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Coupling Proteomics to Metrology: Identification and Absolute Quantification of Food Allergens

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Declaration

I hereby declare that the contents and organisation of this dissertation constitute my own original work and does not compromise in any way the rights of third parties, including those relating to the security of personal data.

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2025

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I would like to dedicate this thesis to the ones who are my home — to those who showed me that love gives meaning, and determination gives shape.

Acknowledgment

At the end of this Doctoral thesis work, I would like to sincerely thank you to all the people who, in different ways, have accompanied me to this result.

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Finally, to those who taught me that passion and determination are the true forces behind every meaningful step—thank you!

Summary

Food allergies represent an increasing challenge in the current scientific landscape, with significant implications at the clinical, regulatory, and industrial levels. Throughout the doctoral project, challenges related to the biological complexity of food allergens, the diversity of involved food matrices, and the impact of technological processing on their detectability were addressed. The work was structured around the development of an integrated analytical approach, capable of combining the molecular characterization of emerging allergens with their accurate quantification in real-world applications. This path led to the definition of a comprehensive methodological model aimed at understanding immunological mechanisms and at developing reliable tools for managing allergen risk in clinical, industrial, and regulatory contexts. Specifically, the first project focused on the identification of an emerging allergen, galactose-α-1,3-galactose (α-Gal), associated with red meat allergy syndrome (Alpha-Gal Syndrome, AGS). The study made it possible to systematically investigate, for the first time, a fraction of milk that had so far been little explored in this context: the milk fat globule membrane (MFGM). Within this component, proteins potentially involved in the immune response of sensitized individuals were identified. Analysis performed through liquid chromatography coupled with mass spectrometry revealed that the immunological interaction observed in the sera of AGS patients was attributable to the glycan portion of the proteins, rather than to their peptide backbone. This project thus provides the first direct experimental evidence of the presence of the α-Gal epitope in proteins from the lipid fraction of milk, suggesting that dairy products—alongside red meat—may also represent a potential risk for sensitized individuals. The second project focused on the accurate quantification of allergenic proteins present in trace amounts in red wine, deriving from the use of animal-derived fining agents such as egg white and gelatin. The main challenge of the project concerned the development of an analytical method capable of operating within a complex matrix such as wine, while ensuring precision, reliability, and metrological traceability of the results. To achieve this, an advanced approach based on high-resolution mass spectrometry was adopted, enabling the quantification of peptide markers of the target proteins through an analytical chain traceable to the International System of Units (SI). This work led to the development of a robust and innovative method, grounded in metrological principles, which offers a new and more reliable perspective for managing allergen risk in enological products. Overall, the work provides an innovative contribution to molecular allergology, proposing an integrated approach that supports both research and practical application in allergen risk management.

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1.1 The biochemistry of food allergenic proteins

1.1.1 The structure of proteins

Proteins are complex biological macromolecules composed by chains of amino acids linked by peptide bonds, which are formed when an amino acid's carboxyl group and another amino acid's amino group come together during a condensation reaction that results in the loss of a water molecule (Alberts *et al*., 2002). All the information required for a protein to correctly fold into its "native" conformation is contained in its **primary structure**, which is the linear sequence of amino acids.
Hydrogen interactions between a carbonyl oxygen atom and the amide hydrogen of peptide bonds allow amino acid sequences to fold, forming the **secondary structure** of proteins (*Fig. 1.1*), which mostly consists of alpha-helix and beta-folded sheet structures (Creighton *et al*., 1997). A high number of hydrogen bonds are created to build secondary structures, which then tend to interact and pack tightly inside the protein to form the **tertiary structure**. Its overall three-dimensional shape is maintained by ionic connections, disulfide bonds, and hydrophobic interactions. This folding is essential to the protein's biological activity. Predicting a protein's three-dimensional conformation based just on its amino acid sequence is therefore one of the main objectives of researchers studying protein structure. Recent advances have been primarily attributed to artificial intelligence and machine learning technologies. One such example is AlphaFold, which predicts the three-dimensional conformation of an amino acid sequence with

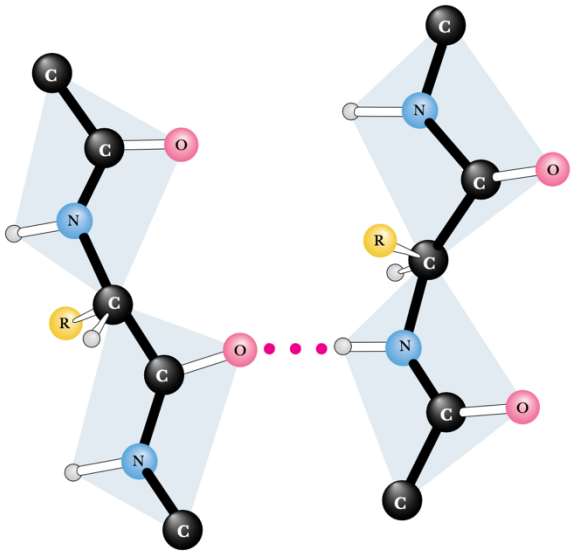


Figure 1.1: Hydrogen bond between the amide proton and carbonyl oxygen of adjacent peptide groups. (Creighton, "Proteins: Secondary, Tertiary, and Quaternary Structure" (1997) chapter 6)

high accuracy by utilizing deep neural networks and a large amount of data on known protein structures (David *et al*. 2022). Proteins fold to build structures that

are as stable as possible; in fact, proteins are only stable when multiple intramolecular hydrogen bonds are formed. No protein is stable if it has a single-layer structure. The **quaternary structure** results from the interaction of several protein molecules, each of which has a unique tertiary structure, to form complexes with multiple subunits (ex. hemoglobulin).
A protein's three-dimensional structure is intimately connected to its biological activity, making it essential for both the protein's function and its allergenicity (Breiteneder *et al*., 2004). As a result, the stability of a protein is essential. It is enhanced by the presence of disulfide bonds. Some food allergens, such as nsLTP, 2S albumin and a-amylase inhibitors in grains, have many disulfide bonds and are more heat resistant (Thompson *et al*., 1999).

1.1.2 Proteins as allergens

The allergenic activity of a protein depends largely on its structure, in particular on its stability and resistance. The epitope is defined as an antigenic region of a protein that can bind specific antibodies (Breiteneder & Mills, 2005). A single protein can contain several epitopes, each recognized by different antibodies. Epitopes can be sequential (or linear) if they are defined by a specific linear sequence of amino acids (for example, Arg-Glu-Ser); conformational if they depend on the three-dimensional structure of the protein. Some processed or prepared foods may still cause allergic reactions because linear epitopes can be recognized even when the protein is partially denatured, such as during digestion. However, since many allergenic proteins (like pollen or food proteins) maintain their native shape when exposed to the immune system, the majority of IgE antibodies recognize conformational epitopes. Several studies have emphasized that identifying both linear and conformational epitopes in proteins may be crucial in determining whether a child will outgrow or retain a food allergy (James *et al*., 1992; Sicherer *et al*., 1999; Vila *et al*., 2001). For instance, Cooke and Sampson (1997) observed a different pattern of IgE reactivity to linear and conformational epitopes of ovomucoid in children with persistent egg allergy compared to those likely to developed tolerance. They suggested that the development of IgE antibodies against linear epitopes may be linked to the persistence of egg allergy. Moreover, the resistance of epitopes to denaturation or digestion is especially important in food allergies, as these epitopes remain intact long enough to interact with the immune system. This highlights the importance of protein structure, as evidenced by the presence of disulfide bonds, which stabilize proteins (Bannon *et al*., 2004).
Allergens can be introduced into the body through various routes, including contact, inhalation, or ingestion. Inhaled allergens include dust mites, with their most common allergens derived from mite proteins such as Der p 1 (*Dermatophagoide pteronyssinus*) and Der f 1 (*Dermatophagoide farinae*); pollen proteins from plants like birch and grasses; and animal dander, including Can f 1 found in dogs and Fel d 1 in cats. Contact allergens, present in certain substances like para-phenylenediamine (PPD), can provoke allergic reactions when they touch the skin.
As for food allergens, fourteen major ones have been identified: celery, gluten-containing cereals (such as glyadin in wheat), crustaceans (tropomyosin), eggs

(ovomucoid), fish (parvalbumin), lupin, milk (beta-lactoglobulin and alpha-lactalbumin), mollusks, mustard, peanuts (Ara h 1, Ara h 2, Ara h 3), sesame, soybeans (Gly m 4), sulfur dioxide and sulfites (if present in concentrations greater than ten parts per million), and tree nuts such as almonds, hazelnuts, walnuts, Brazil nuts, cashews, pecans, pistachios, and macadamia nuts (Jug r 1 in walnuts). In contrast to inhalation allergies or food allergies to tree nuts, peanuts, or shellfish, food allergies to milk, eggs, or wheat typically tend to resolve more quickly. (Sicherer *et al*., 1999; Sicherer *et al.,* 2001; Sampson *et al.,* 2000; Burks *et al.,* 2012). Numerous factors, including the protein's structural stability and the epitope's shape, influence how an allergy resolves. For instance, peanut proteins, including Ara h 2, are particularly strong and persistent allergens because they are resistant to heat and digestion. Additionally, it may also depend on the maturation of the immune system, which may over time stop producing IgE antibodies specific to the allergen or generate more IgG antibodies that compete with IgE and inhibit the allergic reaction.

1.1.3 The impact of food thermal processing and food matrix on allergens' structure

Food allergens are typically small proteins with molecular sizes ranging from 10 to 70 kDa (Vickery *et al.,* 2011). They exhibit high water solubility and are resistant to enzymatic degradation and heat treatment (Costa *et al.,* 2022). However, in some cases, the allergenic determinant may not be protein-based but instead represented by carbohydrate structures known as Cross-Reactive Carbohydrate Determinants (CCDs). These carbohydrate moieties result from a common post-translational modification of proteins called glycosylation, which can influence protein functionality, structural stability, solubility, and transport. CCDs are found in the glycoproteins of plants, non-primate mammals, and invertebrates, but they are absent in humans (Van Ree *et al.,* 2000).

A study has demonstrated that CCDs also play a role in the cross-reactivity between the peanut allergen Ara h 1 and egg antigens from *Schistosoma mansoni*, highlighting the importance of glycan groups for this binding (Igetei *et al.,* 2017; Kamath *et al.,* 2023). An example of a carbohydrate determinant is galactose-alpha-1,3-galactose (alpha-Gal). Individuals exposed to glycoproteins in tick saliva after a tick bite produce IgE antibodies against alpha-Gal, which can bind to this carbohydrate in red meat, whether it is attached to proteins or lipids. Recently, alpha-Gal has also been identified in whey proteins from milk (Perusko *et al.,* 2019) and in proteins associated with milk fat globules (Aiuto *et al.,* 2024). It has been proven that alpha-Gal from glycolipids, rather than glycoproteins, can cross the intestinal monolayer and trigger an allergic reaction. Although rarely documented, allergies such as red meat allergy (alpha-gal syndrome) have been associated with specific lipids or glycolipids (Anumolu *et al.,* 2019; Roman Carrasco *et al.,* 2020). Food allergens may change in conformation after heat treatment or as a result of a matrix effect due to the complexity of the food they are present in.

1.1.3.1 Effect of thermal processing on food allergenicity

Raising the temperature induces conformational changes in a protein (Besler *et al.,* 2001). As the temperature rises, the kinetic energy of molecules increases, impacting the non-covalent bonds within the protein, such as ionic bonds, hydrogen bonds, Van der Waals forces, and hydrophobic interactions. Consequently, the protein tends to unfold and lose its three-dimensional structure (*Fig 1.2*) due to the disruption or alteration of these bonds (Rees *et al.,* 2001). Heat interferes with the interactions that maintain the protein's native conformation, such as hydrogen and disulfide bonds. However, in the food industry, heat treatments like boiling, cooking, pasteurization, and sterilization are widely used to reduce bacterial load, extend shelf life, and improve food quality (Rahaman *et al.,* 2015). These processes can alter, mask, or destroy conformational epitopes, affecting immune recognition and allergenicity, while sequential epitopes remain intact. Heat can also induce the formation of advanced glycation end products (AGEs), such as in the Maillard reaction, a non-enzymatic process involving the condensation between amino groups of proteins and reducing sugars. Additionally, it can modify the digestibility of proteins in the gastrointestinal tract and their absorption, further influencing their allergenic potential (Renzone *et al.,* 2015; Schulten *et al.,* 2011).

1.1.3.2 Effect of food matrix on food allergenicity

Foods consist of proteins, fats, carbohydrates, and fibers, which often interact with one another. These interactions can either mask or highlight allergenic epitopes, thereby modifying the immune system's capacity to detect allergens, potentially increasing or decreasing the risk of an allergic reaction (Van Wijk *et al.,* 2005). For instance, the fats abundantly present in tree nuts and peanuts (Lehrer *et al*., 2002) may shield proteins during digestion, increasing their allergenicity.

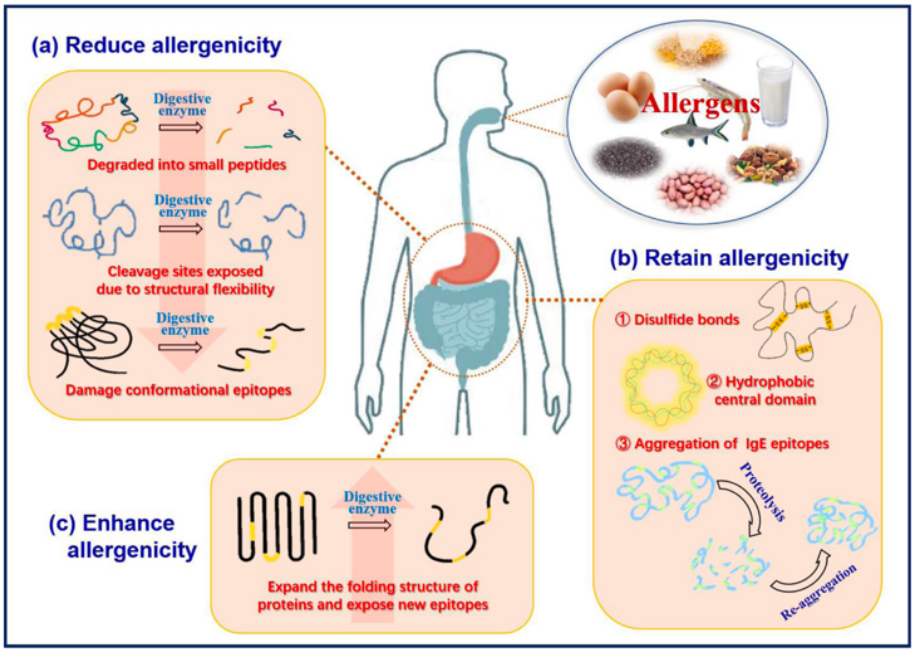


Figure 1.2: Structural characteristic of food allergens. (Ballegaard & Bøgh, "Intestinal protein uptake and IgE-mediated food allergy. Food Research International 163 (2023) 112150)

Given that allergenic proteins can cause severe allergic reactions in even minute levels, this could help to explain why some allergies are persistent (Al-Muhsen *et al.*, 2003). In certain situations, such as with red wine, these interactions can change the allergenic potential. Glycerol, polysaccharides, aldehydes, ketones, organic acids, esters, sugars, soluble proteins, vitamins, minerals, and polyphenols are just a few of the many substances found in wine, a hydroalcoholic solution (Miraldi *et al.*, 2024). For instance, polyphenols have the ability to interact with proteins, possibly changing their structure and hiding or changing their epitopes. Likewise, tannin-protein interactions can result in protein denaturation, which impacts the immune system's ability to recognize allergens. Accordingly, food proteins' allergenicity can be modified by tannin-protein complexes (McRae *et al.,* 2011).

1.2. The chemical characterization of food allergens

One of the major challenges in molecular allergology is the characterization of the protein confering allergenic properties upon interaction with an atopic (allergy-prone) immune system. An allergen is defined as a molecule that triggers an allergic reaction by inducing an abnormal immune response in susceptible individuals. Therefore, the allergenicity of a substance encompasses both its ability to **sensitize** an unexposed individual and to **provoke an allergic response** in those already sensitized to a specific protein (Jenkins *et al.,* 2005). These compounds are not inherently harmful, but in sensitized individuals, they can elicit a type I hypersensitivity reaction mediated by immunoglobulin E (IgE) antibodies. Although the molecular basis of these reactions is not yet fully understood, factors such as the level of exposure and the intrinsic properties of the allergen itself influence its allergenic potential. Most recognized allergens, whether of food or inhalant origin, are proteins (Aalberse *et al.,* 2000). The structure and properties of proteins play a critical role in inducing the allergic response, as factors such as stability, conformation, and the presence of specific amino acid sequences (epitopes) determine their allergenic potential.

1.2.1 Food Allergy

The Austrian scientist Clemens von Pirquet first used the term "allergy" in 1906 to refer to hypersensitive reactions, which he described as unintended and aberrant immune system reactions (Bendiner et al., 1981; Coombs et al., 1968). As stated in the 2023 position paper on nomenclature by the European Academy of Allergy and Clinical Immunology (EAACI), an allergy is "an abnormal or exaggerated reaction to exogenous stimuli involving various types of hypersensitivity reactions, including antibody, immune, cell-mediated, tissue-induced, or metabolic mechanisms, resulting in respiratory, cutaneous, ocular, gastrointestinal, and other symptoms, including anaphylaxis" (Jutel et al., 2023). Food allergies stand out among the other forms of allergies because of the intricacy of their mechanisms, as allergens enter the body and have the potential to cause more severe systemic reactions. In this study I focused specifically on food allergies, exploring their

distinctive characteristics and the risks arising from the accidental ingestion of hidden allergens in food products.

The incidence of food allergies is rising worldwide, affecting approximately 2-4% of adults and 5-8% of children (Ballegaard & Bøgh, 2023). Certain food allergies are more likely to resolve during childhood, while others tend to persist into adulthood (Sicherer *et al.,* 2004; Sicherer *et al.,* 2010). For instance, milk allergy has a prevalence of about 2% to 3% in the pediatric population, with most children outgrowing it by the age of five. Another common food allergy in early childhood is egg allergy, which affects approximately 1% of children; similarly, many children tend to outgrow this allergy by ages five to seven. In contrast, allergies to tree nuts, including walnuts and peanuts, are on the rise, with an estimated prevalence of 1% to 2% among children. In adults, this figure can reach up to 3%, and these allergies are known to persist over time (Host, 2002; Savage *et al*., 2007; Keet *et al*., 2009). The prevalence of food allergy is increasing in some regions of the world (Sigurdardottir *et al.,* 2021). As a result, there is an increasing focus on understanding the mechanisms and causes of food allergies and tolerance to enhance diagnosis and develop preventive or therapeutic measures.

1.2.2 Food allergy classification

The immunological mechanisms and type of hypersensitivity reaction involved in food allergies determine their classification in different classes: IgE-mediated, non-IgE-mediated and cell-mediated food allergy. In the **IgE-mediated**, the immunoglobulins E (IgE) antibodies are involved. The symptoms can occur within minutes to a few hours of exposure and may include hives, swelling, vomiting, diarrhea, respiratory symptoms (such as wheezing or shortness of breath), and anaphylaxis. Instead, in the non-IgE-mediated allergies, the gastrointestinal system is frequently affected, leading to disorders such food protein-induced enteropathy (FPE), food protein-induced allergic proctocolitis (FPIAP), and food protein-induced enterocolitis syndrome (FPIES). Symptoms may include vomiting, diarrhea, and poor growth or failure to thrive in infants. **IgE-mediated and non-IgE-mediated reactions can coexist** in some allergic reactions. In this case, the symptoms can vary and may include both immediate and delayed reactions. Conditions such as eosinophilic esophagitis (EoE) fall into this category, where eosinophils, a type of white blood cell, build up in the esophagus in response to food allergens. In the **cell-mediated food allergy**, the predominant population of cells involved are the T-cells (such as Th1, Th2, Treg, CD8+) and the symptoms are primarily gastrointestinal, including conditions like celiac disease, where ingestion of gluten leads to immune-mediated damage to the small intestine(Jutel *et al*., 2023). In this study, we examined only the IgE-mediated food allergy.

1.2.3 The molecular mechanism of IgE-mediated food allergy

Two steps defined the hypersensitivity reaction type 1 (*Fig 1.3*): the *sensitization phase* where the immune system is primed to recognize a specific allergen and the

elicitation phase upon subsequent exposure to the allergen (Chinthrajah *et al*., 2016). During the *sensitization phase*, an individual with a genetic predisposition to allergies is exposed to an allergen for the first time. Antigen-presenting cells (APCs), including B lymphocytes (B cells) and dendritic cells (DCs), capture the allergen in the mucosal lining or skin. After processing the allergen, these APCs display its fragments on their surface bound to major histocompatibility complex class II (MHC II) molecules identified by naïve CD4+ T-helper (Th) cells. In the presence of specific cytokines, such as interleukin-4 (IL-4) and interleukin-13 (IL-13), these Th cells differentiate into Th2 cells. Th2 cells secrete IL-4 and IL-13, which promote the class switching of B cells to produce IgE antibodies specific to the allergen (Varricchi *et al*., 2020; Berin *et al*., 2016). The sensitization phase begins when the Fc portion of IgE binds to mast cells (MC) or basophils, specifically to their high-affinity receptor (FcεRI). The elicitation phase, on the other hand, occurs after exposure to the same allergen or one with a comparable structure. IgE antibodies bound to FcεRI receptors on sensitized mast cells and basophils undergo a cross-linking reaction following this second exposure, which activates MC and basophils. This leads to degranulation that induces the release of granules that contain a variety of inflammatory mediators, including heparin, histamine, and tryptase. Major mediator histamine binds to histamine receptors in many tissues, resulting in the formation of mucus, bronchoconstriction, vasodilation, and enhanced vascular permeability. Other mediators such as prostaglandins, leukotrienes, and cytokines (e.g., IL-4, IL-5, IL-6, IL-13, and tumor necrosis factor-alpha [TNF-α]) are also released or synthesized *de novo* and contribute to the inflammation and recruitment of additional immune cells to the site of allergen exposure (Tordesillas *et al*., 2017).

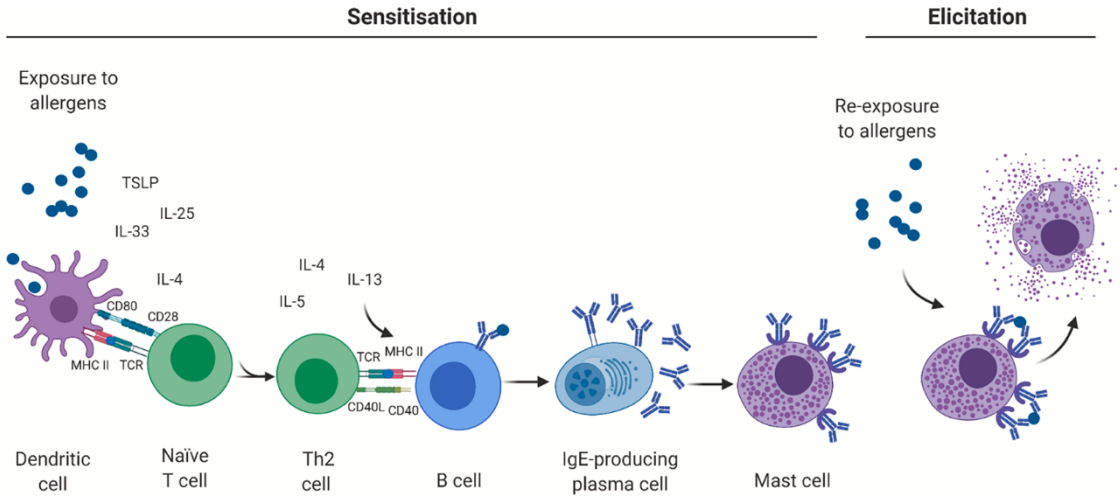


Figure 1.3: Molecular mechanism of IgE-mediated food allergy divided into the sensitisation and elicitation phases. (Ballegaard & Bøgh, "Intestinal protein uptake and IgE-mediated food allergy". Food Research International 163 (2023) 112150)

1.2.4 The approaches in food allergy prevention and treatment

Currently, the primary approach for managing food allergies involves strict avoidance of allergens. However, this long-term strategy can significantly reduce the quality of life for both patients and their families (Dunn Galvin *et al*., 2017; Lau *et al.,* 2014; Muraro *et al*., 2014). Emerging immunotherapy techniques, such as oral immunotherapy (OIT), represent a major advancement in the treatment of food allergies, which result from a complex interplay of genetic, environmental, and nutritional factors (van Ginkel *et al.,* 2018). In the treatment of food allergies, new immunotherapy approaches like oral immunotherapy (OIT) are a significant advancement (van Ginkel *et al*., 2018). OIT involves the gradual introduction of the allergenic food in increasingly larger doses to "train" the immune system to tolerate the allergen. However, maintaining this state requires continued exposure to the allergen, as its interruption can lead to a resurgence of allergic sensitivity (Wood RA *et al*., 2017). Genetics alone cannot explain the significant increase in food allergies. Environmental elements are also important, including delayed introduction of allergic foods and more stringent hygiene regulations (Sampath *et al.,* 2021). For these reasons, OIT is significant because it addresses the multifactorial nature of food allergies by re-educating the immune system in addition to managing symptoms. Palforzia, a biologic drug made from peanut allergen powder, was the first therapy approved for the management of peanut allergy through OIT (Dougherty *et al.,* 2021).

