & Corredig, 2012; Datta, Elliott, Perkins, & Deeth, 2002).

5. Conclusion

Heat treatment employed to produce UHT milk induces the assembling of the milk proteins in supramolecular aggregates, which are distributed between supernatant and insoluble dispersed residue. The formation of sediment gets through a supramolecular reversible architecture that, therefore, dynamically changes along the storage. The casein fragments are involved in the supramolecular aggregates formation when enzymatic reactions occurred in UHT milk during the storage, due to a residual enzyme action and as result of chemical changes, temperature and pH-depended. Indeed, along the storage, the protein fraction in the supernatant showed a great variability, especially the casein fraction, whereas the insoluble residue showed a composition with a constant casein/whey proteins ratio of about 80/20. Our results suggested the existence of a dynamic arrangement of milk proteins between the soluble and insoluble forms, that anyway, along the storage, lead to the sediment formation. For this purpose two main step could be proposed: in

the first step, the reactions are induced by heat treatment with the formation of heat-induced supramolecular aggregates (disulfide and not disulfide bonded) at higher energy; the second step, which occurs during the storage, is a spontaneous re-arrangement of milk proteins up to the formation of supramolecular insoluble aggregates with a well-defined proportion (casein/whey proteins ratio of about 80/20). Therefore, the milk protein rearrangement shifts across three systems: soluble forms, insoluble/dispersed architecture and sediment formation.

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Conclusions and Future Perspectives

The study of protein structures, which can arrange themselves in supramolecular architectures, both as induced or spontaneous event, is a new topic in food science.

The study of the food structure with a bottom-up approach, starting from the molecular organization, passing through the arrangement of supramolecular structures, can help to understand the machinery that rules the interactions among the food constituents and that can explain some of the food matrix properties.

The technology affects the food matrix causing the protein rearrangement, a mechanism that implicates known and still unknown effects on chemical-physical, technological and sensory characteristics and, which have a possible role also in the protection or delivering of other food ingredients, including those with a biological activity.

Publications

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