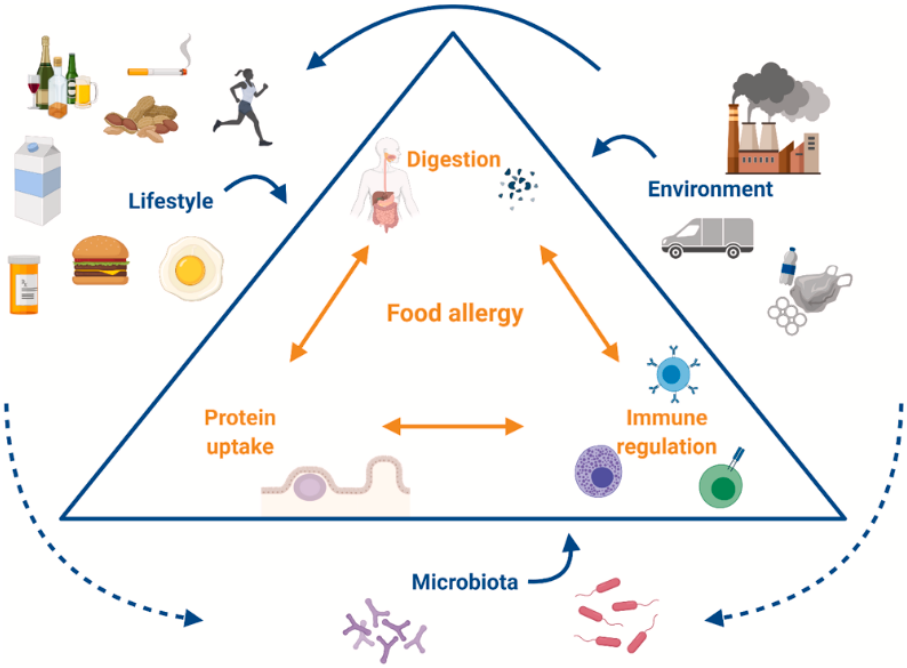


Figure 1.4: Lifestyle and environmental factors involved in food allergy development. (Ballegaard & Bøgh, "Intestinal protein uptake and IgE-mediated food allergy. Food Research International 163 (2023) 112150)

1.2.5 The problem of allergen's threshold in food allergy

The determination of a threshold value is considered one of the most complicated challenges in the field of food allergy and, moreover, the absence of precise data in this context also complicates the risk assessment due to traces of allergens in foods. A threshold dosage is the smallest quantity of an allergic meal that can cause modest symptoms in the most sensitive people, such as mild urticaria, erythema, or oral angioedema. However, because of things like potentiating effects from physical

activity, alcohol, or food processing (e.g., cooking or fermentation), which can change the allergenic epitopes and thereby change the allergen potential, the amount required to cause a reaction can differ significantly among people with the same allergy (Hourihane, 1997). National and international programmes such as ThRALL (Thresholds for Allergenic Reactions in Living Life), iFAAM (Integrated Approaches to Food Allergen and Allergy Risk Management), EuroPrevall (European Food Allergy and Anaphylaxis Network) and FAIR (Food Allergy, Ingestion Risk) place a strong focus on determining minimal allergenic dream levels, offering recommendations and resources for the treatment of food allergies. The lack of straightforward analytical techniques to identify allergen residues in foods, however, contributes to the continued lack of accurate estimations (Burney *et al.,* 2010; Mills *et al*., 2016; Ballmer-Weber *et al.,* 2015). Both the food business and allergy consumers are concerned about two significant difficulties that arise from the difficulty of identifying allergenic thresholds. Patients' dietary options are severely limited as a result of the uncertainty, which drives them to avoid items that may contain trace levels of the allergen in addition to those that actually contain it.This restriction often results in the elimination of entire categories of foods from their diet. Moreover, this behavior is further influenced by the presence of Precautionary Allergen Labelling (PAL), such as 'May contain traces of allergen'. Without clear threshold guidelines, consumers tend to avoid products with PAL warnings altogether, even if these products may be safe for them (Barnett *et al.,* 2011).

1.2.6 Food allergens labeling legislation

EU Regulation 1169/2011, together with the related European Commission Communication (C(2017)4864/F1), requires that 14 allergenic components (celery, cereals containing gluten, crustaceans, eggs, fish, milk, shellfish, mustard, peanuts, soya and sulphur dioxide) must be labelled when used in pre-packaged foods. However, despite this regulatory framework, allergen labelling requirements are not standardised globally and can differ significantly between countries (Fiocchi *et al.,* 2021). This lack of uniformity creates a problem for consumers, particularly when allergens are present in trace amounts due to inadvertent cross-contact during food processing or in catering environments. For this reason, the most widely adopted practice by food manufacturers is the use of precautionary allergen labelling (PAL). PALs are used to inform consumers of the potential unintended presence of allergens due to cross-contamination, even though the allergens are not intentional ingredients in the product (Holleman *et al*., 2021). Unfortunately, there aren't any widely recognized standards for correctly implementing Precautionary Allergen Labeling (PAL). In addition, companies that may implement PAL based on different levels for allergen presence—often without well-defined criteria—create confusion. The absence of standardization and the lack of quantitative information regarding the concentration of possibly present allergens or the actual level of risk are the results of this sort of labeling. Despite its important informative role for allergic consumers, its effectiveness as a food labeling tool is compromised by its excessive or improper use (Gupta *et al*., 2021). It is therefore crucial to invest

in new, more precise and sensitive analytical methods and standardized methods to provide the food industry and allergic patients with a more useful tool. Therefore, it is essential to establish a standardized unit of measurement, such as milligrams of allergenic proteins per kilogram (mg/kg) or liter (mg/L) of food, in order to improve food safety, communicate risks and ensure regulatory compliance. Consumer safety is enhanced by this standardization, which also makes food labels more reliable and clear and reduces the ambiguity and confusion that are frequently connected to PALs. Customers will be better able to evaluate their personal risk if they have access to more open information.

1.3. Analytical methods for Allergens Detection and Quantification in Food Matrices

In the field of analytical chemistry, trace allergen identification and quantification are essential for ensuring food safety and public health. This is because allergenic proteins in the extracts are contained inside a complex matrix that also includes carbohydrates, vitamins, salts, polyphenols, and other proteins. Changes in protein shape or epitope exposure might impact immunological activity as a result of interactions between proteins and matrix components. Proteins can also be altered by food processing techniques that cause denaturation, aggregation, or precipitation, which results in low extraction levels and protein insolubility. Therefore, several parameters, such as the allergens' extractability in relation to solubility and extraction buffers, as well as the methodology employed for their analysis, affect the ability to detect allergens. Among the analytical technique widely used for the quantification of trace amount of proteins in food the Enzyme-Linked Immunosorbent Assay (ELISA), and Mass Spectrometry (MS) are the most well-known methods that have been developed to achieve high sensitivity and specificity in allergen identification.

1.3.1 The Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is a common immunological technique that relies on antigen-antibody interactions to measure allergens. This approach is renowned due to its ease of use, high productivity, and ability to measure protein allergens directly. Highly specific antibodies can be used to enhance the sensitivity of ELISA, which can be set up for both qualitative and quantitative analysis. However, there are concerns because antibodies have the potential to produce false positives by cross-reacting with non-target proteins. Additionally, the components of a very complex matrix, such blood or red wine, can interfere with the assay and lower its sensitivity and accuracy. Red wine is one example, whose complexity primarily stems from its high tannin and polyphenol content. According to a study by Koestel (2016) on the measurement of ovalbumin in red wine, there is a strong relationship between the amount of tannin present and the ELISA signal's inhibition: the more tannins present, the more noticeable the signal loss. This clearly illustrates how the matrix effect can adversely affect the results. Despite ELISA's capability to yield quantitative results, it is generally less precise than methods such as mass spectrometry (MS) (Koeberl *et al.*, 2014).

In order to address the many drawbacks of the currently accepted methods for allergen analysis, in the last ten years, alternative non-immunological methods have been studied, with a focus on mass spectrometric method. Specifically, the combination of mass spectrometry with liquid chromatography separation has shown potential in the identification, characterization, and, more recently, quantification of food allergens (Monaci *et al*., 2009).

1.3.2 Mass Spectrometry (MS)

MS is an advanced analytical technique, known for its high sensitivity and ability to analyze complex mixtures, making it an exceptionally powerful tool for detecting allergens (Heick & Popping 2011). The mass spectrometer consists of three primary components: the ionization source, the mass analyzer, and the detector. Among ionization methods, two techniques—electrospray ionization (ESI) (Yamashita *et al.*, 1984) and matrix-assisted laser desorption/ionization (MALDI) (Karas et al., 1987)—are the most commonly used for converting large, non-volatile, thermolabile compounds into gas-phase ions. This discussion will focus on the ESI source, where ions are generated at atmospheric pressure by introducing a solution-based sample through a narrow capillary subjected to a potential difference relative to a counter electrode. The electrostatic atomization of the solution produces an aerosol of charged droplets, which contain both solvent and analyte molecules with a positive or negative charge, determined by the polarity of the applied voltage (Glish *et al*., 2003). Depending on the particular type of analyzer being used, the analyzer makes it easier to measure ions. Ions are released as a beam from the ion source and travel through the analysis field to the detector in beam analyzers. On the other hand, ions are contained inside the analysis field in trap analyzers. The quadrupole is probably the most popular mass analyzer among them, particularly when used in conjunction with gas or liquid chromatography. In a quadrupole, the mass-to-charge ratio (m/z) of ions traveling in a dynamic electric field is directly proportional to the mass separation and can apply electric fields in only two dimensions (x and y) allowing the ions to travel perpendicular to the field (along the z-axis). This feature makes ion trapping possible and makes a number of analytical methods easier. Ion traps offer sophisticated fragmentation modes, like collision-induced dissociation (CID), which facilitate in-depth analyses of molecular structures. Tandem mass spectrometry (MS/MS) is an even more important part of mass analysis. Ions having a particular m/z ratio, also known as precursor or parent ions, are first separated from the other ions produced by the ion source in this procedure (Busch et al., 1988). It is very useful for the separation of complicated mixtures since these isolated ions are subsequently broken apart to create product ions. In 2000, Makarov introduced the Orbitrap mass analyzer, which was initially put on the market in 2005 as a component of a hybrid instrument known as the "LTQ Orbitrap" that combines the high-resolution capabilities of the Orbitrap with a low-resolution linear ion trap (Makarov et al., 2006). Later, an upgraded model known as the "LTQ Orbitrap Velos" was created, which included a Higher-energy Collisional Dissociation (HCD) cell for more effective ion fragmentation and an S-lens to enhance ion transmission by up to ten times (Olsen et al., 2009). Thanks to the compact design of the Orbitrap, a benchtop instrument called the Exactive was subsequently created. However, the Exactive lacked mass selection capability, limiting it to "All Ion Fragmentation" (AIF), where the entire mass range is fragmented (Geiger et al., 2010). The next advancement was the Q Exactive Orbitrap, which combined the robustness of a quadrupole mass filter with the high-resolution capability of the Orbitrap analyzer (Michalski et al., 2011;

Kelstrup et al., 2012). With the Q Exactive, precursor ions could be selectively isolated and fragmented (Figure 4), using the quadrupole as a mass filter for precursor ion selection and the Orbitrap analyzer to record high-resolution full scans and MS/MS spectra, with an HCD cell handling fragmentation (Michalski et al., 2011).

The MS technique provides comprehensive molecular information, allowing for the precise identification of specific allergenic proteins and peptides. It has been the preferred technique for the proteomic studies of protein characterization rather than quantification, due to the difficulty of ionizing large macromolecules such as proteins. But the most recent generation of analyzers, such as the Q Exactive Orbitrap, has made it feasible to develop MS methods that can provide details on allergenic proteins in food, both qualitatively and quantitatively. The principle of MS-based quantification techniques is the use of internal standards, which is based on comparing analyte MS signal intensities with standard values (Gallien *et al.,* 2012). The most reliable internal standard is the isotopically labeled analyte since its chromatographic elution, ionization response, extraction recovery, and spectrum will all be comparable. Internal standard (blank, analyte, calibration standard) is added to samples in practice at a known consistent amount. There are two ways to approach this quantification method: in the one, the intact protein functions as the analyte and, thus, the standard; in the second, the analyte is a peptide that is the end product of proteolytic enzymes like trypsin breaking down proteins. Despite its numerous advantages, MS requires extensive sample preparation, specialized equipment, and considerable expertise, which can limit its accessibility in comparison to methods like ELISA (Enzyme-Linked ImmunoSorbent Assay).

1.3.2.1 Identification of new allergens using mass spectrometry

Mass spectrometry (MS), in the field of food allergies, is particularly useful for identifying proteins in complex mixtures without any prior information about their existence (Bianco *et al*., 2022). MS allows for a much broader identification spectrum than, for example, traditional techniques such as immunoblotting or ELISA, which rely on specific antibodies to identify known allergens. Due to the limitations of traditional methods in accurately identifying allergens, mainly in processed food where the temperature or heat could lead to the formation of new epitopes, researchers are increasingly adopting MS-based techniques as confirmatory tools for the precise identification and characterization of food allergens (Monaci *et al.,* 2009). Two are the approaches commonly used for the identification of new allergens using MS: top-down and bottom up.

The bottom-up approach involves enzymatic digestion of the protein extract. Because of its excellent purity and ability to produce peptides that fall within the mass range of the majority of MS analyzers, trypsin is the most widely employed enzyme. It cleaves peptide bonds at the carboxyl side of arginine (R) and lysine (K) residues, except when they are followed by proline. Ideal peptides for mass spectrometry are produced by this selection; these are usually 7–20 amino acids long. The efficiency of trypsin in generating peptides containing basic residues at the C-terminus also enhances their ionization in electrospray ionization (ESI) mass spectrometry. This leads to increased signal intensities, which are especially helpful in PRM since precise quantification of the targeted peptides is necessary (Mansuri *et al.,* 2024). The protein sequence coverage can also be increased by using a mixture of enzymes to produce a large number of shorter peptides (Nardiello *et al*., 2018; Morsa *et al*., 2019). Before digestion, a separation of the protein sample is

usually performed using one- or two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein-specific bands can be removed and digested independently, making digestions with MS and MS/MS—whether or not they are combined with LC—simpler and easier to define. "Target" or "untargeted" approaches are used in bottom-up proteomics, depending on the particular objectives of the investigation. Since the sequences of many allergenic proteins are already known, the targeted approach is frequently chosen since it enables the prediction of signature peptides by in silico analysis. Data-dependent acquisition (DDA), an untargeted method, is employed when sample information is scarce. This technique makes it easier to acquire a large number of tandem mass spectra, which may subsequently be examined using software that is now accessible to interpret amino acid sequences. Data-independent acquisition (DIA) methodically records all fragment ions within a preset m/z range, in contrast to DDA, where the device chooses ions for fragmentation depending on their intensity or abundance. This allows for the identification and quantification of every detectable analyte in the sample, irrespective of its m/z value or abundance. DIA is particularly advantageous for comprehensive protein studies that require extensive coverage and quantitative precision, especially when characterizing a sample matrix without targeting specific proteins (Ka Wan Li *et al*., 2020). Conversely, for smaller-scale studies that demand high sensitivity and accuracy—such as our current investigation—DDA is the preferred method.

In contrast, the top-down method evaluates intact proteins directly, without requiring prior digestion, enabling the identification of various isoforms and post translational modifications (PTMs). The process involves the ionization of intact proteins in the gas phase and then measuring the relevant ions' masses with high resolution using a mass spectrometer through direct fragmentation (Han et al., 2008).

1.3.2.2 High Resolution Mass Spectrometry (HRMS) coupled to High Resolution Liquid Chromatography (HPLC)

Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ is the high-resolution mass spectrometer utilized in this thesis (*Fig. 1.5*). In order to select precursor ions according to their mass-to-charge ratio (m/z), this device combines an Orbitrap mass analyzer, which enables precise, high-resolution mass detection, with a quadrupole mass filter. It is compatible with tandem MS/MS acquisition as well as full-scan MS. Before being sent to the Orbitrap for examination, certain precursor ions can be broken up in the high-energy collisional dissociation (HCD) cell in MS/MS mode. After being injected into the Orbitrap, the ions circle a center electrode in the shape of a spindle, producing an image current that is then transformed into a mass spectrum using Fourier transform (FT) analysis. A heated electrospray ionization (HESI) source connects the mass spectrometer to a high-performance liquid chromatography (HPLC) apparatus.

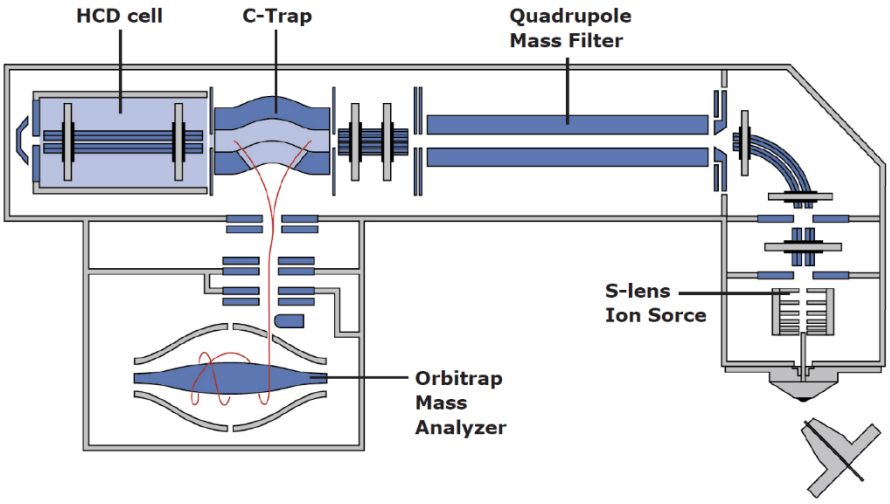


Figure 1.5: Schematic representation of the Thermo Scientifc™ Q-Exactive Hybrid Quadrupole Orbitrap™ mass spectrometer. Source: thermofisher.com

Electrospray ionization (ESI) is the most commonly used ionization technique for polar and hydrophilic molecules in liquid chromatography–mass spectrometry (LC-MS). SI serves primarily as a transfer interface, enabling the coupling between the high-pressure liquid phase (e.g., HPLC, operating at ambient pressure) and the high-vacuum environment of the mass spectrometer (typically around 10^{-11} bar). In this process, charged droplets generated at the capillary tip undergo successive evaporation and Coulomb fission, ultimately releasing gas-phase ions that can be analyzed by MS in either positive or negative ion mode. In this technique, molecules of interest are introduced into a narrow capillary, which is held at a high voltage (3–6 kV) relative to a counter-electrode. The electric field created between the capillary tip and the electrode causes the formation of a "Taylor cone," leading to the nebulization of the liquid flow and the production of small droplets (Taflin *et al.,* 1989). The droplets are deformed and subsequently fragmented into smaller droplets with sizes of around 0.1 µm when exposed to the electric field. The solvent concentration of the droplets drops as they get smaller, while the charge density rises. Desolvated molecule ions are eventually ejected straight from the droplet surface by the electric field's increasing strength (Chong *et al*., 2018). After that, the ions are sent to the mass analyzer. Two essential elements are relevant to this thesis: the Orbitrap, which acts as both a mass analyzer and a mass detector, and the quadrupole, which acts only as a mass filter to select the ions of interest. Using their mass-to-charge (m/z) ratio, ions can be separated at high resolution in the Orbitrap. The data obtained is then analyzed by specialized software that registers the ions according to their relative abundance and creates a mass spectrum. The quadrupole consists of four parallel metal rods that receive a direct current (DC) voltage, with voltages of the same polarity supplied to diagonally opposite rods. The ions are forced to follow oscillatory trajectories by the resulting electric field, and the trajectories change according to the m/z values. Only ions with a particular m/z value can be selectively transmitted by adjusting the applied field; ions with higher or lower m/z values depart from the stable trajectory and are lost before they reach the detector. The selected ions can then be either transferred directly to the Orbitrap for analysis (Full MS Mode) or sent to the Higher-energy Collisional Dissociation (HCD) cell for fragmentation prior to Orbitrap analysis (MS/MS Mode). Once injected into the Orbitrap chamber, ions begin to orbit around the

central spindle-shaped electrode. Their axial motion induces an image current, which is detected and processed using Fourier Transform (FT) analysis, ultimately generating high-resolution mass spectra (Michalsky *et al.,* 2011). Data acquisition for the thesis was also carried out utilizing the Parallel Reaction Monitoring (PRM) mode, a targeted MS/MS technique that use the Orbitrap to identify all product ions with high resolution and mass accuracy and the quadrupole to isolate particular precursor ions. This method is especially well-suited for absolute quantification of peptides in complicated mixtures because it combines the analytical strength of high-resolution detection with the selectivity of the quadrupole.

1.3.3 Advantages and disadvantages of the different analytical approaches

Each of these analytical techniques (ELISA and MS analysis) offer unique advantages and face specific challenges in the quantification of trace allergens. ELISA is useful due to its ease of use and capacity for protein quantification and MS offers a comprehensive molecular analysis with high sensitivity. The choice of technique depends on the specific requirements of the analysis, including the type of allergen, the matrix of the sample, and the available resources. LC-MS/MS is regarded as the "gold standard" for protein and peptide identification and quantification by the European Network of Laboratories for the Detection of Food Allergens (ENFADL) which aims to harmonize these analytical methods throughout the European Union (Breidbach *et al.,* 2022). Because MS can directly quantify allergen traces in food with a sensitivity that allows achieving country-specific recommended reference doses, it offers a viable alternative to PAL (Fiocchi *et al.,* 2021). These values are frequently employed as minimum target values when developing an allergen quantification method in order to establish an adequate limit of detection (LOD) and/or limit of quantification (LOQ).

2. Aim of the study

The goal of this PhD project is to use mass spectrometry to analyze the research pipeline in the field of food allergies. This project was divided into two related research objectives: the first was to identify and characterize novel allergens, and the second was to apply a metrological approach to quantify known allergens in complex food matrices. The first step in the allergy discovery process is the extraction of proteins from a food matrix. The extracted proteins are subjected to immunoenzymatic assays such as immunoblotting. These reactive proteins are then identified by mass spectrometry. Once a protein has been verified as an allergen, its presence and concentration in the food matrix are further studied. In this case, accurate quantification is necessary to assess the allergenic risk. A thorough metrological methodology is necessary for the quantification of allergenic proteins inside complex matrices. A crucial component is the precise characterization of the measurement, which can be challenging in biological systems with limited protein or peptide reference materials. The measurement is expressed as milligrams of protein per kilogram or liter of product in accordance with EU Regulation 168/2002. Starting with a reference material, an unbroken chain of calibration must be established in order to achieve quantification in these terms. Here, uncertainty values are gradually transferred to peptides and proteins until a consistent measurement is achieved, using a combination of NIST amino acids as a reference point.

In summary, this PhD project encompasses two core areas of study:

- **Characterization and discovery of new food allergens** utilizing mass spectrometry to identify novel allergenic proteins and understand their structure and immunogenic properties. The project is “Discovery of new peculiar allergen (CDD) by means Mass Spectrometry”
- **Quantification of known allergens in complex food matrices with a metrological approach** applying advanced mass spectrometry to measure allergen levels with precision, addressing the critical need for a traceable, standardized quantification protocol. The project is “Quantification of trace amount of proteins using Mass Spectrometry in red wine”

By integrating these two research areas, this project aims to advance the study of food allergens, offering insight into the complex technical and scientific challenges posed by proteomic analysis and metrological principles in allergen quantification.

3. Case study 1:

Discovery of the galactose-alpha-1,3-galactose allergen by HPLC-HRMS in milk fat globule proteins

3.1. Introduction

3.1.1 Biochemistry of the galactose-α-1,3-galactose (α-Gal) allergen

Galactose-α-1,3-galactose (α-Gal) is a glycan determinant synthesized by the enzyme α-1,3-galactosyltransferase (α-1,3 GT), and is abundantly expressed on glycoproteins of most mammals, including non-primate mammals, prosimians, and New World monkeys. In contrast, α-Gal is not synthesized by humans, Old World monkeys, and apes due to a point mutation that occurred approximately 28 million years ago, rendering the α-1,3 GT enzyme inactive (Macher and Galili, 2008). This mutation altered the expression of the α-1,3-galactosyltransferase gene (GGTA1), resulting in the enzyme's inability to transfer a galactose residue via an α-1,3 linkage onto the terminal lactosamide of disaccharides in glycoproteins and glycolipids, thus preventing the formation of the α-Gal epitope (Hilger et al., 2019). Studies by Koike et al. have suggested that the inactivation of the GGTA1 gene likely coincided with the emergence of other glycosyltransferases capable of producing products with biological functions that substitute for the α-Gal epitope (Koike *et al*., 2002). Studies by Galili and colleagues (1984) have demonstrated that α-Gal epitopes are present on the cell walls of certain gut microbiota strains, such as *Escherichia coli* O86 and *Klebsiella pneumoniae* 18033. Anti-α-Gal antibodies are present in all individuals, regardless of blood group. These antibodies are very low during the first 3-6 months of life, gradually increase, and reach levels comparable to adults by 2-4 years of age. The production remains constant throughout life, reflecting continuous antigenic stimulation by the intestinal flora. It is estimated that about 1-5% of all human IgG and IgM antibodies are directed against the α-Gal epitope (Hilger *et al.,* 2019).

3.1.2 Alpha-Gal syndrome

Alpha-Gal syndrome is a delayed allergic reaction caused by the α-Gal epitope. It differs from typical IgE-mediated allergies because it is due to a carbohydrate epitope rather than a protein one and symptoms appear 3-4 hours after ingestion of red meat. The amount of allergen ingested also influences the severity of allergic reactions (Iweala *et al.,* 2020). High-fat mammalian meats are more likely to trigger severe and frequent allergic responses. This is likely because lipid-bound α-Gal can penetrate the intestinal barrier and initiate an immune response, indicating that glycolipids, in addition to glycoproteins, might act as allergens (Roman-Carrasco *et al*., 2019). Recent findings by Chakrapani et al. (2022) have further substantiated the role of glycolipids in triggering basophil activation in patients with Alpha-Gal Syndrome (AGS), although glycoproteins, particularly those derived from pig kidney and beef extracts, are still recognized as the primary allergens. (The α-Gal syndrome was first identified in 2004 during clinical trials for cetuximab, a monoclonal antibody, when some patients, mainly in the southern U.S., experienced rapid hypersensitivity reactions. These reactions were traced back to pre-existing specific IgE antibodies against α-Gal caused by cetuximab's glycosylated Fab portion. Further studies between 2006 and 2008 by Platts-Mills and Commins

(2009) linked unexplained hypersensitivity reactions, such as generalized urticaria and anaphylaxis, to meat consumption hours earlier. Intradermal tests and specific IgE blood tests confirmed a strong positive reaction to red meat and cetuximab, suggesting an association with α-Gal (Commins *et al.,* 2009). The syndrome was later linked to tick bites, specifically from the Lone Star tick, which significantly increased α-Gal-specific IgE levels in affected individuals. This connection between tick bites and meat allergies helped to better understand the onset of the syndrome. Research has shown that α-Gal epitopes are not only found in red meat but also in bovine milk, though in smaller quantities. Recent studies (Armstrong *et al.*, 2020; Wilson *et al.,* 2019), including one large cohort study involving 2,500 AGS patients in the United States, have revealed that approximately 10–20% of AGS patients also exhibit allergic reactions to milk. The most commonly reported symptoms after consuming bovine milk in these patients are abdominal pain and urticaria, which tend to have a delayed onset (Commins *et al.,* 2020).

While α-Gal-proteins in meat have been extensively studied, the presence of α-Gal epitopes in dairy products has only recently been explored (Commins *et al.,* 2016). Modified inhibition radioimmunoassay (RIA) techniques have detected α-Gal proteins in heavy milk cream but not in skimmed or low-fat milk (Mullins *et al*., 2012). Research by Perusko et al. (2021) identified bovine γ-globulin, lactoferrin, and lactoperoxidase as α-Gal-carrying proteins that are recognized by the IgE antibodies of AGS patients, leading to basophil activation. Additionally, similar glycosylated proteins containing α-Gal were recently found in sheep milk, further expanding the understanding of potential sources of α-Gal in dairy products (Perusko *et al.*, 2021).

3.2. Materials and Methods

3.2.1 Alpha Gal protein extraction

3.2.1.1 Extraction of the major milk's protein fractions

The extraction process of the protein fractions of cow's milk allows the separation of proteins associated with fat globules, whey proteins and caseins.
The milk sample was centrifuged at 5000 xg at 6°C for 30 minutes to remove somatic cells and impurities normally present in milk. After centrifugation, the pellet containing the somatic cells was eliminated and the fat pad formed during centrifugation was mixed back into the milk. To promote mixing between the aqueous part and the fat, the milk was heated to 37°C and vortexed repeatedly.
The milk sample was then subjected to ultracentrifugation (189,000 xg at 6°C for 70 minutes), which in vacuum conditions allows reaching a rotation speed such as to cause the sedimentation of particles much smaller or denser than those ordinarily separated in centrifugation at atmospheric pressure. After ultracentrifugation, milk is divided into three phases: the fat pad in the upper part, whey proteins in the intermediate phase and caseins in the lower phase. The three fractions were collected separately. Unlike caseins and whey proteins, the extraction of proteins associated with fat globules required the application of a protocol for the enrichment of the protein fraction with respect to the lipid component.

3.2.1.2 Extraction of Milk Fat Globule Membrane (MFGM) Proteins

To 300 mg of fat (obtained after ultracentrifugation) were added 500 µL of Molloy buffer (5mM Tris-HCl pH 8.8, 6,5M urea, 2,2M thiourea, 1% p/v ASB-14). The samples were vortexed and left to shake at room temperature for one hour, vortexing every 15 minutes, finally they were centrifuged at 21,000 x g for 30 seconds. After centrifugation, the formation of a white-yellowish pad is observed in the upper phase and a supernatant below containing the proteins. Using a glass Pasteur, the supernatant was collected and transferred. In order to limit the presence of non-protein interferents (salts, lipids, polysaccharides, DNA, etc.) and concentrate the sample, precipitation with methanol/chloroform was performed. 800 µL of methanol, 400 µL of chloroform, and 600 µL of MilliQ water were added to 200 µL of fat globule-associated protein sample, vortexing after each solvent was added. The sample was centrifuged at 10,000xg for 2 minutes, resulting in the formation of three phases: an upper phase containing chloroform and lipids, an interphase represented by a very thin layer of proteins (sample of interest), and a lower phase containing methanol and water. The upper phase was removed, taking care not to touch the intermediate layer, and then 800 uL of methanol was added. The sample was vortexed again and centrifuged at 10,000xg for 3 minutes, the supernatant was discarded, and the pellet was left under a hood to allow evaporation of residual solvents. The dried pellet was resuspended in 60 µL of Laemmli buffer (60 mM TRIS HCl pH 6.8, 2% SDS, 10% glycerol).

3.2.1.3 Protein's quantification

The protein concentration of the sample after methanol/chloroform treatment was determined by spectrophotometric assay (2D-Quant kit,GE Healthcare). Quantification was performed by measuring two volumes of each sample in duplicate. A bovine serum albumin (BSA) standard solution (2 mg/mL) was used to generate the calibration curve. Both the unknown concentration samples and the calibration curve were processed under the same conditions.
Each sample was treated with 500 µL of precipitant and 500 µL of co-precipitant, followed by centrifugation at 14,000×g for 5 minutes. After removing the supernatant, 100 µL of copper solution and 400 µL of deionized water (ddH_2O) were added to the pellet. Subsequently, 1 mL of working color reagent—prepared by mixing color reagent A and color reagent B in a 100:1 (v/v) ratio—was added to start the colorimetric reaction. The tubes were incubated at room temperature for 15 minutes, and absorbance was then measured at 480 nm using a Beckman Coulter UV-DU730 spectrophotometer. The sample concentration was determined using the equation derived from the concentration/absorbance values of the BSA calibration curve.

3.2.1.4 Enrichment of MFGM proteins using magnetic beads

The BioMag Goat Anti-Human IgG beads (5.2 mg/ml) (Polysciences) were washed twice with 500 µL of PBS. Subsequently, the washed beads were blocked using TBS buffer containing 0.3% Tween-20 and incubated under agitation at 4°C for 15 minutes, repeating the process twice. After removing the TBS solution, the anti-Human IgG-specific magnetic beads were incubated with 6 µL of α-gal IgG antibody for 6 hours, at 4°C, under rotation. The antibody-bead complexes were then separated from the supernatant and washed twice with 500 µL of PBS. To enable the capture of α-gal glycosylated proteins, fat globule membrane-associated proteins (60 µg) diluted in 500 µL of PBS were added to the magnetic beads and incubated overnight at 4°C. The antibody/bead/protein complexes were separated from the supernatant and washed twice with 500 µL of PBS to remove unbound components. For protein elution, the beads were incubated with 100 µL of elution buffer containing 1% (w/v) SDS, 100 mM Tris-HCl (pH 7.4), 10 mM DTT, and 8 M urea (Bonifacino *et al.,* 2001), at 95°C, for 10 minutes (*Fig. 3.1*). The eluted proteins were subsequently analyzed via immunoblot assay to confirm the successful purification of α-gal glycosylated proteins associated with the fat globule membrane. The corresponding protein bands were excised and sent for LC-MS/MS analysis.

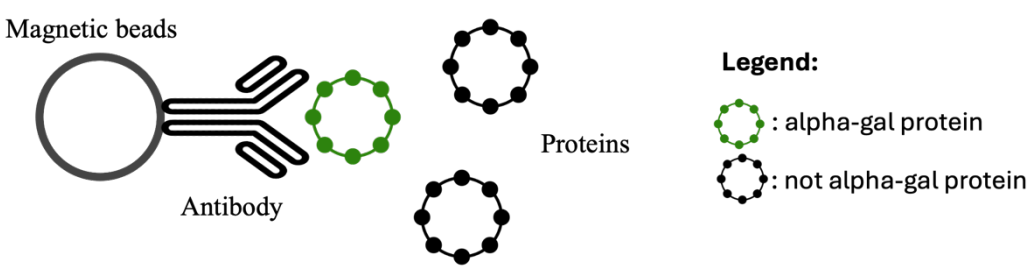


Figure 3.1: Magnetic BioMag beads schematic mechanism.

3.2.2 Protein's characterization and clinical presentation of AGS (alpha gal syndrome)

3.2.2.1 SDS-PAGE

One-dimensional lithium dodecyl sulfate polyacrylamide gel electrophoresis (1D-LDS-PAGE) was performed under reducing conditions on a NuPAGE Novex 4-12% Bis–Tris 10-well precast gel (Invitrogen) with 600 mL of NuPAGE MES Running Buffer (30 mL of 20X MES made up to 600 mL with milliQ water) with an XCell SureLock Mini-Cell system (Invitrogen). 5 µg of protein extract was solubilized in NuPAGE Lithium Dodecyl Sulfate (LDS) Sample Buffer with the addition of the reducing agent Dithiothreitol (DTT) (NuPAGE Sample Reducing Agent 10X), in a final volume of 20 µL per well. The extract was then incubated at 70°C for 10 min before loading. Within the same electrophoresis run, 5 µL of molecular weight marker (Mark12TM, Invitrogen) was loaded. The run was performed at 200 V, 125 mA, 25 W, at RT for 45 minutes using the Amersham Pharmacia Biotech EPS 3501XL power supply. To visualize the protein bands on the gel, the proteins were fixed with the Fixing buffer containing 30% methanol and 10% orthophosphoric acid in water for 2 hours and then stained with the Colloidal Coomassie Blue BCC dye (80% stock solution, 20% methanol; *Table 3.1*). The gel was left to stain overnight (o/n) with gentle agitation and the following day the dye was removed and replaced with water several times until the excess dye was eliminated. The gel image was acquired with a resolution of 600 dpi with the ChemiDoc MP System densitometer (Bio-Rad).

Substance	Quantity
Coomassie G-250 (Biorad)	0,15% (w/v)
Phosphoric Acid H_3PO_4 (85%)	2,4% (v/v)
Ammonium Sulphate $(NH_4)_2SO_4$	10% (w/v)
Ultrapure water	To final volume

Table 3.1: Stock solution composition of Blue Coomassie Colloidal colorant

3.2.2.2 Western Blot

For western blot experiments, 2 µg of protein extract were loaded into each well. At the end of the electrophoresis run (LDS-PAGE) (*see paragraph 3.2.2.1*), proteins were transferred from the gel onto a nitrocellulose membrane with a pore diameter of 0.2 µm. NuPAGE Transfer Buffer (Invitrogen) was used with the addition of 10% methanol and the XCell IITM Blot Module (Invitrogen) was used as the electrophoresis cell, placing the gel between the filter paper and the nitrocellulose membrane, previously saturated with the transfer buffer (as shown in *Fig. 3.2*). Above the nitrocellulose, another filter paper was placed and the whole thing was placed between 4 blotting pads, also previously immersed in the transfer buffer to create a "sandwich" that was placed in the blotting chamber and, subsequently, completely saturated with the buffer. The parameters set for the transfer were: 35 V, 150 mA, 30 W, at RT, for 1 hour. At the end of the transfer, the nitrocellulose membrane was stained in Ponceau Red (0.2% Ponceau Red, 3%

acetic acid TCA) for 15 minutes, with gentle agitation at room temperature, then destained in water and finally left to dry.

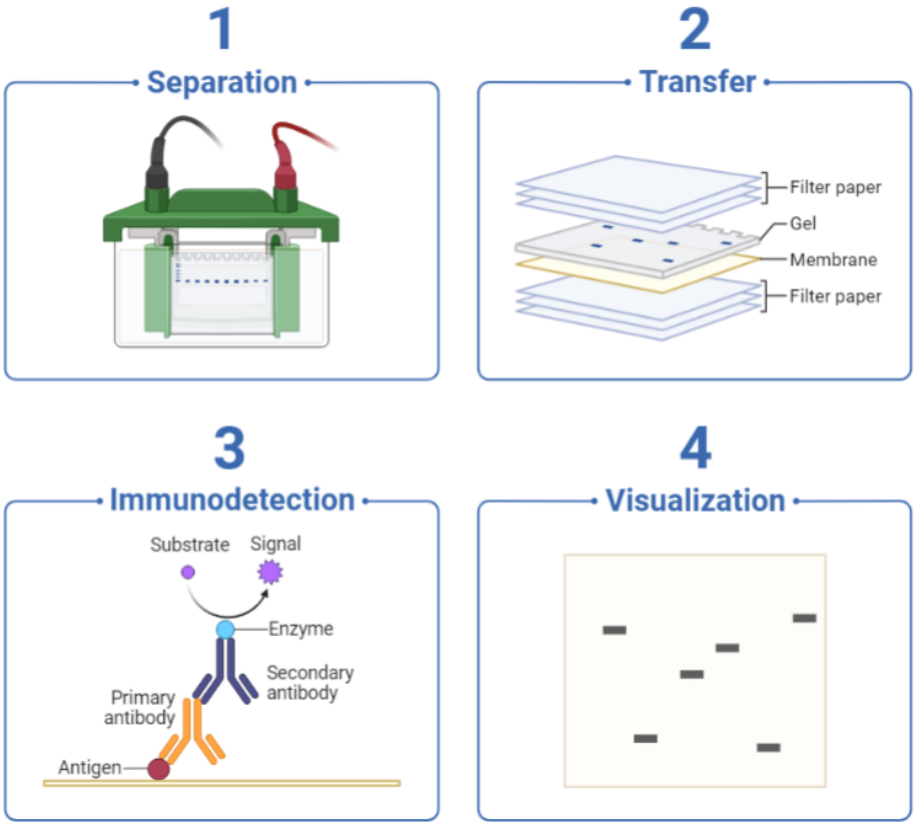


Figure 3.2: Workflow of Western blotting. In step 1, the protein to be analyzed is separated by gel electrophoresis. The proteins are then transferred to a membrane (2) and analyzed by immunodetection. In this process, a primary antibody binds to the protein of interest and an enzyme-coupled secondary antibody binds to the primary antibody. After addition of the appropriate substrate, a signal can be detected (3) and the proteins are visualized as bands on the membrane (4)
(Figure created with biorender.com by BIOMOL site).

3.2.2.3 Immunoblotting

Immunoblotting is an immune-enzymatic technique capable of identifying the antigenic specificity of proteins that have been separated by electrophoresis and transferred onto a nitrocellulose membrane. This method allows for the assessment of patient sera reactivity toward these proteins. The primary antibody presents in the serum binds directly to the target protein (*Fig 3.2*). To detect this interaction, a secondary antibody conjugated with an enzyme is necessary, recognizing the Fc fragment of the primary antibody. The immunoreactive bands are visualized through a colorimetric reaction between the enzyme-conjugated secondary antibody and an appropriate substrate. The appearance of colored bands on the nitrocellulose membrane indicates serum reactivity against a specific protein.

In this study, immunoblotting was applied to evaluate the reactivity of both the monoclonal anti-α-Gal antibody and patient sera toward the protein extract. The protocol was slightly modified depending on the case.

The key steps were:

- Blocking non-specific binding sites
- Incubation with serum or monoclonal antibody (primary antibody, AbI)

- Incubation with anti-IgE/anti-IgM secondary antibody (AbII)
- Reactivity development

After protein transfer, the membrane was cut into strips corresponding to the lanes of each sample loaded onto the gel and placed into designated incubation trays. The strips were then decolorized through multiple washes with 1X TBS buffer. All buffers used in the procedure are summarized in *Table 3.2*.

Buffer	Composition
TBS 10X	0.5M Tris, 1.5M NaCl. pH 7.4
Blocking	TBS 1X, Tween20 0.3% v/v
Washing	TBS 1X, Tween20 0.05% v/v
Incubation	TBS 1X, Tween20 0.05% v/v, vegetal gelatin 0.05% w/v

Table 3.2: Buffer composition used for immunoblotting protocol

Each strip was incubated with 1 mL of blocking solution to prevent non-specific binding for 15 minutes, a step repeated twice. Subsequently, the membranes were incubated with either the monoclonal anti-α-Gal antibody, conjugated with horseradish peroxidase (HRP) and diluted 1:1000, or patient sera diluted 1:10, both in the incubation solution. In the first case, no secondary antibody was required due to the direct HRP conjugation of the monoclonal antibody. The trays containing the membranes were covered with aluminum foil and placed on a rocker overnight at 4°C. The following day, the membranes were washed three times for 10 minutes with a washing buffer. The membranes incubated with patient sera were subsequently incubated at room temperature for one hour with the secondary antibody (AbII) anti-IgE, diluted 1:10,000 in the incubation solution. In contrast, the membranes incubated with the monoclonal anti-α-Gal antibody did not require a secondary antibody, as the primary antibody was already conjugated with the substrate for reactivity development. After incubation, the membranes incubated with patient's sera, were washed three times for 10 minutes with washing buffer. Two different detection kits were used depending on the AbI-AbII antibody pair (*Table 3.3*). The Opti-4CN (Bio-Rad) kit was employed for visualizing the bands recognized by the anti-α-Gal/anti-IgM pair. This kit utilizes horseradish peroxidase (HRP), a metalloenzyme that oxidizes the substrate to an insoluble, brown-colored product. The reaction solution was prepared by diluting the Opti-4CN diluent 1:9 in milliQ water, followed by the addition of the Opti-4CN substrate at a 1:50 ratio just before the reaction. The color reaction developed within 5–20 minutes. For the detection of bands recognized by the serum/anti-IgE pair, the Alkaline Phosphatase Conjugate Substrate (Bio-Rad) kit was used, based on alkaline phosphatase activity. The reaction solution was prepared by diluting the AP color development buffer (stock 25X) in milliQ water. Just before development, color reagent A (nitro-blue tetrazolium in aqueous dimethylformamide, magnesium chloride, DMF) was added at a 1:100 ratio, along with color reagent B (5-bromo-4-chloro-3-indolyl phosphate in DMF) at the same ratio. The reaction developed within approximately 5 minutes under a chemical fume hood. For both kits, after signal development, the

membranes were transferred into water to stop the reaction and then allowed to air dry.

Ab I (Primary antibody)	**Ab II (Secondary antibody)**	**Development Kit**
α-Gal monoclonal Antibody	/	Opti-4CN kit: - Opti-4CN diluent 1:10 - Opti-4CN substrate 1:50
Patient's sera	Anti IgE	Alkaline Phosphatase Substrate Kit: - AP Color Development Buffer (25X) - AP Color Reagent A and B, 1:100

Table 3.3: Antibody and development Kit for the immunoreactive bands

3.2.2.4 Patient's Selection

To assess the allergenic potential of protein extracts, sera from patients with confirmed red meat allergy and α-Gal positivity were used (*Table 3.4*). The selected subjects had a history of delayed allergic reactions (mean of 3.40 ± 1.58 events per person), occurring several hours after the consumption of red meat or innards. Clinical manifestations ranged from widespread urticaria (100% of cases) to gastrointestinal symptoms (60% of cases), hypotension, angioedema (50% of cases), and anaphylaxis episodes. In some cases, reactions were managed with antihistamines and corticosteroids, while severe cases required epinephrine administration. None of the patients had an allergy to cow's milk. The most implicated food was pork meat.

Patient ID	Sex	Age	Alpha Gal IgEs [KUA L^{-2}]
α 1	M	37	3.08
α 2	F	74	0.37
α 3	F	69	86.5
α 4	M	68	15.01
α 5	F	74	2.54
α 6	M	66	11.60
α 7	M	57	>100
α 8	M	58	31.50
α 9	F	26	1.17
α 10	M	67	8.95
Negative Control	M	48	<0.10

Table 3.4: Patient ' characterization; M: male; F: female

Serological analysis confirmed sensitization to α-Gal, with variable levels of specific IgE. All patients tested positive for α-Gal-specific IgE (26.08 ± 35.87 KUA L^{-1}), with a mean total serum IgE level of 389.99 ± 429.94 KU L^{-1}. Tryptase levels were within the normal range in all patients, with a mean value of 7.18 ± 3.78 μg L^{-1}. Among the identified risk factors, eight out of ten patients reported prior tick bites before the onset of AGS, while no evidence of such exposure was found in the remaining cases. Dietary recommendations included avoiding innards, fatty red meat, and other potential sources of α-Gal (milk, dairy products, etc). Additionally, patients were advised to monitor cofactors such as the consumption of nonsteroidal anti-inflammatory drugs (NSAIDs), alcohol intake, and physical activity in the hours surrounding red meat consumption. As a negative control, a subject without a cow's milk allergy and with a negative RAST for milk proteins was selected. A monoclonal anti-α-Gal antibody was used as a positive control.

3.2.2.5 Immunoprecipitation

The glycosylated proteins bovine thyroglobulin and bovine xanthine oxidase (XO) were used in immunoprecipitation assays. Glycosylation patterns of these proteins were chosen because they are pertinent to the investigation of antigen-antibody interactions in patients with AGS. In order to investigate the immunological response, serum samples from three AGS patients (α2, α3, and α5) were treated for one hour at room temperature with thyroglobulin concentrations ranging from 1, 3, 30, and 60 μg. At the same time serum from three other AGS patients (α1, α2, and α7) was incubated with xanthine oxidase (3, 30, and 60 μg) under the same conditions. By taking this step, the antibodies in the patient's serum were able to bind themselves specifically to the target proteins' glycosylated epitopes. Following immunoprecipitation, electrotransferred milk fat globule membrane proteins (MFGPs) were first blocked on nitrocellulose membranes for 30 minutes using a blocking solution to avoid nonspecific binding. Potential antibody-protein interactions were subsequently detected by incubating the membranes with the immunoprecipitated sera for a whole night at 4 °C and the immunoblotting was carried out to visualize the outcomes. This method made it possible to assess the antibodies' reactivity and specificity against the chosen glycosylated proteins found in the serum of AGS patients.

3.2.3 Protein's identification

3.2.3.1 Band preparation

The reactive band corresponding to the protein recognized either by the monoclonal antibody or by patient sera was excised using a steel scalpel and transferred into a 0.5 mL Eppendorf tube. The sample was then treated with 50 μL of 10 mM DTT in 50 mM NH_4HCO_3 and incubated for 45 minutes at 56°C. After incubation, it was cooled to room temperature, and the liquid was removed. Subsequently, 50 μL of 55 mM iodoacetamide (IAA) in 50 mM NH_4HCO_3 was added. The sample was vortexed and incubated for 30 minutes at room temperature in complete darkness, as IAA is photosensitive. After removing the liquid, 10 μL of MilliQ water was added, vortexed, and left for 15 minutes. Following liquid removal, 100 μL of 50% acetonitrile (ACN) was added, vortexed, and incubated for 15 minutes. This step was repeated twice. The liquid was then removed again, followed by the addition

of 20 µL of pure ACN. The samples were gently shaken for 5 minutes before removing the liquid. This step was repeated until the gel band became white and opaque. The gel band was then rehydrated with 30 µL of 50 mM NH_4HCO_3 and gently agitated for 5 minutes. Subsequently, 30 µL of pure ACN was added, followed by agitation for an additional 15 minutes. Finally, the liquid was removed, and the sample was dried using a SpeedVac concentrator (Concentrator 5301, Eppendorf AG, Hamburg, Germany) for 30 minutes to dehydrate the gel band in preparation for subsequent digestion.

3.2.3.2 Deglycosylation of alpha gal proteins

Deglycosylation is a technique used to remove oligosaccharide structures from a sample, in this case, to facilitate enzymatic digestion and the identification of the protein via LC/MS. Typically, N-glycans are released from proteins through the enzymatic action of peptide N-glycosidase F (PNGase F), which selectively cleaves N-glycans between an asparagine (Asn) residue and the sugar core. This enzymatic release requires a prolonged incubation (10–24 h) at 37°C (Eckard *et al.,* 2018). In this study, a total of 30 µL of PNGase F was added, using the enzyme at the concentrations 500 U/500 µL. The sample was gently agitated and incubated for one hour at 37°C. After incubation, 30 µL of 20 mM NH_4HCO_3 buffer (pH 7.2) was added, and the sample was left to incubate overnight. The following day, the excess liquid was removed from the sample, followed by the addition of 30 µL of pure acetonitrile (ACN). The sample was then agitated for 15 minutes. After removing the liquid, it evaporated using a SpeedVac for 30 minutes.

3.2.3.3 Tryptic digestion of alpha-gal proteins

Protein digestion is performed to cleave proteins into peptides, allowing for their identification via mass spectrometry (MS). In most cases, the preferred protease is trypsin, as it is highly specific and capable of generating peptides of an optimal length for MS identification. Trypsin is a protease that specifically cleaves peptide bonds at the carboxyl side of basic residues, lysine (K) and arginine (R). However, cleavage efficiency can be hindered by the presence of adjacent acidic, aromatic, or proline residues. In MS analysis, missed cleavage sites must also be considered, as incomplete digestion can generate peptide fragments with uncleaved sites. For digestion, 5 µL of a 75 ng/µL trypsin proteomic grade (Promega) solution in 25 mM NH_4HCO_3 buffer was first added to the dehydrated gel band, followed by 10 µL of 25 mM NH_4HCO_3 buffer. The sample was then incubated overnight (o/n) at 37°C under gentle agitation. After the first hour of incubation, an additional 10 µL of 25 mM NH_4HCO_3 buffer containing 10% formic acid was added to ensure the band remained covered. At the end of the digestion process, the sample was immediately frozen for further analysis.

3.2.3.4 High-resolution protein identification via HPLC-HRMS

Protein identification was carried out using an Orbitrap Q Exactive Plus mass spectrometer, which was coupled to a UHPLC binary pump system (Vanquish,

Thermo Fisher Scientific, Waltham, Massachusetts, USA). The chromatographic separation was performed on a BioBasic C18 HPLC column (1 × 150 mm, 5 μm; Thermo Scientific), which served as the stationary phase. For the mobile phase, a gradient elution method was applied using 0.1% (v/v) formic acid (FA) in MilliQ water as A solvent and 0.1% (v/v) formic acid in acetonitrile (ACN) as B solvent. To guarantee the best elution of the retained peptides, the chromatographic flow rate was 50.0 μL/min with a gradient that progressively raised the percentage of Solvent B from 5% to 70% over 50 min. A final step was then performed at 80% for 5 min. The autosampler was set at 6 °C to maintain sample integrity, and the column temperature was kept at 55 °C. Each injection included 4.0 microliters of the sample. Data from mass spectrometry were obtained using Full MS-ddMS2 mode. The instrument was configured to acquire Full MS spectra across an m/z scan range of 150–1800, ensuring comprehensive peptide mass coverage. The resolution was set to 70,000, with a maximum injection time (IT) of 200 ms and an automatic gain control (AGC) target of 5×10^5, optimizing sensitivity while preventing detector saturation. Additionally, unassigned charge states were excluded, refining data acquisition to relevant precursor ions. The device dynamically chose the 12 most intense precursor ions found in MS1 for further fragmentation in order to perform MS/MS fragmentation. In order to improve spectrum diversity and avoid continually fragmenting the same ion, the fragmentation spectral resolution was adjusted to 17,500 with a dynamic exclusion window of 20 seconds. Target ions could be precisely selected since the isolation window was set at 2.0 m/z. To ensure effective peptide dissociation, fragmentation was carried out using a normalized collision energy (NCE) of 28. The maximum IT for MS/MS was 200 ms, while the AGC target was set to 2×10^4, balancing sensitivity and scan speed for optimal peptide identification.

3.2.3.5 Data analysis: Parameter optimization and glycoprotein identification

A popular program for marker-free protein identification and quantification, MaxQuant (v. 2.0.3.0), was used to process Data Dependent Acquisition (DDA) files from mass spectrometry analysis. The UniProt Bos taurus repository was the database used for the search; it contained both reviewed and unreviewed protein sequences, guaranteeing a thorough examination of bovine proteins. The search was conducted using a custom contaminant list because the objective was to exclusively detect proteins of bovine origin. Bovine proteins were rigorously excluded to prevent misidentification and guarantee that only genuine hits were taken into consideration. Peptide identification was optimized by carefully adjusting the search parameters. To guarantee that all cysteine residues were regarded as alkylated, cysteine S-carbamidomethylation was specifically specified as a fixed change. Meanwhile, oxidation of methionine and N-terminal acetylation were included as variable modifications, allowing for natural post-translational modifications that could affect protein structure and function. Additionally, since trypsin was used for protein digestion, the algorithm was allowed to recognize up to two missed cleavage sites, accounting for potential incomplete digestion events.

To further refine the identification of glycosylated proteins, an additional Asparagine (Asn) to Aspartate (Asp) modification was included as a variable modification for protein bands derived from enzymatic de-glycosylation. This accounted for potential deamidation events, a key factor in glycoprotein analysis. The fragment ion mass tolerance for MS/MS spectra was set to 20 ppm (parts per million), ensuring high-resolution mass accuracy and minimizing the risk of false identifications. For a protein to be considered as confidently identified, it needed to meet the following stringent criteria:

- Detection of at least three peptides associated with the protein.
- A false discovery rate (FDR) threshold of 0.01%, both at the protein and peptide level, ensuring minimal false positives.
- A minimum score of 20 for both modified and unmodified peptides.

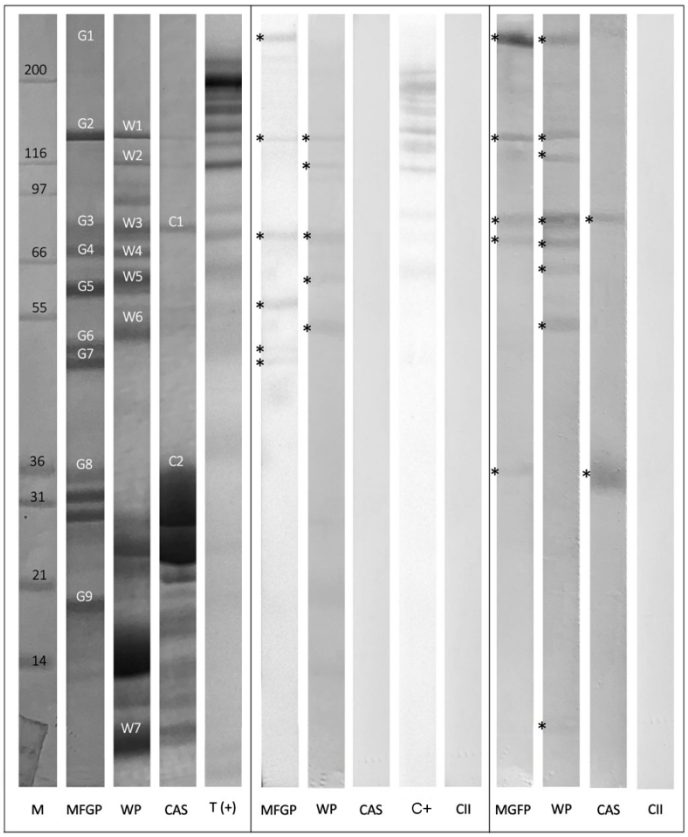
A slightly looser criteria of >10 was permitted for proteins identified by unstained bands removed from the top of the gels, however only proteins scoring greater than 35 were included in the final dataset. This adjustment took into consideration the bands' decreased intensity and possible detection challenges.

3.3. RESULTS

3.3.1 MFGM proteins are recognized by anti-α-Gal antibodies and exhibit immunoreactivity in AGS patients

LDS-PAGE and immunoblotting experiments were performed to separate and analyze the reactivity **of** milk fat globule membrane proteins (MFGP), whey proteins (WP), and caseins (CAS) with anti-α-Gal IgG antibodies and sera from 10 AGS patients. This approach enabled the identification of multiple immunoreactive bands in both MFGP and WP extracts, indicating the presence of α-Gal-positive proteins within milk-derived fractions. Specifically, the bands (*Fig. 3.3*) that exhibited reactivity were:

- **MFGP bands:** G1, G2, G3, G5, G6, and G7.
- **WP bands:** W1, W2, W3, W5, and W6.



Legend:
M: marker
MFGP: milk fat globule proteins
WP: whey proteins
CAS: caseins
T (C+): Tireoglobulin, positive control
CII: secondary control

Figure 3.3: LDS page of MFGP, WP and CAS; Immunoblotting with anti α-Gal. Source: Aiuto et al., 2024, Mol. Nutr. Food Res. 2300796

To further characterize the proteins, present in these immunoreactive bands, LC-HRMS analysis was performed (*Table 3.5*). This high-resolution mass spectrometry analysis allowed the identification of lactoferrin (LF) and lactoperoxidase (LPO) in band W3**,** as well as several immunoglobulin (Ig)-like domain-containing proteins in bands W2, W3, W5, and W6. One of the most striking findings of this study was the identification of Xanthine Oxidase (XO) in multiple immunoreactive bands (W1, G1, G2, and G3). XO is a highly expressed oxidoreductase enzyme found in milk and mammalian tissues, and its glycosylation profile makes it a strong candidate for containing α-Gal epitopes. Notably, band G1 was not detectable by standard protein staining techniques (such as Coomassie staining), yet it was

strongly recognized by both anti-α-Gal IgG and AGS patient sera. This suggests that even low-abundance α-Gal-containing proteins can be highly immunogenic in sensitized individuals, further underscoring the importance of mass spectrometry-based proteomics in the detection of clinically relevant allergens that may otherwise be overlooked using traditional protein visualization techniques**.** Other MFGP-reactive bands (G3, G5, G6, and G7) predominantly contained butyrophilin (BT) and lactadherin (LA), two well-characterized milk glycoproteins involved in immune regulation.

N° Band	Uniprot Entry	Protein name	Protein Score	N° of peptides	Protein coverage [%]
G1	P80457	Xanthin dehydrogenase	130.480	16	16.0
	Q8WNR8	Perilipin	52.718	8	27.9
	Q27960	Sodium-dependent phosphate transport protein 2B	52.365	5	6.6
	Q4GZT4	Broad substrate specificity ATP-binding cassette transporter ABCG2	43.296	7	15.0
	P18892	Butyrophilin subfamily 1 member A1	25.794	4	11.0
	A0A4W2HXW4	3-hydroxyacyl-[acyl-carrier-protein] dehydratase	12.606	2	1.1
G2	P80457	Xanthine dehydrogenase	317.140	30	30.1
	P18892	Butyrophilin subfamily 1 member A1	59.231	10	24.7
	A0A4W2I0L9	ATP-binding cassette sub-family G member 2	34.445	4	6.0
G3	G5E513	Ig-like domain containing protein	31.553	9	23.2
	G5E5T5	Ig-like domain containing protein	129.030	10	22.4
	A0A3Q1M193	Glycoprotein 2	260.530	8	17.3
	P18892	Butyrophilin subfamily 1 member A1	145.460	10	24.1
	C7FE01	Lactoferrin	55.906	8	12.8
	P80457	Xanthine dehydrogenase	32.807	5	3.8
G4	P81265	Polymeric immunoglobulin receptor	211.620	18	35.1
	A0A3Q1M193	Glycoprotein 2	92.215	10	23.8
	P18892	Butyrophilin subfamily 1 member A1	106.110	15	41.3
	P26201	Glycoprotein IIIb	91.212	6	12.9
	G5E513	Ig-like domain-containing protein	95.157	9	33.3
	A0A3Q1LWT4	Acyl-CoA synthetase long chain family member 1	79.564	10	18.0
	J7K1V4	Lactoferrin	75.774	12	18.6
	F1MHI1	Perilipin	53.926	7	25.5

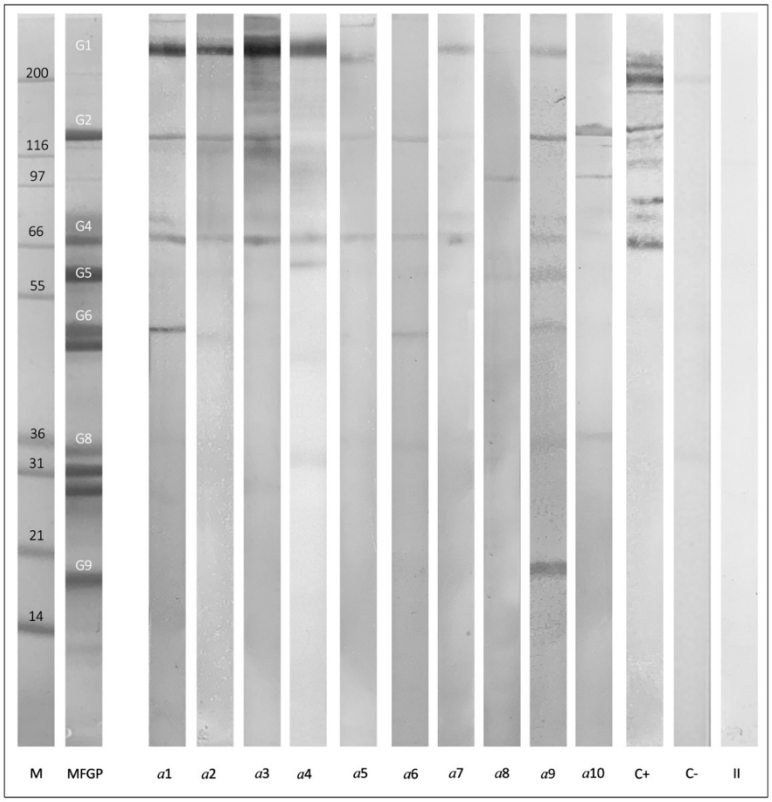
	Q27960	Sodium-dependent phosphate transport protein 2B	32.389	5	9.7
	Q95114	Milk fat globule-EGF factor 8 protein (Lactadherin)	32.227	4	17.0
	A0A3Q1MK38	Terpene cyclase/mutase family member	52.104	5	8.8
	P80457	Xanthine dehydrogenase	30771	5	3.8
G5	P18892	Butyrophilin subfamily 1 member A1	252.710	19	31.4
	Q95114	Milk fat globule-EGF factor 8 protein (Lactadherin)	50.477	6	19.0
G6	Q95114	Milk fat globule-EGF factor 8 protein (Lactadherin)	198.570	22	57.2
	Q9TUM6	Perilipin-2	189.240	19	59.5
	P18892	Butyrophilin subfamily 1 member A1	31.353	5	13.3
G7	Q95114	Milk fat globule-EGF factor 8 protein (Lactadherin)	231.350	13	35.3
	Q8HZM7	Perilipin	55.801	4	18.0
G8	P02663	Alpha-S2-casein	41.439	6	18.0
	P18892	Butyrophilin subfamily 1 member A1	47.768	5	12.5
G9	B5B0D4	Major allergen beta-lactoglobulin	116.590	11	65.2
	Q5E9I6	ADP-ribosylation factor 3	47.494	7	45.9
	Q8HZM7	Perilipin	35.690	5	18.9
G10	P80457	Xanthine dehydrogenase	37.778	5	6.4
	A0A3Q1MGL5	SRCR domain-containing protein	16.338	2	23.1
	A0A4W2HXW4	3-hydroxyacyl-[acyl-carrier-protein] dehydratase	13.596	2	1.4
	Q27960	Sodium-dependent phosphate transport protein 2B	14.233	2	2.7
G11	P80457	Xanthine dehydrogenase	97.052	11	13.0
G12	P80457	Xanthine dehydrogenase	167.660	20	21.4
G13	P80457	Xanthine dehydrogenase	103.510	11	9.4
	Q27960	Sodium-dependent phosphate transport protein 2B	35.245	2	2.2
G14	G5E5T5	Immunoglobulin heavy constant mu	157.780	12	32.1
	F1MZQ4	Butyrophilin subfamily 1 member A1	65.440	7	15.8
G15	A0A4W2DWX4	Butyrophilin subfamily 1 member A1	94.962	13	25.9
G16	P0DOX5	Immunoglobulin gamma-1 heavy chain	97.277	10	31.2

	Q95114	Milk fat globule-EGF factor 8 protein (Lactadherin)	34.165	5	16.7
G17	P01834	Immunoglobulin kappa constant	59.743	5	67.3
	A5PK49	Ig-like domain-containing protein	30.416	4	12.8
G18	A0A4W2I0L9	ATP-binding cassette sub-family G member 2	11.869	2	3.1
	P80457	Xanthine dehydrogenase	17.852	3	1.9
	Q27960	Sodium-dependent phosphate transport protein 2B	42.271	2	2.2
G19	P80457	Xanthine dehydrogenase	292.240	32	27.0
G20	G5E513	Immunoglobulin heavy constant mu	84.106	10	29.2
	P81265	Polymeric immunoglobulin receptor	65.441	9	17.6
	P18892	Butyrophilin subfamily 1 member A1	51.220	8	20.0
G21	F1MZQ4	Butyrophilin subfamily 1 member A1	63.366	7	17.5
G22	P18892	Butyrophilin subfamily 1 member A1	143.590	16	35.6
G23	Q9TUM6	Perilipin-2	83.058	8	28.9
	P18892	Butyrophilin subfamily 1 member A1	45.368	6	12.7
G24	Q95114	Milk fat globule-EGF factor 8 protein (Lactadherin)	137.390	16	39.2
	P18892	Butyrophilin subfamily 1 member A1	31.275	5	9.9
G25	P21163.2	Peptide-N(4)-(N-acetyl-beta-D-glucosaminyl)asparagine amidase PNGase F	227.360	16	52.5
	P18892	Butyrophilin subfamily 1 member A1	49.619	5	11.2
W1	P80457	Xanthine dehydrogenase	323.310	20	18.2
W2	A0A4W2CZN6	C3 complement	308.810	32	20.3
	A0A3Q1M3L6	Ig-like domain-containing protein	106.250	7	29.7
W3	C7FE01	Lactoferrin	323.310	45	66.5
	G5E513	Ig-like domain-containing protein	307.500	16	54.2
	G3X6N3	Serotransferrin	117.080	22	39.9
	P80025	Lactoperoxidase	187.390	22	39.9
	A0A3Q1M3L6	Ig-like domain-containing protein	44.510	4	19.3
	B3VTM3	Lactotransferrin	45.075	7	13.0
	A0A3Q1LNN7	Albumin	32.956	5	9.2
W4	P81265	Polymeric immunoglobulin receptor	134.530	11	25.0
	A0A4W2DZ09	Serotransferrin	133.090	15	33.5

	E1BMJ0	Serpin family G member 1	95.139	5	17.9
	A0A4W2CZN6	C3-beta-c	79.946	10	9.0
	A0A3Q1M032	Ig-like domain-containing protein	92.036	4	16.8
	A0A4W2DDL5	Albumin	60.754	8	18.9
	A0A4W2GX34	Lactoperoxidase	33.890	5	9.2
W5	P02769	Albumin	323.310	41	64.7
	A0A4W2CZN6	C3 complement	244.450	28	22.2
	Q2KJF1	Alpha-1B-glycoprotein	75.560	9	36.2
W6	A0A3Q1M3L6	Ig-like domain-containing protein	148.910	10	52.9
	G3N0V0	Ig-like domain-containing protein	49.249	6	25.2
	Q9TTE1	Serpin A3-1	75.075	7	25.3
	A0A4W2HXY3	Serpin A3-1	33.062	5	17.9
	A0A140T8A9	Kappa-casein	30.867	3	23.7
	A0A3Q1NG86	Alpha-S1-casein	30.935	3	18.0
	P02754	Beta-lactoglobulin	32.047	3	22.5
	A0A4W2FAA0	Antithrombin-III	32.029	5	11.8
	P08037-2	Isoform Short of Beta-1,4-galactosyltransferase 1	79.095	2	5.1
W7	P02754	Beta-lactoglobulin	31.959	4	32.1
	P00711	Alpha-lactalbumin	144.240	3	24.4
	P80195	Glycosylation-dependent cell adhesion molecule 1	31.741	2	12.4
C1	P24627	Lactotransferrin	323.310	47	61.9
	P18892	Butyrophilin subfamily 1 member A1	41.743	3	12.2
	G5E513	Ig-like domain-containing protein	32.696	3	9.3
C2	P02662	Alpha-S1-casein	323.310	8	42.2
	P80195	Glycosylation-dependent cell adhesion molecule 1	93.318	2	12.4
	A0A140T8A9	Kappa-casein	190.770	4	30.5
	A0A452DHW7	Beta-casein	62.074	5	18.5
	P02754	Beta-lactoglobulin	61.784	5	37.1
	P02663	Alpha-S2-casein	62.470	3	13.5

Table 3.5: Identification of the proteins immunorecognized by anti-alpha-Gal IgG and/or by the pool of alpha-Gal syndrome patient's sera in MFGP (band from G1 to G25), WP (from W1 to W7) and CAS (C1 and C2). Source: Aiuto et al., 2024, Mol. Nutr. Food Res. 2300796

A critical aspect of this study was the comparison between bands recognized by anti-α-Gal IgG and those identified using AGS patient sera (*Fig. 3.4*). Additional bands were exclusively detected by AGS patient sera (G4, G8, W4, W7, C1, and C2).



Legend:

M: marker
MFGP: milk fat globule proteins
α1-10: patients with positive immunoreactivity
T (C+): Tireoglobulin, positive control
C-: pool of patients with negative immunoreactivity
II: secondary control

Figure 3.4: Recognition of MFGP by AGS patients; immunoblotting of MFGP with the sera of 10 AGS patients. M: molecular weight marker; C+: thyroglobulin; C-: patient not assuming meat. Negative control; CII secondary antibody. Source: Aiuto et al., 2024, Mol. Nutr. Food Res. 2300796

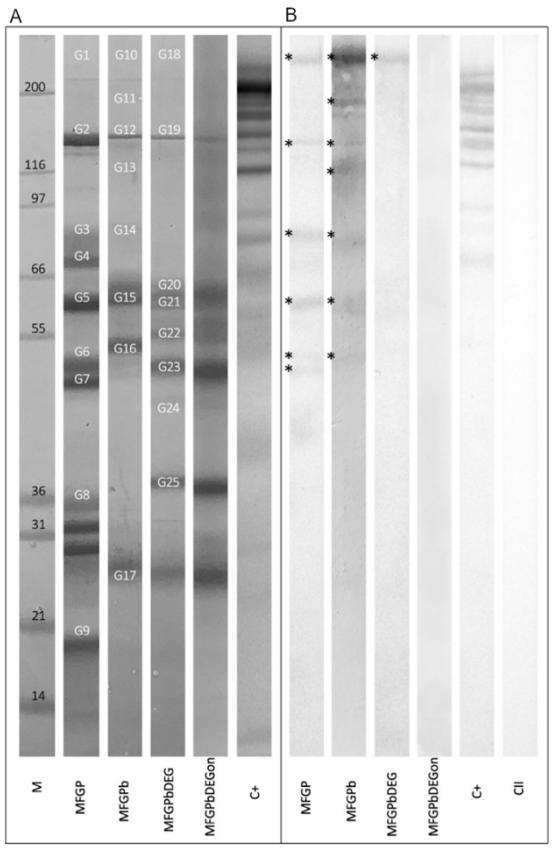
In particular, the three main milk fat globule membrane proteins (XO, BT, and LA) were found in Band G4. BT, LPO, and proteins with Ig-like domains were among the previously identified α-Gal-glycosylated proteins found in bands G8, W4, and C1. Common milk allergens, such as caseins and β-lactoglobulin, were found in bands W7 and C2. This discovery implies that individuals with AGS might have a more comprehensive immune response that goes beyond the traditional α-Gal epitopes, possibly identifying other milk glycoproteins or cross-reactive antigens. Moreover, in addition to α-Gal, structural motifs or post-translational modifications (PTMs) may also cause immune system reactions in AGS patients, which calls for greater research.

This finding suggests that AGS patients may have a broader immune response beyond classical α-Gal epitopes, potentially recognizing additional milk glycoproteins or cross-reactive antigens**.** Furthermore, the AGS patients' immune system may react to structural motifs or post-translational modifications (PTMs) beyond α-Gal, warranting further investigation.

3.3.2 Deglycosylation and BioMag beads-enrichment as key steps for confirming α-Gal glycosylated proteins

α-Gal-glycosylated proteins were enriched using BioMag Goat Anti-Human IgG beads conjugated with anti-α-Gal IgG antibodies**,** allowing for the selective

isolation of the proteins of interest. The enriched proteins were subsequently separated via LDS-PAGE (*Fig. 3.5* A, lane MFGPb) and as expected, were recognized by anti-α-Gal IgG antibodies**,** confirming the presence of α-Gal epitopes**.** In addition to high molecular weight bands, the protein profile of the spiked sample showed bands G16, G17, and G25, which contained PNGase F, the enzyme involved in deglycosylation, and anti-α-Gal heavy and light IgG chains that were partially released from the beads during protein elution. Of these, bands G17 and G25 exhibited no immunoreactive response, whereas band G16 displayed a mild positivity, most likely as a result of lactadherin (LA). The presence of α-Gal epitopes on these proteins was confirmed in large part by the deglycosylation process. N-linked glycans, which are normally bonded to the nitrogen atom of asparagine (Asn) side chains within the consensus sequence Asn–X–Ser/Thr (where X represents any amino acid other than proline), are selectively eliminated by the enzyme PNGase F. Following PNGase F's removal of the N-glycan, the asparagine residue deaminates and becomes aspartic acid (Asp). This modification can be detected by comparing the protein profile before and after deglycosylation**,** where asparagine should be present in the intact sample but replaced by aspartic acid in the deglycosylated form**.** Notably, this alteration in the epitope structure renders it unrecognizable by the monoclonal antibody**.** After deglycosylation, anti-α-Gal IgG antibodies no longer detected any bands**,** except for a faint signal in band G18**,** where Xanthine Oxidase (XO) was present (*Fig. 3.5* A, lane MFGPb-DEG). To assess the impact of incubation time on deglycosylation efficiency, multiple deglycosylation experiments were conducted**,** revealing that immunoreactivity was completely abolished only after an extended overnight PNGase F treatment (*Fig. 3.5*).



Legend:

M: marker
MFGP: milk fat globule proteins
MFGPb: MFGP enriched alpha-gal glycosylated
MFGPbDEG: MFGPb deglycosylated
MFGPbDEGon: MFGPb deglycosylated overnight
C+: Tireoglobulin, positive control
CII: secondary control

Figure 3.5: LDS page and immunoblotting of MFGP; MFGP after anti alpha-Gal IgG/beads incubation (MFGPb) and MFGPb deglycosilated. Source: Aiuto et al., 2024, Mol. Nutr. Food Res. 2300796

To identify which asparagine residues were glycosylated with α-Gal**,** N-deglycosylated proteins that had lost reactivity were further analyzed (*Fig. 3.5* B, lane MFGPb-DEG). The presence of newly generated tryptic peptides**,** where aspartic acid (Asp) replaced the original asparagine (Asn)**,** provided strong evidence of α-Gal glycosylation sites prior to proteolytic digestion (*Table 3.6*)**.** LC-HRMS analysis of band G22 revealed that butyrophilin (BT) contained a peptide where Asn_{215} was converted to Asp_{215} following deglycosylation**,** strongly suggesting that Asn_{215} was originally glycosylated with α-Gal**.** Similarly, in band G24**,** lactadherin (LA) exhibited Asn_{227} modified to Asp_{227}**,** confirming the presence of N-linked α-Gal glycosylation on this residue.

	THEORETICAL DATA		LC-MS/MS EXPERIMENTAL DATA	
α-gal MFGMP	**N-glycosylated triplets**	**triplets already known from literature**	**Peptide-containing triplet before enzymatic de-glycosylation**	**Peptide-containing modified triplet (N-->D)**
XO (P80457)	N_{644}ET	not	not found	not found
	N_{704}NS	not	not found	**704-713 (D_{704}NS)**
	N_{904}LS	yes (in goat)	903-912 (N_{904}LS)	not found
	N_{1073}SS	yes (in human)	not found	not found
	N_{1288}NT	not	1283-1290 (N_{1288}NT)	not found
BT (P18892)	N55VS	yes (in cow)	not found	not found
	N215VS	yes (in cow)	not found	**215- 221 (D215VS)**
	N337MT	not	not found	not found
LA (Q95114)	N_{59}ET	yes (in cow)	not found	not found
	N_{144}NS	not	138-149 (N_{144}NS)	not found
	N_{227}NS	yes (in cow)	not found	**221-232 (D_{227}NS)**
	N_{390}NS	not	382-395 (N_{390}NS)	not found

Table 3.6: Deglycosylation site for xanthine oxidase, butyrophillin and lactadherin. XO: Xanthine Oxidase; BT: Butirophilin; LA: Lactoadherin; MFGMP: milk fat globule membrane proteins. Source: Aiuto et al., 2024, Mol. Nutr. Food Res. 2300796

3.3.3 Immunoprecipitation confirms patient-specific recognition of α-Gal epitopes

Immunoprecipitation (IP) is a widely used technique for selectively isolating antigen-antibody complexes, allowing for the assessment of specific immune responses. To confirm that patient immunorecognition was specifically directed against α-Gal epitopes, immunoprecipitation assays were performed using sera from three patients (α2, α3, and α5) with varying concentrations of bovine thyroglobulin (1, 3, 30, and 60 μg) (*Fig. 3.6* A). According to the data, patient α3 needed 60 μg of thyroglobulin to fully suppress immunorecognition, while the other two patients only needed 3 or 30 μg. These results imply that patients' antibody concentrations or affinities vary. To further assess the specificity of the immune response, the same experiment was conducted using bovine xanthine oxidase (XO) with sera from three additional patients (α1, α2, and α7) (*Fig. 3.6* B).

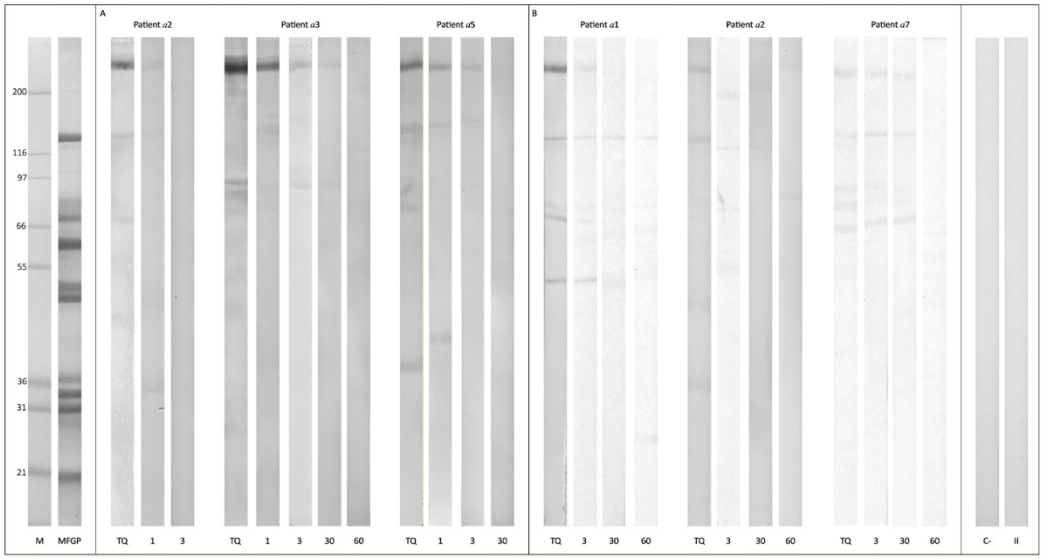


Figure 3.6: Immunoprecipitation using AGS patient's sera and bovine thyroglobulin.
Source: Aiuto et al., 2024, Mol. Nutr. Food Res. 2300796

In this instance, 60 μg of XO was needed by every patient to completely immunoprecipitate their sera, suggesting a distinct binding efficiency in contrast to thyroglobulin. Interestingly, in both inhibitory experiments, patient α2 required 60 μg of XO but only 3 μg of thyroglobulin, indicating that their antibodies had a higher affinity or specificity for thyroglobulin. These findings validate that patient antibodies selectively detect α-Gal epitopes on numerous glycoproteins and demonstrate the usefulness of immunoprecipitation in evaluating antigen-specific immune responses. Understanding immunological sensitivity and response variability may be affected by the fact that different patients have various immune responses to α-Gal, as evidenced by the different antigen concentrations needed for total suppression.

4. Case study 2:

Quantification of trace amounts of proteins using HPLC-HRMS in red wine

4.1. Introduction

4.1.1 Trace amount of allergens in food and the problem of the minimum elicitation dose

Trace allergens in food can be extremely dangerous to allergic patient's health. Anaphylaxis and other potentially fatal reactions can be brought on by even trace levels of allergenic proteins. Because of biological heterogeneity, it is challenging to establish a universally safe threshold. Although some people with food allergies can tolerate low concentrations of an allergen, the majority can respond to very low levels, like a few milligrams or even micrograms. Furthermore, because the inclusion of fats, carbs, or other proteins can alter the reactivity and absorption of allergenic proteins, the food's composition can also determine how severe an allergic reaction is. When 14 specific allergens are purposefully included as ingredients on food labels, they must be declared in accordance with European Union Directive 2006/142/EC. Nevertheless, accidental contamination, which can happen at any point during the food production process, is not covered by this law. Zero risk is essentially impossible to attain, despite efforts to minimize these hazards (Remington *et al.* in 2020). Therefore, analysis of over 3400 low-dose oral clinical challenges revealed that unintentional allergens in food make it impossible to eliminate risk entirely (Turner *et al.,* 2017).

Even with stringent dietary restrictions, allergic individuals continue to experience reactions (Gupta *et al*., 2018; Michelsen-Huisman *et al*., 2018). Elimination of trace allergens becomes more difficult by the usage of shared processing equipment and the intricacy of food supply networks. Uncertainty may arise because regulators must make choices on an individual basis in the absence of reference dosages or defined action levels at the regulatory level (DunnGalvin *et al*., 2015; Hattersley *et al.*, 2014; Madsen *et al.,* 2010). Therefore, even with the best of intentions, it is nearly impossible to guarantee that all allergen residues are eliminated from shared equipment (Crevel *et al*., 2014). The accuracy and precision of mass spectrometry is particularly useful when dealing with complex or processed foods. These foods may exhibit variable allergenic potential due to processing or novel ingredients. In such cases, traditional methods such as ELISA, which rely on detecting known allergenic epitopes, may be insufficient or unreliable for accurate assessment (Monaci et al., 2009). For this reason, MS-based techniques are essential in both research and regulatory settings to ensure food safety and accurate labeling.

4.1.2 Legislation and Labelling of fining agents in wine

Quantifying residues of protein finishing agents in wine is crucial especially in relation to label transparency and consumer safety. Winemakers often use these protein-based processing aids, however, traces of these proteins may still be present in the finished product, with significant consequences for consumers with allergies or dietary restrictions (vegetarians or vegans). Precise quantification is becoming increasingly important to enable accurate labeling and allowing consumers to make informed choices. This is especially significant for two main reasons. The market for vegan-friendly goods is growing, leading some winemakers to look for substitute plant-based fining agents or to explicitly label wines that contain ingredients derived from animals. Furthermore, individuals who have dietary allergies are susceptible to allergens in even small doses, which can result in extremely severe allergic reactions (Gupta *et al.,* 2021; Besler, M. *et al*., 2001). In the clarification process, the most commonly used protein adjuvants, which can cause allergies, are casein and egg white. Precisely because residues of these adjuvants can be dangerous, it is essential to accurately measure these residual proteins. EU Regulation 1169/2011 requires the declaration of specific allergens, including those used as technological adjuvants in wine, when their presence in the finished product could endanger consumers. To meet these regulatory requirements, quantification techniques must be sufficiently sensitive to detect these allergens at the threshold levels specified by the guidelines (Fiocchi *et al.,* 2021). Before December 8, 2023, alcoholic beverages were excluded from the need that all items include an ingredient list and nutritional data. Nevertheless, the European Union has modified this regulation, making it necessary for a wine to include the producer's name, the region of origin, the amount of alcohol it contains, the batch number, and information about allergies. Specifically, any presence of milk or egg proteins in wine needs to be disclosed. Regulation 579/2012 states that the most stringent levels for the disclosure of allergenic proteins on wine labels, employing ELISA as a quantitative analytical method, are 0.25 mg/L and 0.50 mg/L for detection and quantification, respectively.

4.1.3 Wine making, wine chemical composition and the use of fining agents

Wine is an alcoholic beverage made from the fermentation of grapes or other fruits. Numerous elements, such as the kind of grapes used, the fermenting process, and the place of production, affect the creation and qualities of wine. Red wine is produced by fermenting grape skins, which give the wine its color and tannins. It is made from grape types with dark colors. Because of its wide variety of chemical components and their dynamic interactions, wine is a biologically complex matrix. Wine's complexity is influenced by a variety of processes, including aging, fermentation, and environmental influences on flavor, fragrance, color, and stability. It is constituted from water (80-90%), ethanol, acids, phenolic compounds, carbohydrates, proteins, and a variety of volatile and non-volatile compounds. Among phenolic compounds there are flavonoids (anthocyanins, flavanols, favonols) which contribute to color, astringency and bitterness; non-flavonoids (resveratrol, phenolic acids) which provide health benefits and contribute to antioxidant capacity (Haslams *et al.,* 1989). Tannins are the major components that

produce sensation of astringency and contribute to the “structure” of wine. The wine astringency is defined as the drying and puckering sensations in the mouth, but when it combined with other elements like alcohol and sugar content, it’s thought to be a pleasant quality (Gawel, 1998). Understanding the tannin structure of the matrix is crucial for producing better wine because it affects the final product. They change significantly during the winemaking process, particularly once "grape" tannins are transformed into "wine" tannins, which are chemically more complex (Cheynier *et al.,* 2012). Tannins from oak barrels used to age wine are known to be “hydrolyzed tannins” and these wines contain traces of them. They have a core of glucose, or another polyol esterified with either gallic acid (gallotannins) or ellagic acid (ellagitannins) (Smith *et al.,* 2015). The most common tannins in red wine, up to 4 g/L, are “condensed tannins” that derive from the skin, pulp and seeds of the grape during the winemaking process. They consist of repeating units of flavan-3-ol, such as catechin, epicatechin, epigallocatechin and epicatechin gallate (Herderich and Smith 2005). Wine tannin’s structure is well known and more complex. During winemaking, yeasts, enzymes, and fermentation byproducts (such as acetaldehyde) alter the structure of the condensed tannins derived from grapes. Therefore, the chemical reactions that occur in wine after fermentation cause the tannins to progressively change from a purple to a brick red color, and they also usually lead the tannins to become less harsh (Smith *et al.,* 2015).

4.1.4 Egg white proteins and gelatin

Processing aids are designed to reduce the concentration of tannins. Egg white and gelatin, for example, are able to bind tannins and create complexes that precipitate in the wine. These stable complexes that precipitate can then be subsequently eliminated by filtration or sedimentation. These proteins preferentially bind to higher molecular weight tannins, which facilitates the precipitation of tannins. Enzymes such as pectinases and proteases may also enhance the breakdown of grape cell walls, releasing bound tannins, which can then be selectively removed from the must. This not only reduces astringency but also helps to stabilize the wine’s color by interacting with anthocyanins.

Different types of protein-based fining agents exist such as gelatin, isinglass, casein and egg albumen: a) Gelatin is a hydrocolloid derived from collagen and contains many amino acids that can form hydrogen bonds and hydrophobic interactions with tannins (Blade & Boulton, 1988); b) Isinglass is a collagen-based fining agent sourced from fish swim bladders and it’s able to remove tannins thank to its strong protein-tannin interactions (Clark *et al.,* 2004); c) Casein is a milk protein that efficiently forms insoluble complexes with tannins by binding to them through its polar and non-polar amino acid residues (Zoecklein *et al.,* 1995); d) Albumin derived from egg whites and has a strong affinity for tannins, especially because it has a lot of binding sites that help with precipitation (Dakis *et al.,* 2014).

Gelatin: Gelatin is a positively charged protein derived from animal collagen. In contrast, tannins in wine generally have a net negative charge at the pH of the wine. When gelatin is introduced, the opposite charges attract, forming large protein-

tannin complexes that are insoluble in wine and precipitate. These complexes are removed by filtration or racking (Marangon *et al.* 2011). Therefore, the wine becomes softer and more palatable as the excess tannins are reduced. Gelatin also interacts with other phenolic substances to help keep the wine's color stable. Usage levels are typically from 2.5 to 6 g/hL (equivalent to 25–60 mg/L) (Ribéreau-Gayon *et al.,* 2000).

Egg white: According to the OIV definition, egg white proteins—commercially referred to as "albumin"—are the most commonly utilized finishing agents in the manufacture of red wines. They bind to phenolic compounds that are negatively charged because they are positively charged. Between 3 and 15 g/hL (or 30 to 150 mg/L) of these proteins are typically added to red wine. Bitterness and astringency are mostly caused by the smaller, more reactive tannin molecules that albumin preferentially binds to. Without excessively sacrificing the taste and structure that tannins seek, this selective binding helps mitigate the bitterness of young red wines. Increasingly, lysozyme is an enzyme widely utilized in winemaking for its antibacterial qualities rather than mainly as a finishing ingredient. Gram-positive bacteria are strongly inhibited by this enzyme. The peptidoglycan layer found in the cell walls of Gram-positive bacteria, including Oenococcus and Lactobacillus (lactic acid bacteria), can be hydrolyzed by it. It functions by rupturing the lactic acid bacteria's cell walls, which stops them from growing or even kills them. This shields the wine from bacterial deterioration and stops or regulates malolactic fermentation. Additionally, lysozyme helps keep the wine fresh and clear by stopping malolactic fermentation, which turns malic acid into lactic acid. The typical range for the amount of lysozyme supplied is 25–50 g/hL (250–500 mg/L).

4.1.5 Metrological approaches in biochemistry

The intricacy of the biological systems under study contributes to the issue of irreproducibility in science. Inaccurate results can arise from the propensity to work in concentrations (e.g., mg/ml) without taking the molecule's activity or the complexity of the test system into consideration. This is since, when a molecule is added to an experiment, a milligram of an enzyme or protein from manufacturer X may not necessarily have the same activity as a milligram of the identical molecule from manufacturer Y (Coxon *et al., 2019*). Metrology aims to produce accurate findings by standardizing data from various producers or labs. Because biological molecules like proteins, enzymes, and DNA have such a high degree of structural and functional heterogeneity, it is very difficult to standardize measurements and produce accurate and repeatable results in biological systems. There are few reference materials for these biomolecules, and when they are, they rarely ever fully represent the intricacy of biological matrices. Additionally, biological measurements are often influenced by interactions within complex matrices, such as food products like wine. These interactions can alter the signal or detectability of the analyte in unpredictable ways, complicating both the interpretation and standardization of results. Therefore, to create a metrological approach that enables the acquisition of exact measurements, it is crucial to fully evaluate the

characteristics of biological samples (Glish & Burinsky 2008). For example, implementing the use of certified reference materials (CRMs) and biological matrix-specific reference standards provides a known standard against which to compare sample measurements, improving accuracy. Sensitivity and specificity can also be increased by using sophisticated analytical methods including mass spectrometry, nuclear magnetic resonance (NMR) spectroscopy, and high-performance liquid chromatography (HPLC) (Coxon *et al*., 2019). Accurate labeling for traces of allergens is essential in wine, especially for egg residues that can cause allergic reactions in sensitized people. Therefore, the use of highly sensitive and reliable techniques becomes vital to ensure consumer safety (Penas *et al.,* 2015). Gelatin, on the other hand, is not typically thought to pose a serious allergy risk. Compared to other fining agents like egg white (albumin), gelatin is believed to have a minimal propensity for causing allergies. This is because of its limited solubility, large molecular weight, and the way it interacts with wine, which lessens the possibility that it may trigger an immunological reaction. Even though gelatin may leave behind residues in wine, research has typically shown that these residues are negligible and unlikely to cause allergy reactions. However, the presence of gelatin, even in trace amounts, remains a concern for vegans and vegetarians who seek products free from animal-derived components. Therefore, it is equally important to quantify gelatin residues accurately to cater to these consumer preferences (Karim *et al*., 2008). Reliable results depend on precise and accurate measurements, which metrology guarantees. The first step to get these accurate measures traceable directly to the SI, is to define a common measurand. In the third edition of the "International Vocabulary of Metrology" (VIM 3), published in 2007, measurement is defined as "the quantity that is intended to be measured". A standard measurement can improve accuracy and precision, reducing measurement uncertainty. This is also essential for comparing results from different laboratories. Since all measurements are based on the same reference, variability is minimized. According to Martinez-Esteso (2020), in the field of food safety, the "mass of total protein of the allergenic ingredient per mass of food" is stated as the common and unambiguous measurand. These are the terms in which the research project's outcome should be expressed.

4.1.6 IDMS: a primary technique for accurate results in biochemistry measurements

The high-precision analytical method known as isotope dilution mass spectrometry (IDMS) is used to quantify chemical compounds in different matrices. The technique consists of adding a known quantity of an internal standard, or labeled isotope such as ^{13}C and ^{15}N, into one of the amino acids in the reference peptide (Heumann, K. G. 2004; Vogl & Rosner 2012). Despite having a different isotopic makeup, the tagged isotope is chemically identical to the native analyte. Mass spectrometry is used to quantify the isotopic ratio that is created between the analyte and the internal standard once the analyte is added (Vogl, J. and Pritzkow, W. 2010). One of the most exact and accurate analytical techniques is IDMS. The use of an isotopically labeled internal standard greatly improves measurement precision

by lowering methodological and instrumental variability. Furthermore, assuring results are globally recognized and comparable is crucial since it lowers measurement errors related to different analytical process steps (Taylor, P. D. P. 2001). There are some critical steps in the development of this method to ensure reliable results such as the peptide selection and the setting of a MS analysis design.
Selection of signature peptides: Selecting appropriate signature peptides from tryptic digested proteins is crucial in configuring an LC-MS/MS analytical method for quantifying trace amounts of proteins. The peptides must be unique to the protein of interest. Otherwise, the protein of interest might be overestimated. The peptides must be proteotypic with a maximum length of 12 amino acids because this size range balances stability, ionization efficiency, and chromatographic behavior (Coon, J. J. *et al*., 2018). Some amino acids should be avoided because they are prone to oxidation or instability such as cysteine, methionine, or tryptophan residues. Because of their reactive nature, these residues can result in an inconsistent measurement. Additionally, these peptides should be unaffected by mutations and must not carry post-translational modifications, such as N-glycosylation sites (NXS or NXT motifs) or proline residues following tryptic cleavage sites. This ensures that the peptide sequence remains unaltered during analysis (Liebler, D. C., and Zimmerman, L. J. *et al.,* 2013). The chosen peptides should be found in regions of the protein that are conserved and less vulnerable to genetic alterations. This guarantees that every sample has the same peptide sequence. To make sure the chosen peptides uniquely identify the target proteins, programs like the Basic Local Alignment Search Tool (BLAST) are employed. Consequently, during experimental verification, the peptides should be assessed according to peak form, signal intensity, and elution time. It's also crucial that peptides do not exhibit crosstalk during transitions, which can interfere with accurate quantification (Elias and Gygi *et al.,* 2007). This rigorous selection and verification process ensures the accuracy and specificity of the LC-MS/MS method for quantifying trace proteins in complex mixtures.
MS analysis design: The Orbitrap Q Exactive is particularly effective for quantifying trace proteins in complex samples due to its ability to narrow down the selection of precursor ions and focus on a specific m/z range. This device selects precursor ion masses precisely by combining a quadrupole mass filter and an orbitrap mass analyzer. Both parallel reaction monitoring (PRM) and selected ion monitoring (SIM), which are based on tandem mass spectrometry, are methods for doing mass analysis. As the *figure 4.1* shows, the quadrupole isolates a certain m/z range in SIM mode, which contains the desired precursor ions (1). Following the accumulation in the C-trap (2), these ions are subsequently subjected to orbitrap mass analyzer (3) analysis. Similar steps are performed in PRM mode, where the quadrupole focuses exclusively on a relevant m/z range (1). Subsequently, ions are sent to the HCD via the C-trap, where they are fragmented and accumulated (2). After being transferred to the C-trap (3), these fragment ions are subsequently injected into the orbitrap mass analyzer for analysis (4). In this mode, the quantification is based on the peak areas of the selected fragment ions (Gallien *et al*., 2012).

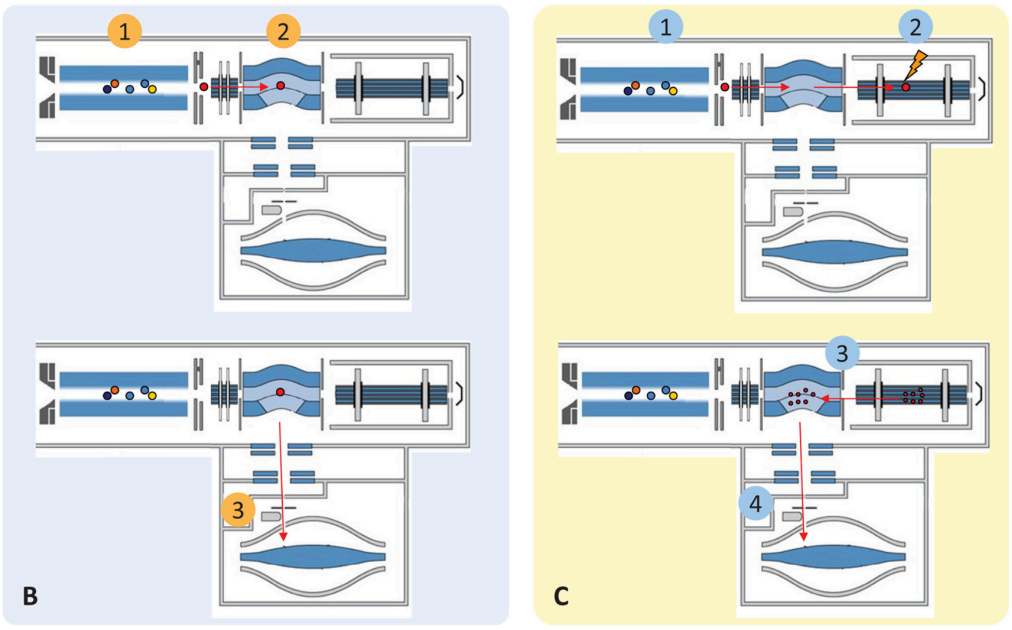


Figure 4.1: Targeted proteomic workflow employing the quadrupole-orbitrap mass spectrometer
Source: Gallien et al., 2012 Targeted Proteomic Quantification on Quadrupole-Orbitrap Mass Spectrometer. Molecular & Cellular Proteomics 11.12

4.2. Materials and Methods

4.2.1 Preparation of samples

Egg albumen (Albumina—Everintec, Pramaggiore, VE, Italy) and animal gelatin (Italgelatine, S.p.A., Santa Vittoria d'Alba, CN, Italy) were the adjuvants employed in the study. They were weighted and resuspended in 50 mM NH_4HCO_3 to obtain a solution with a final concentration of 1mg/mL. We worked on 100 µL of this solution. For the reduction step, 200 mM of dithiothreitol (DTT) was added to a final concentration of 2.5 mM and the mixture was incubated for 15 minutes at 60°C. For the alkylation step, iodoacetamide (IAA) was added and left for 30 minutes at 25°C. Protein digestion was performed by adding trypsin (sequencing grade modified trypsin) in 25 mmol/L NH_4HCO_3 to each sample at a 1:40 mass ratio and incubated at 37°C with slow shaking. One µl of a solution of 5% formic acid was added to stop the enzymatic protein digestion.

4.2.2 Fining agent's proteins identification

The peptides after protein digestion were separated by a BioBasic™ C18 HPLC Column (1 × 150 mm, 5 µm; Thermo Scientific). The mobile phases used were 0.1% (v/v) formic acid in MilliQ water (A) and 0.1% (v/v) formic acid in acetonitrile (ACN) as solvent B. The flow rate was set at 57.0 µL/min with a gradient that progressively increased the percentage of Solvent B from 5% to 70% in 50 min, followed by a final step at 80% for 5 min to ensure optimal elution of the retained peptides. The column compartment was set at 55 °C. The autosampler was set at 4 °C. The injection volume was 2 µL. Mass spectra were acquired in an Orbitrap Q Exactive Plus (Vanquish Thermo Fisher Scientific, Waltham, Massachusetts, USA) using a Full MS-ddMS2 mode. The resolution of the full scan was set at 70,000, the maximum IT was 200 ms, the AGC target was 1x106. Up to 12 of the most intense ions in MS1 were selected for fragmentation in the ddMS2 mode. The fragmentation spectra resolution was set at 17,500 for the MS/MS spectra, with a dynamic exclusion of 20 s and an isolation window of 2.0 m/z; the normalized collision energy was set at 28 (HCD mode), the maximum IT at 200 ms and the AGC target at 2x104. All the Data Dependent Analysis (DDA) files were analyzed using MaxQuant (https://maxquant.org) version 2.5.1.0 against the UniProt "Sus scrofa" and "Gallus gallus" database (reviewed and unreviewed). The search parameters were set as follows: S-carbamidomethyl derivate on cysteine as a fixed modification, oxidation on methionine, Acetyl (N-term), as variable modifications and two missed cleavage sites for trypsin digestion. For gelatin peptides, the possibility for proline hydroxylation (Hydroxyproline) was added as a variable modification, to account for the presence of hydroxyproline in collagen-derived sequences. The criteria for the protein identification included a minimum of 1 peptide, a False Discovery Rate (FDR) of 0.01% for both proteins and peptides, and a score of 0 for unmodified peptides and 5 for the modified one with a MS/MS matching tolerance of 20 ppm. Only proteins identified with scores greater than 20 were considered.

Following the identification of the proteins present in the adjuvants, specific target peptides representative of the identified proteins were selected. The criteria used for peptide selection are described in detail in Section 3.1.1. The selected peptides were purchased from Thermo Scientific, both in unlabeled form and isotopically labeled with stable isotopes (^{13}C and ^{15}N) on specific amino acid residues.

4.2.3 Optimization of the chromatography and mass spectrometry parameters by Flow injection Analysis (FIA) for the peptide quantification

A preliminary Flow Injection Analysis (FIA) was performed to optimize the ion source parameters of the mass spectrometer and to identify the most sensitive transitions for PRM (Parallel Reaction Monitoring) acquisition.

Subsequently, the chromatographic parameters were optimized, including the composition of the mobile phases and the gradient program, in order to improve peptide separation, analytical sensitivity, and peak shape. LC-HRMS analyses were performed using an HPLC system coupled to a Q-Exactive Orbitrap equipped with a HESI interface, and chromatographic separation was achieved using a Luna Omega Polar C18 column (1.6 µm particle size, 150 x 1 mm). In this context, the FIA technique was employed to directly inject peptides into the mass spectrometer source, bypassing chromatographic separation to focus exclusively on their ionization efficiency. Samples are sequentially injected at regular intervals into a carrier liquid flow that transports them to the detector (Mojano et al., 2017). The flow injection analysis enables highly precise quantitative analyses, as the system can be calibrated on a specific ion (e.g., a characteristic m/z ratio) or an MS/MS transition (e.g., a specific fragment ion generated during fragmentation). The analysis focuses on basic physical and chemical processes that provide high reproducibility even though they are not fully developed at the time of signal detection (Kuznetsov et al., 2018). Labeled and unlabeled peptides were analyzed separately in order to increase signal strength for each peptide and enhance ionization efficiency by optimizing ion source parameters. Additionally, optimizing MS/MS fragmentation is equally critical, as FIA allows the identification of ideal fragmentation conditions for each peptide, taking into account collision energy (CE), which is fundamental for obtaining high-quality fragmentation spectra.

4.2.3.1 Mass spectrometry parameters

The samples of labeled and unlabeled synthetic peptides were prepared at a concentration of 10 mg/L. 0.25 mg of lyophilized synthetic peptide was reconstituted in 250 µL of 5% acetonitrile (ACN) and 0.1% formic acid (FA) to create a stock solution at 1000 mg/L. Subsequently, 10 µL of the stock solution was taken and added to 990 µL of 5% ACN and 0.1% FA to obtain a final solution at 10 mg/L.

Protein	Peptide	Ion (m/z)	Sheet gas (AU)	Aux gas (AU)	Sweepgas (AU)	Spray Voltage (kV)	Cap. Temp	S-lens	Aux. Gas heater	CE (eV)
COL α 2	GHA	405.223 (+2)	35	7	2	3,5	300	60	330	24
	GHA*	410,227 (+2)								24
COL α 1	GFS	426.217 (+2)	35	5	3	3,5	270	50	330	13
	GFS*	430.224 (+2)								13
OVOt	YFG	524.267 (+2)	35	10	2	3,5	270	60	330	19
	YFG*	529.272 (+2)								21
LYS	GTD	523.275 (+2)	35	10	2	3,5	270	60	330	20
	GTD*	528.280 (+2)								20
OVO	HIA	449,247 (+3)	30	10	2	3,5	300	60	330	18
	HIA*	452,587 (+3)								18

Table 4.1: Optimization of the mass spectrometer parameters by FIA – COL α 2: Collagen alpha 2; COL α 1: Collagen alpha 1; OVOt: Ovotransferrin; LYS: Lysozyme; OVO: Ovalbumin.

For each labeled and unlabeled peptide, the final mass method parameters (*Table 4.1*) will be an average of the specific values selected during method optimization.

4.2.3.2 Chromatography parameters

Chromatographic separation was optimized in parallel to ensure that the target peptides were well separated (*Fig. 4.2*). A concentrated mix of synthetic peptides at 1 ppm was prepared.

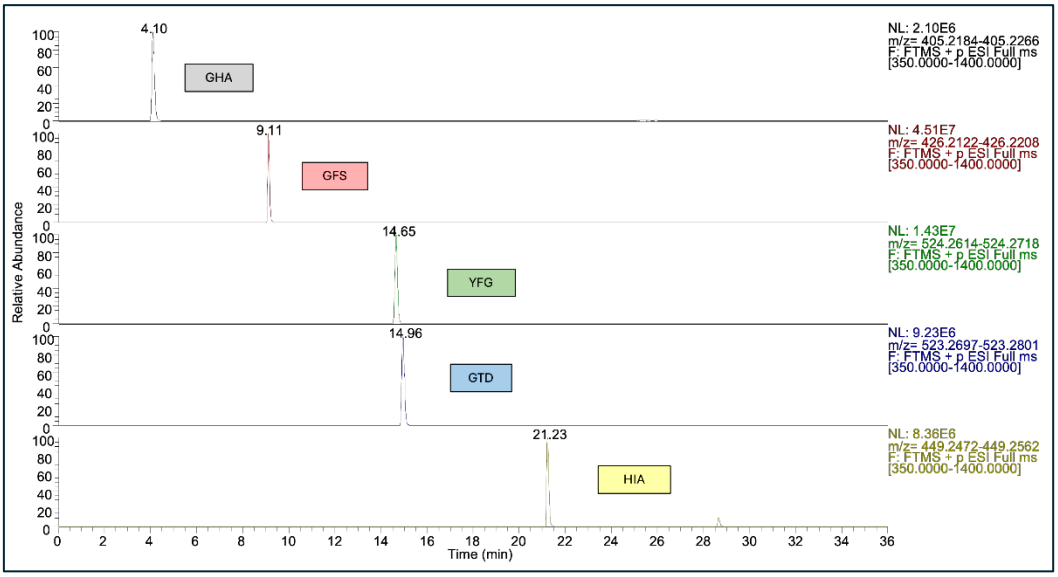


Figure 4.2: Extracted ion current of the synthetized peptide mix at 250 mg/L.

The conditions that provided the highest sensitivity and best peak shape were as follows: mobile phase A consisted of water with 0.05% formic acid, and mobile phase B consisted of 30%ACN/70%MeOH, 0.1% formic acid. The chromatographic separation was carried out using a Luna Omega Polar C_{18} column (1.6 µm particle size, 150 x 1 mm). Gradient conditions were started with 4% v/v solvent B1 and held for 5 seconds, then increased by 6% over 3.5 minutes, reaching 26%. Given that most peptides are between 26 and 28% organic, we developed a linear gradient in which 26% takes 11 minutes (0.18%/min) to reach 28%. The last peptide is about 78%; therefore, we adjusted the gradient to increase from 28% to 70% in 6 minutes (7%/min) in order to rapidly obtain the appropriate organic proportion. Finally, an isocratic at 100% organic was inserted for 5 minutes and then returned to 4% to condition the column. The chromatographic run lasts 45 minutes with a flow rate of 0.1 mL/min, and the peptides were eluted in 30 minutes. There was a 2 µL injection volume.

Curve	**Peptide mix stock solution at 1 mg/L**	**5% ACN / 0,1% FA in Milli Q water**
250 µg/L	25 µL	75 µL
150 µg/L	15 µL	85 µL
100 µg/L	10 µL	90 µL
50 µg/L	5 µL	95 µL
25 µg/L	2.5 µL	97.5 µL

Table 4.2: Sample preparation for peptide calibration curve.

Once the mass spectrometry and chromatography parameters were optimized, a calibration curve (*Table 4.2*) was prepared from the 1 mg/L stock solution of the synthetic peptide mix, ranging from 250 mg/L to 25 mg/L. A full scan followed by a data-dependent MS^2 (dd-MS^2) acquisition was performed. In this step, the instrument acquired a full-scan spectrum over a wide m/z range and then automatically selected (data-dependent) the most abundant ions for fragmentation and identification. This strategy allowed the identification of precursor ions and their corresponding fragment ions, enabling method optimization and increasing the reliability of the analysis. The full scan was carried out with a resolution of 70,000, an AGC (Automatic Gain Control) target of 1×10^6, and a maximum injection time of 100 ms. The scan range was set between 350 and 1400 m/z. The most abundant ions were fragmented with a resolution of 35. 000, an AGC target of 1×10^5, and a maximum injection time of 50 ms. The loop count was set to 5, with an isolation window of 1.0 m/z.

Parameter	***Full scan***
Resolution	70.000
AGC target	1×10^6
Maximum IT	100 ms
Scan Range	350-1400 *m/z*

Table 4.3: Full scan parameters.

Parameter	***dd-MS²***
Resolution	35.000

AGC target	1×10^5
Maximum IT	50 ms
Loop count	5
Isolation window (m/z)	1
Normalized Collision Energy (NCE)	35
Scan Range	350-1400 *m/z*

Table 4.4: dd-MS² parameters.

For the acquisition data in PRM (Parallel Reaction Monitoring) mode, the following parameters were applied: AGC target of 2×10^5, maximum injection time of 100 ms, and an isolation window of 2.0 m/z. The analysis was performed over a total run time of 40 minutes, with a resolution of 35,000 for the MS² spectra. The inclusion list was completed with all the optimized parameters (*Table 4.5*).

Mass (m/z)	CS (z)	Polarity	Start (min)	End (min)	N (CE)	Peptide
405.22247	2	Positive	3.11	5.11	24	GHA
410.22730	2	Positive	3.11	5.11	24	GHA*
426.21650	2	Positive	8.10	10.10	13	GFS
430.22390	2	Positive	8.10	10.10	13	GFS*
524.26654	2	Positive	13.66	15.94	19	YFG
529.27290	2	Positive	13.66	15.94	24	YFG*
523.27490	2	Positive	13.66	15.94	20	GTD
528.28000	2	Positive	13.66	15.94	20	GTD*
449.24720	3	Positive	20.21	22.20	18	HIA
452.58670	3	Positive	20.21	22.20	18	HIA*

Table 4.5: PRM Inclusion list

4.2.4 Preparation of calibration curves for selected peptides

Both labeled and unlabeled synthetic peptides were purchased from Thermo Fisher Scientific and initially solubilized in a dilution solution of 5% ACN and 0.1% FA to obtain a stock concentration of 1 mg/mL. Calibration curves were prepared in matrix, using red Barbera wine as the sample matrix. Each calibration curve consisted of seven concentration points: 50 ng/mL, 100 ng/mL, 250 ng/mL, 500 ng/mL, 1 µg/mL, 2 µg/mL, and 5 µg/mL. Labeled peptides were spiked into each calibration point at a constant concentration of 500 ng/mL. To prepare the calibration points, intermediate stock solutions were first made for both labeled and unlabeled peptides. For each unlabeled peptide, an intermediate solution of 250 µg/mL (M_1 solution) was prepared by diluting 25 µL of the 1 mg/mL stock in 75 µL of 5% ACN / 0.1% FA. From this, two mixed intermediate solutions containing all five target peptides were prepared at concentrations of 50 µg/mL (M_2 solution) and 10 µg/mL (M_3 solution), as follows:

- 50 µg/mL: 20 µL of each peptide M_1 solution were combined.
- 10 µg/mL: 60 µL of the M_2 solution were diluted in 240 µL of dilution solution.
- 5 µg/mL: 50 µL of the M_3 solution were diluted to a final volume of 100 µL.
- 1 µg/mL: 10 µL of the M_3 solution was diluted to a final volume of 100 µL.

The same strategy was used for the labeled peptides and two mixed intermediate solutions (50 µg/mL and 5 µg/mL) were prepared, each containing all five target peptides. The final calibration points for each of the five calibration curves were then prepared by appropriate dilution in matrix, according to the scheme presented in the table below (*Table 4.6*).

Calibration ID	Volume of mix target nonlabelled peptides	Red Wine	Mix of labelled peptides	Final volume	Final conc. µg/mL
1	5 µL of 1 µg/mL peptide mix solution	85 µL	5 µg/mL	100 µL	50 ng/mL
2	10 µL of 1 µg/mL peptide mix solution	80 µL	5 µg/mL	100 µL	100 ng/mL
3	5 µL of 5 µg/mL peptide mix solution	85 µL	5 µg/mL	100 µL	250 ng/mL
4	10 µL of 5 µg/mL peptide mix solution	80 µL	5 µg/mL	100 µL	500 ng/mL
5	10 µL of 10 µg/mL peptide mix solution	80 µL	5 µg/mL	100 µL	1 µg/mL
6	20 µL of 10 µg/mL peptide mix solution	70 µL	5 µg/mL	100 µL	2 µg/mL
7	10 µL of 50 µg/mL peptide mix solution	80 µL	5 µg/mL	100 µL	5 µg/mL

Table 4.6: Preparation of calibration curves for peptide quantification

4.2.5 Assessment of Digestion Variability in Peptides Released from Target Proteins

The variability of protein digestion was assessed across five different experimental trials, evaluating the peptides released from the identified proteins in the two fining agents (collagen alpha-2, collagen alpha-1, ovotransferrin, lysozyme, and ovalbumin). The analyzed samples were processed as described in the 4.2.1 section and analyzed using MaxQuant, applying the parameters detailed in the 4.2.2 section. For each identified protein, the analysis focused on parameters such as protein coverage, total number of peptides released, protein intensity, and protein score assigned by MaxQuant. Similarly, at the peptide level, the number and type of peptides released, peptide intensity, and peptide score were examined. To compare the reproducibility of protein digestion across the five trials, three key parameters:

- **Protein coverage**, which indicates the proportion of the protein sequence detected in the analysis.
- **Peptide intensity**, which assesses the relative abundance of each released peptide and its variability across trials.
- **Peptide length**, which gives information about the degree of protein fragmentation.

Particular attention was given to the intensity of the target peptide. Specifically, the mean intensity of the target peptide, calculated over the five replicates, was compared to the mean intensity of the other peptides identified for the same protein, also averaged over the five replicates. To quantify the deviation of the target peptide from the overall intensity distribution of the other peptides, a **z-score** modified was calculated using the following formula:

$$z = \frac{I - \mu}{\sigma}$$

where:

I is the intensity of the target peptide.

μ is the mean intensity of all identified peptides in the trial.

σ is the standard deviation.

n this study, the z-score formula was intentionally modified by considering the median (m) instead of the mean. This choice was made because the median better reflected the distribution of peptide abundances and the typical intensity profile of the peptides released from the target proteins, reducing the influence of highly intense outlier peptides. A z-score greater than 0 indicates a significant deviation of the target peptide intensity from the overall peptide distribution, providing insights into its stability and reproducibility across different digestion trials.

4.2.6 Aminoacid Hydrolysis

Amino acid analysis was performed using liquid chromatography coupled with isotope dilution mass spectrometry (LC-IDMS). This method was applied to synthetic peptides with a certified purity of 98%. Absolute quantification of the target proteins was ensured through the complete hydrolysis of peptides into their constituent amino acids and traceability to the International System of Units (SI), achieved by using the certified reference material SRM 2389a from NIST. This material consists of an aqueous solution of amino acids in 0.1 mol/L hydrochloric acid (HCl) with known purity. Synthetic peptides were initially dissolved in a solution of 5% ACN and 0.1% FA to prepare a stock solution at a concentration of 1000 mg/L. The final peptide concentration for hydrolysis was optimized to 100 mg/L in a total volume of 1 mL to ensure optimal reproducibility during hydrolysis and sufficiently high and stable analytical signals in the detection system (RSD < 5%) (*Table 4.7*). For each target peptide, 100 µL of the stock solution was diluted with 990 µL of 6M HCl. Additionally, a solution containing 0.01% phenol in 6M HCl was prepared and heated to 40–60°C to prevent crystallization. Phenol was employed as an additive to protect specific amino acids from degradation during hydrolysis (Munoz *et al.*, 2011). To maintain an oxygen-free environment during the process, oxygen was replaced with nitrogen (N_2). The samples were subjected to hydrolysis at 110°C for 24 hours. After hydrolysis, the hydrochloric acid was removed using nitrogen evaporation, and the samples were resuspended in 1 mL of 0.1 mol/L HCl. Isotopically labeled amino acid standards were added prior to hydrolysis, so that they would undergo the same treatment as the amino acids released from the peptides. This approach allows for better monitoring of the protocol, compensating for losses due to degradation during the hydrolysis process.

They were used at a final concentration of 10 mg/L. The amino acids selected for quantification were glycine (GLY), leucine (LEU), alanine (ALA), and phenylalanine (PHE), ensuring the presence of at least two quantified amino acids per peptide (Martinez-Esteso et al., 2021).

N Sample	Peptide stock solution (1000 mg/L)	* Mix aaL (50 mg/L)	HCl 12M	Phenol 0.01% in 6M HCL	H_2O	Final Volume
1	100 µL GHA	+ 100 µL	+ 500 µL	+ 100 µL	+ 200 µL	= 1 mL
2	100 µL GFS					
3	100 µL YFG					
4	100 µL HIA					
5	100 µL GTD					

*Table 4.7: Sample prepration for AA hydrolysis; *mix aaL= mix of GLY, LEU, ALA, PHE.*

4.2.7 Instrumental parameter optimization for PRM Amino Acid Analysis (AAA)

For amino acid analysis, the mass spectrometry source parameters and chromatographic parameters were optimized as for peptide identification. As a result, we were able to create a PRM-based approach that enhanced the analysis's precision and clarity.

4.2.7.1 Mass spectrometry parameters for aminoacid analysis

A mixture of twenty amino acids was prepared by weighing each compound using an analytical balance (USP lower than 1% in the weighed range and readability of 0.01 mg) and dissolving it in 0.1 M HCl to obtain a stock solution at a concentration of 1 mg/mL. Subsequently, 50 µL of the stock solution were diluted in 950 µL of 0.1 M HCl to prepare a working solution with a final concentration of 50 µg/mL. Isotopically labeled amino acids were reconstituted according to the manufacturer's instructions and diluted under the same conditions. Optimization of mass spectrometry parameters was performed via direct infusion (Flow Injection Analysis, FIA) of each amino acid. The exact *m/z* of each analyte was determined using high-resolution mass spectrometry (HRMS), based on its molecular formula and with the aid of Qual Browser software. Each precursor was then fragmented in Full MS/MS mode, and product ions were selected based on their intensity and structural specificity, considering typical fragmentation pathways such as decarboxylation and deamination, while excluding non-specific fragments like simple water losses. For each amino acid, including the isotopically labeled standards, the collision energy (CE) was individually optimized to achieve efficient and reproducible fragmentation, enabling both structural confirmation and accurate quantification.

4.2.7.2 Chromatography parameters for aminoacid analysis

Since amino acids are primarily highly polar molecules, hydrophilic interaction liquid chromatography (HILIC) in conjunction with mass detection offers an alternative for amino acid analysis due to its good retention. We used a sensitive and reproducible HILIC method combined with an Orbitrap Q Exactive for the

detection of 16 amino acids. The chromatographic separation was carried out using an Accucore™ 150-Amide-HILIC (2.1 × 150 mm, 2.6 µm). The eluents consisted in acetonitrile-ammonium formate buffer at pH 2.8 (90:19 v/v) as mobile phase A and water-ammonium formate buffer at pH 2.8 (90:10 v/v) as mobile phase B. Formic acid was used for adjusting pH. In both mobile phases, the final buffer concentration was 20 mM ammonium formate.

4.2.8 Preparation of aminoacid calibration curves

The NIST standard material was dissolved in 0.1 N HCl to create a stock solution with a concentration of 250 µg/mL. Four intermediate standard solutions were prepared from the stock solution with the following concentrations: 1 µg/mL, 5 µg/mL, 10 µg/mL, and 50 µg/mL. These intermediate solutions are obtained by appropriate serial dilution of the stock solution using 0.1 N HCl as a diluent (*Table 4.8*). Using the intermediate solutions, eight calibration points were prepared covering a range from 0.05 µg/mL to 40 µg/mL. Calibration curves were prepared using nominal concentrations ranging from 0.05 to 10 µg/mL. Actual concentrations of each amino acid were adjusted based on certified values provided for the NIST reference material, in order to ensure metrological traceability.

Calibration ID	Volume of internal standard working solution (NIST s2389)	0,1M HCl	Mix aaL	Final volume	Final conc. µg/mL
1	5 µL of 1 µg/mL NIST solution	75 µL	5 µg/mL	100 µL	0.05 µg/mL
2	10 µL of 1 µg/mL NIST solution	80 µL	5 µg/mL	100 µL	0.1 µg/mL
3	10 µL of 5 µg/mL NIST solution	80 µL	5 µg/mL	100 µL	0.5 µg/mL
4	10 µL of 10 µg/mL NIST solution	80 µL	5 µg/mL	100 µL	1 µg/mL
5	10 µL of 50 µg/mL NIST solution	80 µL	5 µg/mL	100 µL	5 µg/mL
6	20 µL of 50 µg/mL NIST solution	70 µL	5 µg/mL	100 µL	10 µg/mL
7	10 µL of 250 µg/mL NIST solution	80 µL	5 µg/mL	100 µL	25 µg/mL
8	16 µL of 250 µg/mL NIST solution	74 µL	5 µg/mL	100 µL	40 µg/mL

Table 4.8: Calibration curves for aminoacid quantification.

4.2.9 Quantification and metrological traceability

To accurately quantify the target peptides, a strategy based on the determination of the concentration of specific amino acids present in their sequences was employed.

Alanine, glycine, leucine, and phenylalanine were selected, as these amino acids are represented—individually or in combination—across the sequences of the target peptides. These amino acids were used as quantifier. For each of them, calibration curves were constructed using certified reference materials (NIST). This strategy ensured metrological traceability to the International System of Units (SI) and, more importantly, enabled the calculation of a scientific measurement uncertainty with each amino acid concentration.
Once the concentration of the amino acid released upon hydrolysis was determined, the corresponding peptide concentration was calculated stechiometrically by dividing the obtained value taking into account the number of residues of that amino acid present in the peptide sequence.

This approach enables consistent transfer of SI traceability, starting from certified reference materials (amino acid standards) to peptides and proteins, while propagating the associated measurement uncertainty throughout the entire analytical chain. In particular, the uncertainty associated with the slope (m) of the calibration curve, built using reference materials, was taken into account in the final quantification process, thus ensuring the metrological robustness of the result.

4.2.9.1 Amino acid quantification

For the quantification of the target amino acids, calibration curves were obtained using a mix of NIST (National Institute of Standards and Technology) standard amino acids (reference material) in combination with isotopically labeled amino acids (internal standards), added at a constant concentration (see section "Amino Acid Calibration Curves"). Chromatographic peak integration of both natural and labeled amino acids was performed using the Qual Browser software (Thermo Scientific). The peak areas obtained from the integration were then used to build the calibration curves in the CCC software (Calibration Curve Calculator www.efsa.europa.eu/en/data-report/calibration-curve-calculator), by calculating the ratio between the area of the natural amino acid and that of the labeled internal standard. The software was used not only to determine the calibration curve parameters (slope and intercept) but also to calculate the uncertainty associated with the slope and the goodness-of-fit index (χ^2). The use of CCC (described in section 2.11.3), which computes the linear regression model using the Weighted Total Least Squares (WTLS) algorithm, allowed for the consideration of the dispersion of experimental data and the errors associated with each calibration point, ensuring an accurate and traceable evaluation of measurement uncertainty in accordance with metrological principles and the ISO GUM guidelines.The parameters obtained were then used for the quantification of the amino acids released during the hydrolysis of the target peptides, and the uncertainty associated with the slope was propagated through to the calculation of peptide concentrations and, subsequently, protein concentrations.

The calibration curve obtained using the CCC software is:

$$y = mx$$

Where:

y represents the ratio between the peak area of the natural amino acid and the corresponding labeled internal standard;
m is the slope of the calibration curve calculated for each quantifier amino acid;
x is the concentration of the target amino acid to be quantified;

The concentration of each amino acid was determined by solving the equation for x and the equation used to determine the concentration of amino acids was the following:

$$[\text{aminoacid}] = \frac{Ratio}{m}$$

The relative uncertainty on the concentration of each amino acid was calculated by propagating the uncertainty on the slope, according to the relationship:

$$\mu_c = \sqrt{\left(\frac{1}{m} \cdot \mu_y\right)^2 + \left(\frac{1}{m^2} \cdot \mu_m\right)^2}$$

Where
y is the ratio of the peak area of the analyte to the internal standard (signal ratio)
m is the slope of the calibration curve (slope)
b represents the intercept of the calibration curve
u_y is the uncertainty about the ratio
u_m is the uncertainty about the slope

4.2.9.2 Uncertainty budget

To ensure the metrological traceability of the quantification performed, an uncertainty budget was developed following the criteria defined in the Guide to the Expression of Uncertainty in Measurement (GUM). In the calculation, both Type A and Type B uncertainty components were considered. Type A uncertainty components were derived from the statistical analysis of experimental data, while Type B components accounted for all sources of variability associated with sample preparation and handling.
Type A uncertainty was calculated based on the output provided by the CCC (Calibration Curve Calculator) software, which returns the uncertainty associated with the slope of the calibration curve for each amino acid and peptide.
Type B uncertainty was estimated by considering the following contributions:

- Uncertainty of the reference material (NIST): derived from the uncertainty reported in the reference certificate related to the concentration of the amino acid.
- Uncertainty in the preparation of the stock solution: due to errors associated with the measurement of the volume used for dissolution.

- Uncertainty in the preparation of diluted solutions (sub-stock solutions) resulting from volumetric errors related to the use of pipettes or measuring instruments during the preparation of dilutions.
- Volumetric uncertainty (pipetting): related to the precision and accuracy declared for each pipette used in the preparation of the calibration curve points.

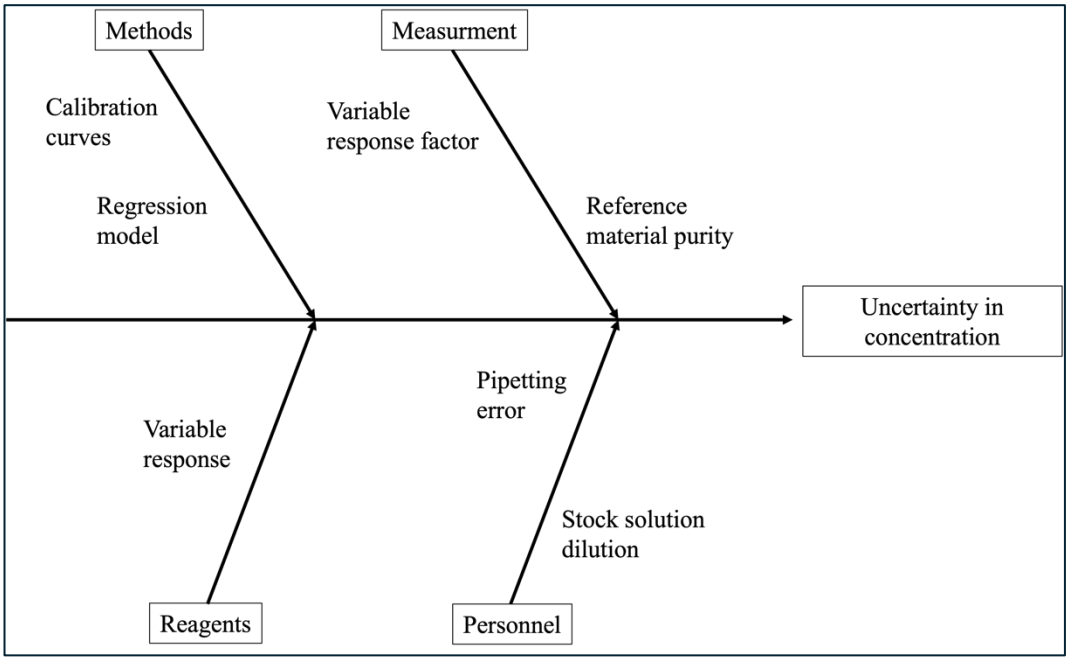


Figure 4.3: Ishikawa plot for the analysis of the main sources of uncertainty in the determination of protein concentration.

The overall relative uncertainty for each amino acid was calculated as a combined uncertainty according to the following formula:

$$\mu_c = \sqrt{\mu_{NIST}^2 + \mu_{prep}^2 + \mu_{dil}^2 + \mu_{pip}^2 + \mu_m^2}$$

Where:
$\mu_{NIST\_}$ = relative uncertainty on the NIST certified value
μ_{prep} = relative uncertainty on the preparation of the stock solution
μ_{dil} = relative uncertainty on sub-mother dilutions
μ_{pip} = volumetric uncertainty related to pipetting for curve point preparation
μ_m= relative uncertainty associated with the slope of the calibration curve

4.2.9.3 From amino acids to peptides quantification

The concentration of the target peptide was determined by dividing the concentration of the corresponding amino acid by the number of residues of that amino acid present in the peptide sequence.

For example:
Target peptide of collagen alpha 1: GHAGLAGAR,
Number of alanine (A) in peptide sequence: 3

Concentration of alanine in the target peptide: 7.769

$$[t\,\mathrm{arg}\,e\;t\;peptide] = \frac{[a\,\mathrm{min}\,o\;acid]}{number\;of\;aa\;residues} = \frac{7.769}{3} = 2.59$$

For peptides containing more than one quantifier amino acid, the average of the concentrations of the individual amino acids was calculated in order to obtain the best estimation of the final target peptide concentration. The relative uncertainty associated with the peptide concentration was also achieved by dividing the relative uncertainty of the amino acid concentration by the number of residues in the peptide sequence.
Subsequently, a specific calibration curve was built for each target peptide to validate the method and verify the linearity of the instrumental response. A relative uncertainty was associated with each calibration point, calculated as the quadratic sum of:

- the uncertainty on the peptide concentration derived from amino acid quantification;
- the uncertainty associated with the preparation of the stock solutions;
- the volumetric error related to pipette use.

The CCC software was also used for the processing of the peptide calibration curves, allowing the calculation of the curve equation, the uncertainty on the slope, and the χ^2 value. To obtain the validation curves using the CCC software, it was necessary to prepare an input template including all experimental data related to the calibration curve points, specifically:

- the concentrations of the peptide standards: **x-axis**;
- the integrated areas of the peptide signals: **y-axis**;
- the uncertainties on the concentrations: error associated with each x-axis point (**x variance**);
- the uncertainties on the instrumental response: standard deviation of the analytical replicates for each y-axis point (**y variance**).

The final relative uncertainty associated with peptide quantification was then obtained by propagating the uncertainty calculated for the amino acids and integrating it with the uncertainties related to the preparation and validation of the peptide calibration curves.

4.2.9.4 Absolute protein quantification

Protein quantification was performed indirectly, based on the concentration of the target peptides generated through enzymatic digestion. Specifically, each peptide was selected to be unique (proteotypic) for the protein of interest and to occur only once within its amino acid sequence. This feature allows the assumption, under

conditions of complete and efficient digestion, of a 1:1 ratio between the peptide and the corresponding protein.
This means that each molecule of the peptide originates from one molecule of the protein.

Accordingly, in the following relationship:

$$[protein] = \frac{[peptide\ signature]}{n}$$

Since the number of copies of the peptide in the protein is equal to 1, it will be like saying that:

$$[protein] = [peptide\ signature]$$

The relative uncertainty on the protein concentration was calculated by propagating the uncertainty associated with the quantification of the peptide, which in turn derives from the propagation of the metrological uncertainties associated with the amino acids. In this way, metrological traceability and uncertainty were consistently transferred throughout the entire analytical chain: from amino acid quantification to peptide validation, up to the absolute quantitative determination of the protein.

4.2.9.5 Validation parameters: Linear regression, LOD and LOQ, precision

For each target amino acid and peptide, the validation parameters required for a quantitative analytical method were evaluated, namely linearity, precision, limit of detection (LOD), and limit of quantification (LOQ).

- **Linear regression**

The linearity of the instrumental response was assessed by constructing calibration curves for each amino acid and peptide, using at least five increasing concentrations. The calibration curve equation was determined by linear regression using the CCC software, and the goodness-of-fit was verified by calculating the coefficient of determination (R^2) and the chi-square value (χ^2).

- **LOD and LOQ**

The LOD and LOQ values were determined according to the following equations:

$$LOD = \frac{3 \cdot \sigma_{low\ concentration}}{m}$$

$$LOQ = \frac{10 \cdot \sigma_{low\ concentration}}{m}$$

Where:
σ_{low} represent the standard deviation of the areas measured for the lowest concentration standard.
m is the slope of the calibration curve.

- **Precision**

The precision of the method was evaluated in terms of intra-day repeatability, by analyzing three independent replicates for each hydrolyzed target peptide. For each replicate, the quantifier amino acid present in the peptide sequence was quantified. Based on the results, the mean, standard deviation, and coefficient of variation (CV%) were calculated for each amino acid and the CV% was used as a measure of precision and compared against internationally accepted thresholds (e.g., EMA, FDA, OIV).

4.3. RESULTS

4.3.1 Approach to quantification: selection criteria for target peptides and product ions

4.3.1.1 Target peptide selection

The selection of marker peptides is a key step in developing a quantitative method based on mass spectrometry. In this study, peptide and fragment markers were identified in an ammonium bicarbonate buffer to which 100 mg/L of each adjuvant (animal gelatin and egg albumen) was added. Subsequently, the sample was subjected to reduction, alkylation, trypsin digestion, and analysis using HRMS-HPLC. The analysis was performed in Full Scan mode, followed by a DDA analysis, and the results were processed with the MaxQuant software.

The selection of peptides focused on specific properties. Peptides must have a maximum length of 12 amino acids to ensure stability, ionization efficiency, and good chromatographic separation. They were chosen to avoid modifications that could overestimate or underestimate quantification. Therefore, they do not contain amino acids prone to oxidation (such as methionine) or reduction (such as cysteine). For absolute quantification, it is important that peptides do not have post-translational modifications such as N-glycosylation sites (NXS or NXT motifs) or proline residues following tryptic cleavage sites. This ensures that the peptide sequence remains unaltered during the analysis (Johnson, PE). The BLAST software (basic local alignment search tool) was used to ensure that the selected peptides uniquely identify the target proteins. The peptides were identified using the UniProt database and selected based on their specificity to the proteins of interest.

For the detection of proteins in "*Gallus gallus"*, the following peptides were selected:

- **HIA** for ovalbumin (P01012|OVAL_CHICK)
- **YFG** for ovotransferrin (P02789|TRFE_CHICK)
- **GTD** for lysozyme (P00698|LYSC_CHICK)

For "*Sus scrofa"*, the selected peptides were:

- **GHA** for collagen alpha-2 type I (F1SFA7_PIG), as it is unique to *Sus scrofa*.
- **GFS** for collagen alpha-1 type I (A0A5G2QQE9_PIG), although it is present in several animal species, it was the only peptide that met all selection criteria.

The selected target peptides generated well-defined peaks with high signal intensity. This careful and rigorous selection process laid the foundation for an accurate and specific metrological approach using the HPLC-HRMS method for the quantification of trace proteins in complex mixtures.

4.3.1.2 Product ions selection

Once the target peptides have been identified, it is necessary to select the product ions representing the fragments generated by the dissociation of the charged peptide (precursor ion) in the collision cell. Each target peptide, both isotopically labeled and unlabeled, was directly injected into the source to determine the specific transitions for each target peptide to be included in the PRM inclusion list.

The transitions selected for each peptide were determined based on the signal-to-noise ratio, reproducibility, and consistency of the signal. Skyline software was used to simulate the fragmentation of precursor ions and confirm that the experimental fragmentation spectra were consistent with theoretical ones. The fragmentation of precursor peptides depends on factors such as their amino acid composition, size, excitation method, and ion charge state. Under common dissociation conditions, precursor ions typically lose water or ammonia molecules and fragment along the amide bonds, generating b and y ions, which respectively retain the N- and C-termini of the peptide (Hunt *et al.*, 1986; Biemann, 1988; Papayannopoulos, 1995).

Product ions were selected for both unlabeled and isotopically labeled synthetic peptides, as shown in *table 4.9*. Three fragments were chosen for each precursor ion. This strategy provides:

1. Enhanced specificity of the PRM method, as three distinct transitions confirm that the detected signal originates from the target peptide.
2. Increased robustness of the method in cases where one transition is affected by interference or low sensitivity, particularly when peptides are analyzed within a complex matrix.

The collision energy selected for each precursor ion was optimized to achieve a good signal intensity for each chosen transition.

Protein	Precursor ion sequence (m/z; charge state)	Product ion sequence (m/z; charge state; fragment type)
Synthetic peptides		
Collagen alpha 2 type 1	GHAGLAGAR (m/z 405.222; +2)	GHAGLAGA (m/z 615.357; +1; y8)
		GHAG (m/z 387.704; +1; y4)
		GHA (m/z 544.320; +1; y3)
Collagen alpha 1 type 1	GFSGLDGAK (m/z 426.217; +2)	GFS (m/z 647.336; +1; y3)
		GFSG (m/z 417.211; +2; b9)
		GFSGLDGAK (m/z 560.304; +2; y4)
Ovotransferrin	YFGYTGALR (m/z 524.267; +2)	YF (m/z 737.393; +1; y3)
		YFG

		(m/z 885.461; +1; y2)
		YFGYT (m/z 517.309; +1; y5)
Lysozyme	GTDVQAWIR (m/z 523.275; +2)	GTD (m/z 673.379; +1; y3)
		GTDVQ (m/z 545.320; +1; y5)
		GTDVQA (m/z 887.474; +1; y6)
Ovalbumin	HIATNAVLFFGR (m/z 449.247; +3)	HIATNAVL (m/z 639,362; +1; y8)
		HIATNAVLF (m/z 526.278; +1; y9)
		HIATNAVLFF (m/z 379.209; +1; y10)
Isotopically Labelled Synthetic peptides		
Collagen alpha 2 type 1	GHAGLAGAR (m/z 410.227; +2)	GHAGLAGA (m/z 615.357; +1; y8)
		GHAG (m/z 554.328; +1; y4)
		GHA (m/z 625.366; +1; y3)
Collagen alpha 1 type 1	GFSGLDGAK (m/z 430.224; +2)	GFS (m/z 655.349; +1; y3)
		GFSG (m/z 421.218; +2; b9)
		GFSGLDGAK (m/z 568,318; +2; y4)
Ovotransferrin	YFGYTGALR (m/z 529.272; +2)	YF (m/z 747.402; +1; y3)
		YFG (m/z 894.470; +1; y2)
		YFGYT (m/z 527,318; +1; y5)
Lysozyme	GTDVQAWIR (m/z 528.280; +2)	GTD (m/z 683.3862; +1; y3)
		GTDVQ (m/z 555.328; +1; y5)
		GTDVQA (m/z 897.481; +1; y6)
Ovalbumin	HIATNAVLFFGR (m/z 452.587; +3)	HIATNAVL (m/z 649,370; +1; y8)
		HIATNAVLF (m/z 536,286; +1; y9)
		HIATNAVLFF (m/z 389,217; +1; y10)

Table 4.9: PRM transition for each target peptide selected by HPLC-HRMS.

4.3.2 Digestion variability

The average protein coverage obtained across the five studies was greater than 50% for all target proteins, with a standard deviation consistently below 20%. This indicates that, although enzymatic digestion is a biological process and therefore inherently variable, peptide release was overall stable and reproducible.

In this study, we focused on target peptides, i.e., peptides selected as quantifiers of the proteins present in the adjuvants. After their selection—based on the theoretical criteria described in Section 4.3.1.1—it was necessary to verify that these peptides were consistently released during each digestion experiment. For this purpose, in each replicate, we analyzed and identified the peptides released from each protein. Results showed that all proteins released their respective target peptides in 100% of the replicates, except for the GHAGLAGAR peptide from collagen α2, which was detected in 4 out of 5 replicates (80%). This exception is related to the structural features of collagen: selecting a suitable peptide for this protein was particularly challenging because most peptides generated by collagen digestion contain proline, an amino acid highly prone to post-translational modification (hydroxylation to hydroxyproline). The potential conversion of proline to hydroxyproline would have introduced a systematic mistake in the quantification if a proline-containing peptide had been chosen, even if it had displayed a greater signal or been detected in 100% of repeats. Since our goal was to develop a robust and traceable quantitative method, we chose to proceed with a peptide that, although released at a slightly lower frequency, could have ensured a more reliable quantification. For each target protein, the distribution of peptide intensities released in the five replicates was evaluated (*Fig. 4.4*). Specifically, we calculated the median of the intensities of all peptides from each protein and compared it with the average intensity of the target peptide. This allowed us to assess whether the response of the target peptide was consistent with the overall behavior of the peptides generated from the same protein.

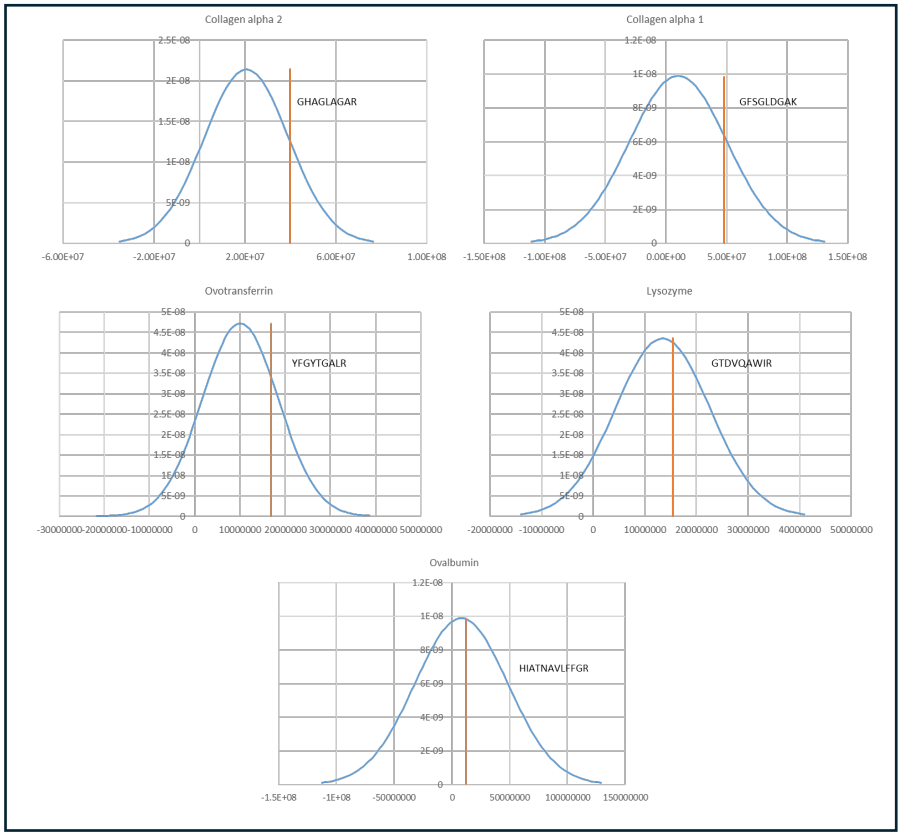


Figure 4.4: Gaussian distribution of the released peptides from target proteins and the evaluation of the target peptide.

To assess the reliability of the data, a modified z-score was calculated using the median rather than the mean as a measure of central tendency. The choice of the median was preferred because in proteomics, peptide intensity distributions often deviate from normality, due to their chemical properties or ionization efficiency, showing disproportionately high signal intensities, regardless of their actual abundance. The median better reflected the central tendency of peptide intensities under these conditions, being less sensitive to outliers.

Target protein	**Protein coverage % (PC)**	**PC Dev.st %**	**Target peptide**	**Frequency**	**Z-score modified**
Collagen alpha 2, type 1	63.9%	11.7%	GHAGLAGAR	80%	1.03
Collagen alpha 1, type 1	57.6%	1.2%	GFSGLDGAK	100%	0.94
Ovotransferrin	59.1%	11.4%	YFGYTGALR	100%	0.84
Lysozyme	62.2%	15%	GTDVQAWIR	100%	0.22
Ovalbumin	55.5%	7.1%	HIATNAVLFFGR	100%	0.09

Table 4.10: Digestion variability parameters evaluated; PC: protein coverage.

The z-score values of the target peptides, as shown in the table, fall between 0.09 and 1.03, meaning that there are no unusual deviations from the distribution of other peptides of the same protein. These findings, which combine a high detection rate,

low z-score values, and careful peptide selection, give support to the target peptides' usefulness as accurate and representative markers of their corresponding proteins.

4.3.6 Analytical method validation

The validation of the analytical method was conducted on both the quantifying amino acids and the target peptides. The validation on amino acids allowed to guarantee the reliability of the absolute quantification based on certified reference materials, while the validation on peptides allowed to transfer the metrological uncertainty from the quantification of amino acids to the determination of the peptides themselves. For each analyte, the required validation parameters were evaluated, namely: linearity, limit of detection (LOD), limit of quantification (LOQ), precision and uncertainty budget.

4.3.6.1 Aminoacid quantification validation

- **Linearity**

Analyte	Calibration ranges (µg/mL)	X^2
ALA	0.045 - 35.6	0.127
PHE	0.084 - 67.4	0.165
LEU	0.064 - 51.2	0.126
GLY	0.038 - 30.28	0.139

Table 4.11: Amino acids range of linearity and chi square (x^2).
ALA: alanine; PHE: phenilalanine; LEU: leucine; GLY: glycine

Table 4.11 reports, for each quantifier amino acid, the actual calibration range and the chi-square value (χ^2) obtained from data processing using the CCC software.
To ensure traceability to international standards, a certified reference material was used for the preparation of the calibration standards. In this case, the available NIST reference material consisted of a mixture containing all the amino acids in a single solution. The calibration ranges differ slightly for each amino acid, depending on the certified concentrations provided in the NIST reference material used for the preparation of the standards. Since the NIST amino acid mix was supplied as a single solution and reconstituted in a single vial, a nominal average concentration of 250 µg/mL per amino acid was initially assumed for the preparation of the calibration curves. This value represented an approximation, used to simplify the preparation of the standards.
However, to ensure the metrological accuracy of the quantification, the actual concentration of each amino acid was subsequently calculated based on the certified values reported in the NIST reference certificate. Specifically, the actual concentration was determined by multiplying the concentration in mmol/L of each amino acid, as indicated in the certificate, by its respective molecular weight.
In order to correct the nominal concentrations used in the calibration curve preparation, a specific conversion factor was calculated for each amino acid. This factor was obtained as the ratio between the actual concentration and the assumed nominal concentration (250 µg/mL). The corresponding correction factor was then

applied to all points of the calibration curve to adjust the nominal concentrations to their actual values and ensure the metrological traceability of the data (*Table 4.12*).

Amino acid	Ipotetic concentration (µg/mL)	Real concentration (µg/mL)	Conversion factor
ALA	250	222.73	0.890
PHE		421.23	1.685
LEU		320.1	1.280
GLY		189.18	0.757

Table 4.12: Correction for the amino acid's concentration using the NIST certificate. ALA: alanine; PHE: phenilalanine; LEU: leucine; GLY: glycine.

The obtained χ^2 values ranged between 0.127 and 0.165, all close to or lower than 1, indicating a good quality of the linear fit (*Fig. 4.5*) between concentration and instrumental response, and confirming the validity of the calibration model used. These values are consistent with the uncertainties associated with the experimental data and with the dispersion observed across the calibration points. Overall, the results confirm the linearity of the analytical method for all the amino acids considered within their respective concentration ranges.

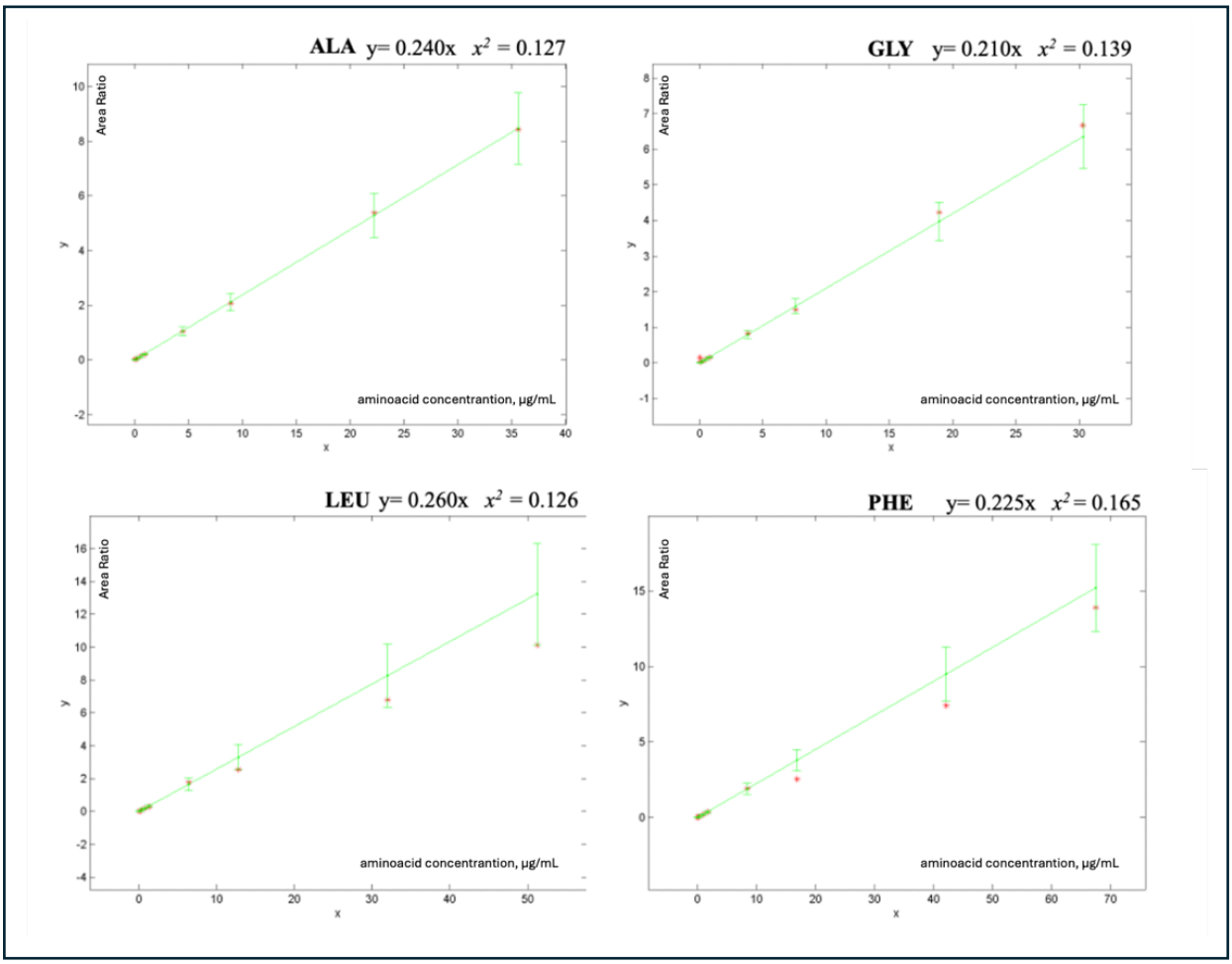


Figure 4.5: Amino acid's calibration curves using CCC software.

- **LOD and LOQ**

Analytes	LOD (µg/mL)	LOQ (µg/mL)
ALA	0.340	1.133
PHE	0.055	0.183
LEU	0.093	0.311
GLY	5.263	17.543

Table 4.13: LOD and LOQ values for each selected amino acid. ALA: alanine; PHE: phenilalanine; LEU: leucine; GLY: glycine.

In the current investigation, the variability of the instrumental response seen at the lower concentration levels of the calibration curve was used to evaluate the limit of detection (LOD) and limit of quantification (LOQ) of the target peptides. The standard deviation of the signal areas corresponding to the lowest concentration point, at the limit of quantification, was used to determine the standard deviation of the background noise. The findings indicate that the LOD values for phenylalanine, leucine, and alanine were less than 0.35 µg/mL. Glycine, on the other hand, has a substantially higher LOD value (5.26 µg/mL) and a matching LOQ of 17.54 µg/mL. The standard deviation and, hence, the limits of detection and quantification increased as a result of the increased experimental variability for glycine that was seen at the low concentration values. Despite this difference, the LOQ values for every amino acid taken into account were consistent with the chosen calibration range, guaranteeing the potential for precise quantification within the experimental range.

- **Precision: repeatability intra-day**

Amino Acid	CV %
ALA	14.6%
PHE	9.2%
LEU	15.2%
GLY	12.7%

Table 4.14: Coefficient of variation calculated for the target amino acid. ALA: alanine; PHE: phenilalanine; LEU: leucine; GLY: glycine.

The precision of the method was assessed by considering each hydrolyzed target peptide as an independent replicate (*Table 4.14*). For each peptide, the quantifier amino acid was hydrolysed and measured in at least three distinct replicates. Based on these data, the mean, standard deviation, and coefficient of variation (CV%) were calculated. The results showed optimal precision for phenylalanine, with CV% values below 10%, while for alanine, leucine, and glycine, CV% values ranged between 10% and 15%, still indicating good method precision. All values fall within the acceptability criteria established by international agencies such as EMA, FDA, and OIV, which consider a CV% below 15% to indicate acceptable precision, and below 10% as indicative of high precision.

These results confirm the reliability of the quantification method and its suitability for application to real samples.

- **Measurement uncertainty**

For each quantifier amino acid and each target peptide, an uncertainty budget was calculated by considering the main sources of uncertainty identified in Section 4.2.9.2 and reported in *figure 4.3*. The relative contributions of each source to the combined uncertainty are reported in *Table 4.15*.

Aminoacid	Uncertainty contribution		μ_c	% Uncertainty contribution
PHE	NIST	0.09	0.0920	95.7%
	Stock solution	0.0008		0.007%
	Diluition	0.0015		0.03%
	m	0.0189		4.2%
LEU	NIST	0.11	0.1146	92.2%
	Stock solution	0.0004		0.002%
	Diluition	0.0118		1.1%
	m	0.0298		6.8%
ALA	NIST	0.07	0.0731	91.8%
	Stock solution	0.0003		0.001%
	Diluition	0.0119		2.7%
	m	0.0172		5.6%
GLY	NIST	0.07	0.0724	93.4%
	Stock solution	0.0003		0.002%
	Diluition	0.0131		3.3%
	m	0.0132		3.3%

Table 4.15: % of uncertainty contribution from amino acid analysis.
PHE: phenylalanine; LEU: leucine; ALA: alanine; GLY: glycine.

Table 4.15 represents the uncertainty contributions affecting the quantification of amino acids. The overall uncertainty is predominantly influenced by the contribution of the NIST reference material, with percentages ranging from 91.8% to 95.7%. This contribution stands out compared to the others (dilutions, stock solution preparation, calibration curve error), which remain extremely low thanks to the high precision of the experimental method.
However, the absolute uncertainty associated with the NIST standards is small and well characterized. The contributions arising from the preparation of the stock solution and the dilutions are negligible (ranging from 0.001% to 3.3%) due to the use of high-precision volumetric instruments and careful solution handling.
The uncertainty associated with the slope of the calibration curve (m) represents a more relevant contribution in the cases of PHE and LEU (4.2% and 6.8%, respectively), but still remains significantly lower than that of the NIST reference material. This confirms that the calibration curves were built with good linearity and low variability in the experimental data, highlighting the reliability of the method. According to the EURACHEM Guide (2014), the uncertainty associated with certified reference materials (CRMs) is classified as a Type B contribution,

meaning that it is not derived from experimental data but rather from documented sources (e.g., certificates), and it has a direct impact on the final result. As observed in this study, although the uncertainty of reference materials is generally very low, it can still account for 90% or more of the total uncertainty budget when the rest of the analytical process is well controlled. Therefore, it represents one of the most significant components of the overall uncertainty.

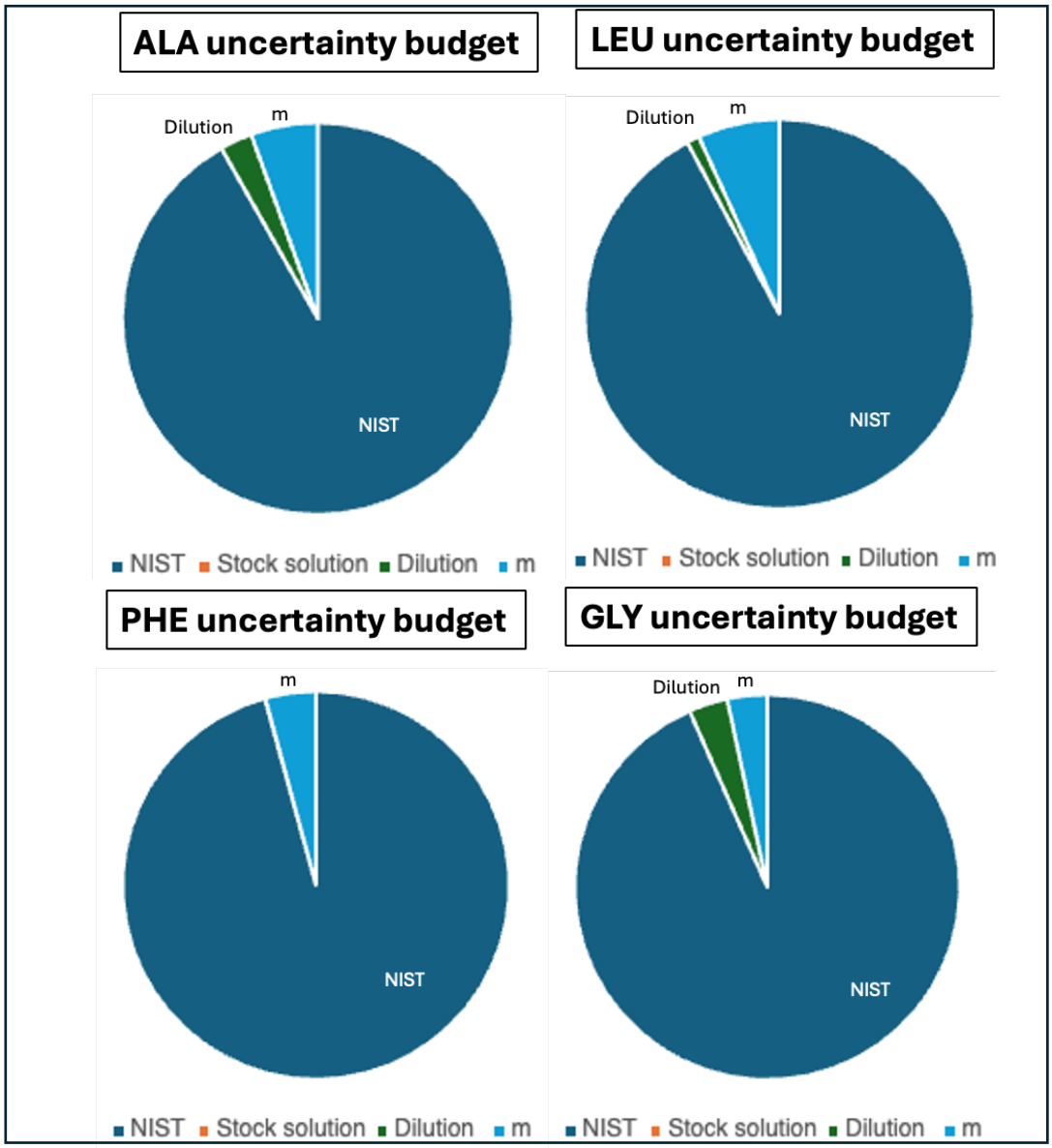


Figure 4.6: Uncertainty budget for individual amino acids analyzed.

4.3.6.2 Peptide quantification validation

- **Linearity**

Analyte	Calibration ranges (µg/mL)	X^2
GHAGLAGAR	0.3 – 5.0	0.242
GFSGLDGAK	0.1 – 5.0	0.191
YFGYTGALR	0.1 – 5.0	0.257
GTDVQAWIR	0.1 – 5.0	0.185
HIATNAVLFFGR	0.5 – 10	0.058

Table 4.16: Range of linearity for each target peptides.

The linearity parameters for each target peptide are reported in *table 4.16*. The adopted calibration range was between 0.1 and 5 µg/mL for the peptides GFS, YFG, and GTD, between 0.25 and 5 µg/mL for GHAGLAGAR, and between 0.5 and 10 µg/mL for the peptide HIATNAVLFFGR, as at lower concentrations it was not possible to obtain a reproducible instrumental response distinguishable from the background noise. This choice was made to ensure the quality of the linear fit and the reliability of the quantitative data. The analysis of the chi-square (χ^2) value, calculated by regression using the CCC software, showed very low values for all the peptides analyzed, confirming the adequacy of the linear fit to the experimental data. Specifically, the χ^2 values obtained ranged from 0.058 to 0.257, indicating good linearity and a dispersion of the data consistent with the uncertainty associated with each calibration curve point (*Fig. 4.7*).

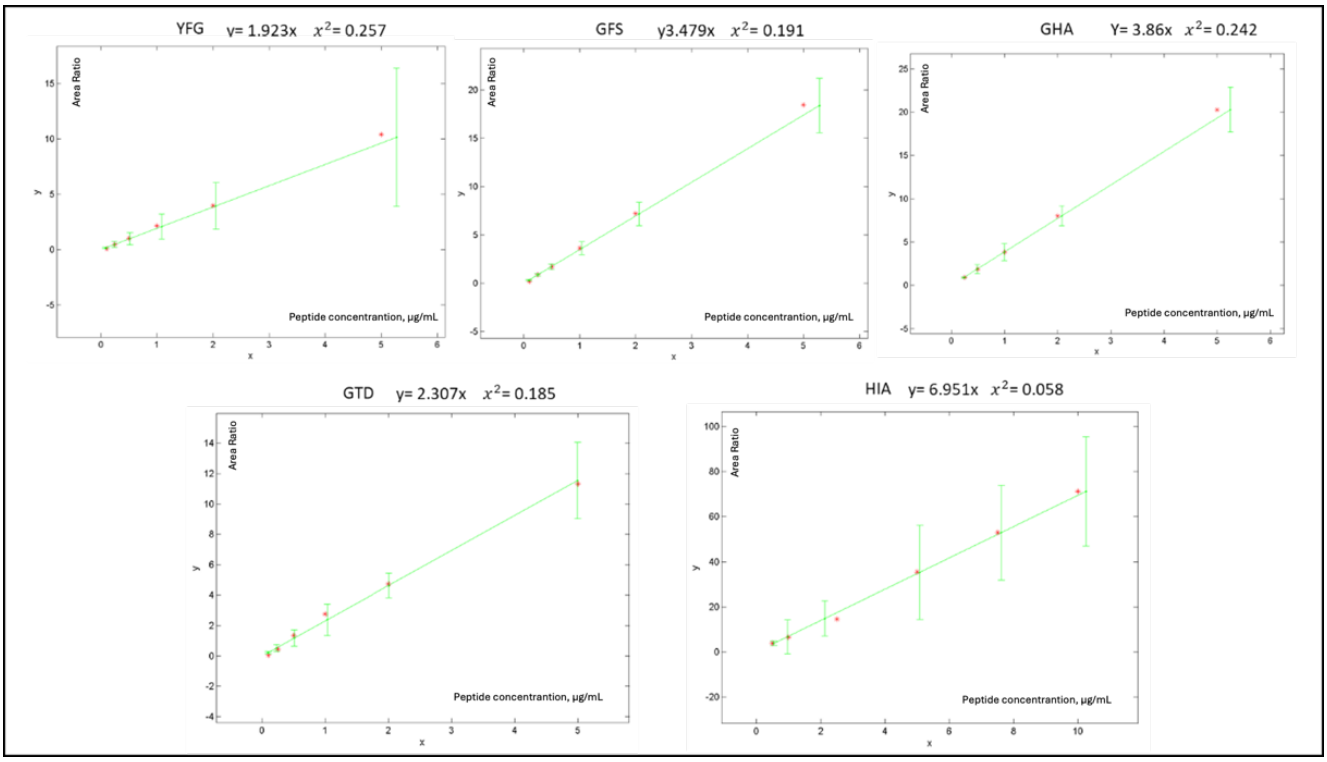


Figure 4.7: Peptide's calibration curves using CCC software.

- **LOD and LOQ**

Analyte	LOD (µg/mL)	LOQ (µg/mL)
GHAGLAGAR	0.015	0.050
GFSGLDGAK	0.004	0.014
YFGYTGALR	0.005	0.019
GTDVQAWIR	0.037	0.123
HIATNAVLFFGR	0.222	0.741

Table 4.17: LOD and LOQ values for target peptides.

The LOD and LOQ values reported in the table highlight the great sensitivity of the method for all the target peptides analyzed. Specifically, the LOD values range from 0.004 µg/mL for the peptide GFSGLDGAK to 0.222 µg/mL for the peptide HIATNAVLFFGR, while the limits of quantification (LOQ) range from 0.014 µg/mL to 0.741 µg/mL. It can be observed that the peptides GFSGLDGAK and GHAGLAGAR show the lowest LOD and LOQ values, indicating a more sensitive and stable instrumental response for these sequences. Conversely, the peptide HIATNAVLFFGR exhibits the highest LOD and LOQ values.

Measurment uncertainty

Peptide	Uncertainty contribution		μ_c	% uncertainty contribution
GHAGLAGAR	Stock solution	0.0008	0.242	/
	Diluition	0.0017		/
	m	0.242		99.9%
GFSGLDGAK	Stock solution	0.0008	0.191	/
	Diluition	0.0017		/
	m	0.191		99.9%
YFGYTGALR	Stock solution	0.0008	0.257	/
	Diluition	0.0008		/
	m	0.257		99.9 %
GTDVQAWIR	Stock solution	0.0008	0.219	/
	Diluition	0.1182		/
	m	0.185		99.7%
HIATNAVLFFGR	Stock solution	0.0008	1.099	/
	Diluition	0.0005		/
	m	1.099		99.9 %

Table 4.18: % of uncertainty contribution by peptide quantification.

The results reported in the *table 4.18* show that, for all analyzed peptides, the percentage contribution to uncertainty is almost entirely dominated by the uncertainty associated with the slope of the calibration curve (m), with values equal to or greater than 99.7%. This behavior is consistent with what was observed in the quantification of amino acids: the error associated with dilutions and stock solution preparation is minimal, thanks to the high precision of the instruments used

(analytical balance and micropipettes). Furthermore, since the peptide calibration curves were constructed based on the concentrations obtained by propagating the uncertainty of the quantifier amino acid, the uncertainty of the certified reference material (NIST) is inherently included within the slope uncertainty (Δm). In summary, the final uncertainty in peptide quantification is almost entirely driven by the propagation of the amino acid's uncertainty, confirming the robustness of the metrological approach adopted.

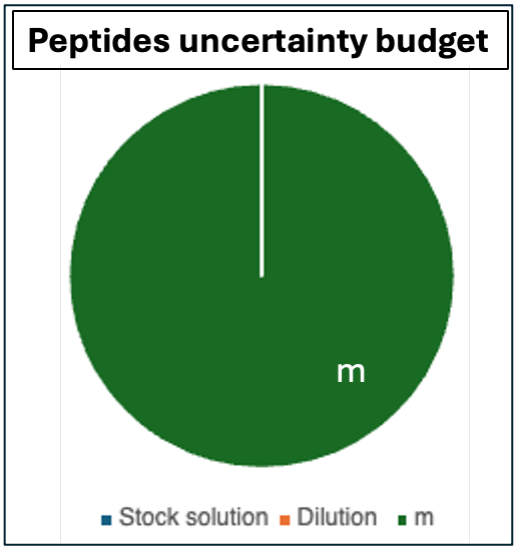


Figure 4.8: Uncertainty budget for every target peptides analyzed.

4.3.4 Peptide and protein quantification

The choice of distinct and representative peptides is crucial to the precision of protein quantification. This step was essential to make sure that the measured signal can be clearly linked to a specific protein. Additional sources of uncertainty could be introduced if the chosen peptides were shared by several proteins. This could result in an overestimation or underestimating of protein levels, which could lead to incorrect conclusions. In order to facilitate precise and straightforward measurement, we made sure that every peptide in this investigation was proteotypic, or distinct to the target protein. Any variation in the measurement of the chosen peptide is then directly reflected in the estimated concentration of the matching protein, making it a trustworthy surrogate marker. The findings of measuring distinct and representative peptides to determine the absolute quantification of target proteins are summarized in *table 4.19*. Based on the measurement of the amino acids released during hydrolysis, a particular signature peptide was chosen for each protein, and its concentration was calculated. Since each peptide appears only once in the corresponding protein sequence, a 1:1 ratio of peptide to protein was assumed in all cases. When more than one quantifier amino acid was used for the same peptide, the final concentration was obtained by calculating the mean of the amino acid concentrations, initially expressed in mmol/L. To express the final result in mg/mL, a unit conversion was applied by multiplying the average concentration (in mmol/L) by the average molecular weight of the quantifier amino acids used. This value was calculated as the mean of the molecular weights of the

individual amino acids considered for quantification. The absolute uncertainty was likewise multiplied by the same conversion factor.

Protein	Target peptide	Aminoacid Quantifier used	[Peptide] (mg/L)	[Protein] (mg/L)	Uncertainty (± mg/L)	CV%
Collagen α 2	GHAGLAGAR	Alanine Glycine Leucine	0.003	0.432	0.031	14.2%
Collagen α 1	GFSGLDGAK	Alanine Glycine Leucine Phenilalanine	0.006	1.023	0.026	13.0%
Ovotransferrin	YFGYTGALR	Alanine Glycine Leucine Phenilalanine	0.004	0.275	0.019	13.0%
Lysozyme	GTDVQAWIR	Alanine Glycine	0.004	0.048	0.003	13.7%
Ovotransferrin	HIATNAVLFFGR	Alanine Glycine Leucine Phenilalanine	0.004	0.118	0.046	13.0%

Table 4.19: Absolute concentration and associated uncertainty of the target proteins calculated based on the quantification of unique signature peptides. Peptide concentrations were determined through the quantification of one or more amino acids released by hydrolysis, with subsequent uncertainty propagation throughout the entire analytical chain: amino acid → peptide → protein. The number of residues refers to the quantifier amino acids present in each peptide sequence. The reported CV% reflects the variability in the quantification of the amino acid and represents the overall precision of protein quantification.

The resulting protein concentrations ranged from 0.048 to 1.023 mg/L, with associated uncertainties between 0.003 and 0.046 mg/L. The CV% values, calculated at the peptide level (and based on the precision of the quantifier amino acids), ranged between 13.0% and 14.2%. These results fall within the acceptability criteria established by international agencies (EMA, FDA, OIV), according to which a CV% below 15% is considered indicative of acceptable precision, especially in complex analyses and at low concentrations.

Furthermore, the consistency of CV% values across all analyzed proteins suggests homogeneity of experimental conditions, both during hydrolysis and instrumental analysis.

5. Discussion and Conclusion

This thesis addressed two distinct but closely interconnected aspects in the field of food allergens characterization: on one hand, the discovery and characterization of novel allergens potentially involved in the immune response of sensitized individuals; on the other, the development of a robust and sensitive analytical method for the absolute quantification of traces of known allergens within the food matrix. These two projects had different objectives and experimental approaches, they are part of the same logical and methodological pathway that underpins research in the field of molecular allergology. The first case study, “Discovery of galactose-alpha-1,3-galactose allergen by HPLC-HRMS”, followed an exploratory approach, focusing on the identification and characterization of potential allergenic proteins through proteomic and immunological techniques. In contrast, the second case study, “Quantification of trace amounts of proteins using HPLC-HRMS in red wine”, was aimed at implementing a methodology that allowed to quantify the presence of very low amount of allergens in food products with high precision, to ensure consumer safety and support regulatory frameworks. Mass spectrometry technology, however in different applications, was the link between the two projects. It played a key role in the first project for the identification of food proteins involved in the immunoreaction of allergic patients; in the second, it allowed to provide results that could be metrologically traced through high-precision quantification. This dual application highlighted the versatility of the technique and the importance of high-resolution mass spectrometry in the field of molecular allergology, from the identification of food allergens to the validation of techniques intended for analytical surveillance in food products.

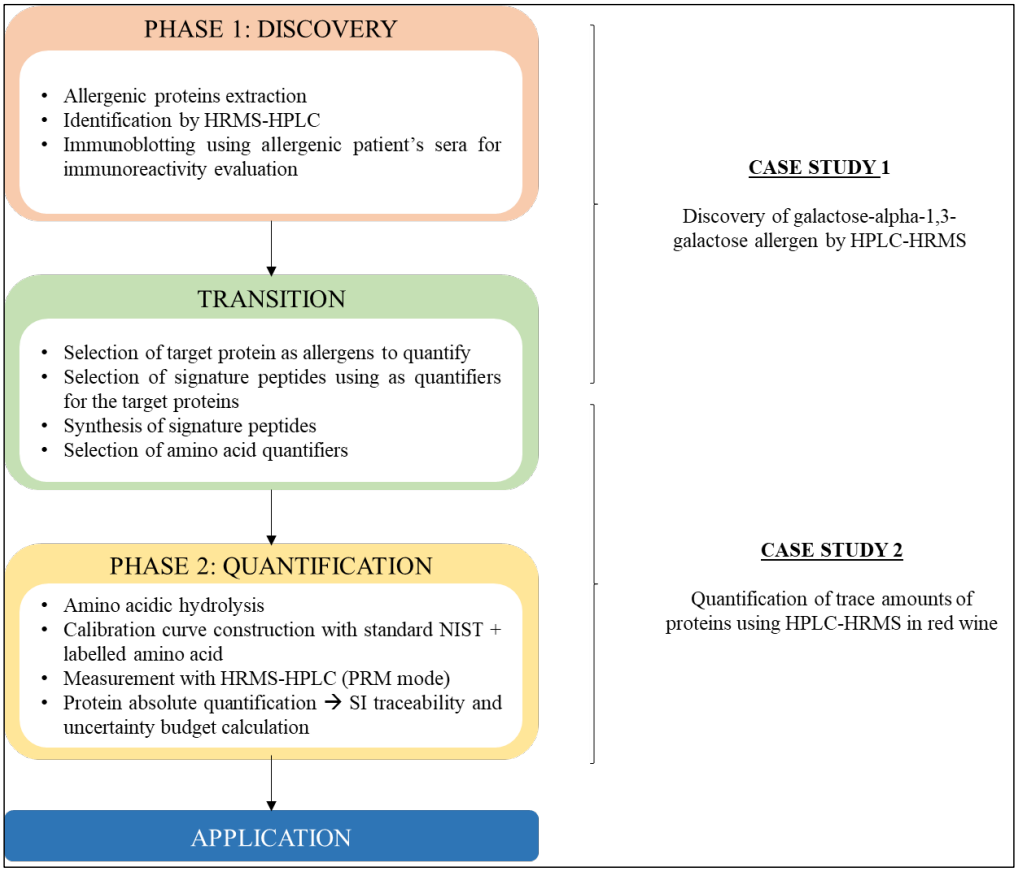


Figure 5.1: Schematic representation of Food allergy research: from Discovery to Quantification.

The **first project** focused on the study of an allergenic determinant: the galactose-α-1,3-galactose (α-Gal), an epitope known to be associated with the "red meat allergy syndrome". Unlike classical protein allergens, α-Gal is a disaccharide present in non-primate mammals that can trigger an IgE-mediated immune response in sensitized individuals, often causing delayed allergic reactions after consumption of meat or animal products. Previous studies on milk allergenicity have primarily focused on well-characterized proteins such as caseins and whey proteins (e.g., β-lactoglobulin, α-lactalbumin, and bovine serum albumin), which are commonly involved in classical IgE-mediated allergic reactions to cow's milk. However, the hypothesis that milk could also represent a relevant dietary source in the context of α-Gal syndrome was only proposed relatively recently. In particular, Commins *et al.* (2012) suggested a possible link between dairy consumption and delayed allergic reactions in patients sensitized to α-Gal, although their analyses focused mainly on serum proteins such as bovine albumin, already known to contain α-Gal residues. In line with these findings, the study by Perusko *et al*. (2021) further investigated the potential presence of the α-Gal epitope in bovine milk whey proteins, reinforcing the hypothesis that milk could represent a risk food for sensitized individuals. Their most recent work (2024) additionally highlighted the clinical relevance of glycosylated α-Gal-containing whey proteins such as bovine gamma globulin (BGG), lactoferrin, and lactoperoxidase. The study showed that these alpha gal carrying proteins are indeed recognized by IgE antibodies from AGS patients, thus contributing to potential allergic reactions following the consumption of cow's milk. This further emphasizes the importance of considering dairy products—and not just red meat—among the potential sources of clinical symptoms

in individuals affected by AGS. However, these studies did not investigate the lipid fraction of milk and, in particular, the proteins associated with the milk fat globule membrane (MFGM). Our study focused specifically on this fraction, identifying α-Gal-containing proteins specifically associated with the MFGM—such as xanthine oxidase, butyrophilin, and lactadherin—which had not previously been considered in the context of α-Gal syndrome. Immunoblotting showed that when glycosylated alpha-gal proteins were deglycosylated, the monoclonal anti-alpha-gal antibody and sera from individuals with alpha-gal syndrome could no longer detect them. Deglycosylation eliminated the sugar components, indicating that the immunological interaction was caused by α-Gal's glycosylation rather than the protein's peptide structure. This conclusion was confirmed by liquid chromatography-coupled high-resolution mass spectrometry (LC-HRMS), which showed that peptides containing aspartic acid (Asp) rather than asparagine (Asn) were generated when the deglycosylated proteins were digested by enzymes. Therefore, we showed in this study that alpha-gal residues are present in the proteins butyrophyllin (BT) and lactadherin (LA), which are already known to be glycosylated in the literature. The presence of a unique glycosylation site on the residue Asn704 of xanthine oxidase (XO), which is changed to Asp704 by enzymatic treatment, was also found for the first time. Xanthine oxidase (XO), one of the proteins analyzed, had a very important role. Several immunoreactive bands identified by patient sera and anti-α-Gal antibodies were found to contain it, even in areas of the gel that Coomassie Blue staining did not highlight. This implies a strong immunological attraction, regardless of the quantity of protein that may be found using standard methods. Lastly, immunoinhibition tests were conducted using sera from three individuals to confirm that XO was directly responsible for the allergic reaction. The reactivity to milk proteins was decreased by immunoprecipitation using XO. Since XO had fewer glycosylated sites than thyroglobulin, which is known to contain α-Gal, a greater amount of XO was needed. Further, also the immunoprecipitation experiment was crucial in verifying the α-Gal epitope's immunologically significant function.

The **second project** focused on the precise measurement of recognized allergenic proteins found in animal gelatin and egg white, two protein fining agents used in winemaking. In this context, the aim was to obtain an accurate and traceable measurement of the target proteins in a very complex matrix. These proteinaceous fining agents, once their function is finished, are generally removed by filtration, but traces of these agents may persist in the final product.
Metrological traceability is defined as “the property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty”, as reported in the International Vocabulary of Metrology (VIM 3). Applying this principle to the determination of the mass fraction of animal proteins in red wine and making it traceable to the International System of Units (SI) represents a challenge in several respects. The first difficulty lies in the clear and unambiguous definition of the measurand, i.e., the quantity to be measured. According to O’Connor’s Technical Report on the “Harmonization of Approaches for informing EU allergen labelling legislation”, the common measurand to be obtained is expressed as mg of total animal proteins per liter of red wine (mg/L) (Martinez-

Esteso *et al.,* 2021). The second challenge is operational and concerns the construction of the measurement chain required to quantify analytically non-directly measurable targets, such as amino acids, peptides and proteins, within a complex matrix like red wine. To achieve this, it was necessary to relate each instrumental signal to a known quantity, through a documented and continuous chain in which each step is traceable to an SI unit. The entire approach was designed in accordance with metrological principles, aligned with the criteria set out in the Eurachem Guide "The Fitness for Purpose of Analytical Methods", and involved the use of high-resolution mass spectrometry in Parallel Reaction Monitoring (PRM) mode, combined with isotopically labelled amino acids and certified reference materials (CRM) from NIST (Martinez-Esteso *et al.,* 2021; Cryar *et al.* 2013). Calibration curves were constructed for four selected amino acids (alanine, glycine, leucine, and phenylalanine), based on their presence in the target peptides (Munoz et al., 2011). The concentration of each amino acid was determined using CRM, and the uncertainty associated with the slope of the calibration curve was calculated using CCC software, following the ISO GUM Guide. These data enabled the traceable quantification of the amino acids released from peptide hydrolysis. Therefore, using the proper propagation of the related uncertainty, the peptide concentration was determined stoichiometrically by dividing the observed amino acid value by the number of corresponding residues in the peptide sequence A 1:1 molar ratio between the peptide and target protein was assumed in order to produce the final measurement result, which was expressed in moles of target protein per milliliter of wine. This assumption was supported by the peptide's unique existence within the protein sequence. The data regarding the amount of marker peptides derive from the analysis of mass spectrometry (MS) signals acquired from the digested protein extract of the wine sample. MS signal traceability was ensured through dedicated calibration curves constructed using synthetic reference peptides, whose purity had been previously characterized via amino acid analysis (AAA). In this way, a continuous chain of metrological traceability was built, from the molecular level of the amino acid up to the intact target protein, thereby ensuring the reliability and SI-traceability of the entire analytical process.

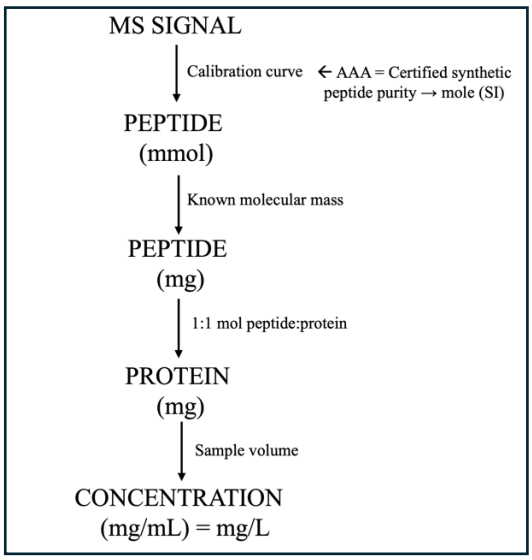


Figure 5.2: Schematic representation of the unbroken chain traceable to the International System ofUnit. Each step of the measurement chain can be traced back to quantities recognised by the SI (mole, gram, litre), guaranteeing the traceability of the final result.

A crucial aspect in developing the method involved the selection of target peptides necessary for the quantification of allergenic proteins. The bottom-up approach, which includes enzymatic digestion of proteins into peptides (Domon *et al.*, 2006; Monaci *et al*., 2009), allows greater sensitivity and specificity for protein identification and quantification. Direct quantification of intact proteins is often impractical due to their structural complexity, large size, and the lack of pure and certified protein standards. The selected peptides thus served as representative analytical surrogates for the target proteins, enabling their indirect determination by mass spectrometry (Bantscheff *et al*., 2017; Domon & Aebersold, 2006). Peptide selection was consistent with the literature, where they have been successfully employed as prototypic markers for the identification and quantification of collagen (GFSGLDGAK and GHAGLAGAR) and of major egg white proteins such as ovalbumin (HIATNAVLFFGR), ovotransferrin (YFGYTGALR), and lysozyme (GTDVQAWIR) in mass spectrometry-based analyses (Pavón-Pérez *et al.*, 2019; Pavón-Pérez *et al.*, 2025; Monaci *et al*., 2010). It was also important to verify the efficient release of these peptides during enzymatic digestion. All selected peptides showed complete release, except for one of the collagen peptides (80%). However, this peptide was preferred over others due to the absence of proline residues, which are prone to modification into hydroxyproline, leading to inaccurate quantification. As part of the method validation, the limits of detection (LOD) and quantification (LOQ) were also determined for the two proteinaceous fining agents considered. These were established based on the most sensitive peptide markers for each target protein. The resulting values were 5 µg/L for egg white and 4 µg/L for gelatin, while the corresponding LOQs were calculated as 14 µg/L and 19 µg/L, respectively. It is important to note that the LOD and LOQ values reported for each fining agent were determined based on the peptide marker showing the best analytical performance, in accordance with common practice for protein identification through targeted proteomics. Directive 2003/89/EC, as amended by 579/2012/EC, mandates the labeling of certain food allergens, including milk and egg derivatives, when present in wine above detectable limits using validated analytical methods The performance standards for analytical techniques for the identification of proteins derived from eggs and milk set a limit of quantitation (LOQ) of 500 µg/L and a limit of detection (LOD) of 250 µg/L, in accordance with OIV COMEX Resolution 502/2012. The OIV officially recognizes the Enzyme-Linked Immunosorbent Assay (ELISA) as the tool for monitoring these thresholds. This method has serious drawbacks, nonetheless, particularly when it comes to red wines, where polyphenols and the intricacy of the matrix can impede the identification of allergens, compromising the test's sensitivity and reliability. As for gelatin, there are no validated analytical methods for either white or red wine. As highlighted by Koestel *et al*. (2016) and Restani *et al*. (2014), there are currently no validated ELISA methods for the detection of gelatin in either white or red wine, primarily due to significant matrix interferences—particularly in red wines. The most sensitive techniques for analyzing allergenic proteins in complex matrices are mass spectrometry-based methods, such as those used in this study. Regarding ovalbumin, LOD values

obtained using mass spectrometry in literature generally range between 2 µg/L and 20 µg/L (Pavón-Pérez *et al*., 2019; Pavón-Pérez *et al*., 2025; Dal Bello *et al.,* 2021). About gelatin, Dal Bello *et al*. (2021) developed a mass spectrometric method able to reach the LOQ of 5 µg/mL in wine, confirming the difficulty of detecting peptides derived from partially denatured proteins.

In this thesis project, the results not only fall within the OIV performance criteria but notably exceed the sensitivity levels reported in the literature for comparable matrices. The approach developed—based on PRM mass spectrometry, combined with metrological traceability and uncertainty propagation—constitutes a robust, reproducible, and highly specific method, potentially applicable in regulatory settings for allergen risk management, beyond the limitations of currently used official methods.

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