Thesis Summary

In recent decades, food allergies have significantly increased in the global population, becoming one of the major public health concerns, especially in industrialized countries. Allergic reactions to food are largely mediated by IgE-dependent mechanisms, involving a specific immune response to proteins that are generally harmless but perceived by the body as threatening. The interaction between allergens and IgE antibodies can trigger severe clinical manifestations and, in some cases, life-threatening reactions. Despite notable progress in clinical diagnosis and management, the molecular complexity of food allergens, their structural variability, and the strong influence of food processing technologies still make their identification and quantification highly challenging. Factors such as thermal stability, glycosylation, resistance to gastrointestinal digestion, and interactions with the food matrix affect the allergenicity of proteins by altering the accessibility and recognition of epitopes by the immune system. At the same time, allergen labeling represents both a fundamental tool for prevention and a highly debated issue. Current European legislation mandates the declaration of 14 categories of allergens, yet it does not establish quantitative thresholds or standardized methods for detecting accidental contaminations, giving rise to the so-called "precautionary allergen labelling" (PAL). This approach, often adopted cautiously by food producers, can lead to confusion among consumers and has sparked a scientific debate about the need for more reliable, quantitative, and traceable analytical methods. The analytical techniques currently in use, such as ELISA or PCR, while offering high sensitivity, present several limitations: they lack robustness in complex food matrices, are affected by thermal treatments, and do not always allow accurate quantification. In response to these critical issues, the use of mass spectrometry (MS) coupled with liquid chromatography (LC-MS) is increasingly gaining ground, thanks to its high selectivity, sensitivity, and ability to simultaneously identify multiple allergens in a single run. Specifically, MS-based methods allow for absolute quantification of allergens and the integration of a metrological approach, ensuring measurement traceability, uncertainty evaluation, and inter-laboratory reproducibility. It is within this context that the present doctoral project was developed, with the main goal of applying advanced mass spectrometry techniques to the identification and quantification of food allergens, through two complementary lines of research: the characterization of novel allergens and a metrological approach to their quantification in complex food matrices.

The **first project** focused on the identification and characterization of an emerging allergen: galactose-α-1,3-galactose (α-Gal). α-Gal is a carbohydrate determinant present on glycoproteins and glycolipids of most non-primate mammals, but absent in humans, higher primates, and a few other species. Its absence in the human body causes α-Gal to be recognized as foreign by the immune system upon exposure, potentially triggering the production of specific antibodies, including IgE. Following bites from ticks (notably Amblyomma americanum in the US and Ixodes ricinus in Europe), some individuals develop IgE-mediated sensitization to α-Gal, which can lead to delayed allergic reactions after consuming red meat (e.g., beef, pork, lamb). This clinical condition, known as Alpha-Gal Syndrome (AGS), differs from classical food allergies due to: the delayed onset of symptoms, typically 3 to 6 hours after ingestion, the carbohydrate nature of the epitope, unlike traditional

protein-based allergens, the mode of sensitization, triggered by a vector (the tick) rather than direct food exposure. Although α-Gal has been previously studied in relation to mammalian tissues, its presence in dairy products, particularly bovine milk, remained largely undocumented and had never been systematically demonstrated experimentally. The scientific literature until now has focused on α-Gal presence in milk serum proteins, but had not explored in detail the lipid fraction or the proteins associated with the milk fat globule membrane (MFGM), which are potentially rich in α-Gal-positive glycoproteins. This study addresses that gap by exploring the presence and immunoreactivity of the α-Gal epitope in the MFGM of cow's milk, using biochemical, immunological, and proteomic approaches. Through classical biochemistry techniques and high-resolution mass spectrometry (HPLC-HRMS), it was possible to identify, isolate, and characterize α-Gal-positive proteins in the protein fraction of the MFGM. Samples were subjected to a targeted extraction protocol, followed by enrichment via magnetic beads and immunoenzymatic analysis. The use of sera from AGS patients revealed a strong immunological response, confirmed by both immunoblotting and immunoprecipitation assays. The core strength of this work lies in the molecular identification of glycosylated α-Gal-containing peptides and proteins via HPLC-HRMS. Analysis of milk samples revealed specific post-translational modifications on three key MFGM proteins: xanthine oxidase (XO), butyrophilin (BT), and lactadherin (LA). Asparagine residues N215 on BT and N227 on LA were confirmed as α-Gal-specific N-glycosylation sites, through the detection of their conversion to aspartate after PNGase F treatment and LC-MS/MS analysis. These results provide the first direct evidence of α-Gal epitopes in high-fat milk proteins, supporting the hypothesis that dairy products—especially those with high lipid content such as cream and butter—may pose a clinical risk for sensitized individuals. This study offers a novel contribution to the literature, which until now had only reported α-Gal in serum protein fractions.

The **second project** focused on the absolute quantification of known allergens in complex food matrices, with a specific case study on red wine. While wine is generally perceived as a "natural" and safe product, it can contain invisible but critical residues for sensitive consumers, particularly those with food allergies. Among the most relevant risks is the use of animal-derived fining agents, such as egg white, casein, fish glue, and gelatin, employed to remove colloids, excess tannins, and protein instability. Although these agents serve no nutritional or preservative function, they may leave residual protein traces in the final product, posing a threat to allergic individuals. Regulation (EU) No. 1169/2011 mandates the labeling of allergens used as ingredients, but not always of fining agents used in processing—unless detectable in the final product. This regulatory framework highlights the challenge of trace-level quantification, which requires highly sensitive, specific, and robust analytical methods capable of working in complex matrices like red wine, rich in polyphenols, ethanol, and other interfering compounds. In the field of enology, few methods successfully combine high sensitivity, robustness, and metrological traceability. The ability to define reliable and comparable limits of quantification (LOQ) based on certified standards and reference materials is a crucial step toward a more informed and regulated approach to allergen risk assessment. The goal of this project was to develop an analytical method able to accurately measure trace levels of proteins from egg white and gelatin, commonly used as fining agents in winemaking. The key innovation of the study lies in the adoption of a rigorous metrological framework—still uncommon

and evolving in applied biology—based on high-resolution mass spectrometry (HPLC-HRMS) coupled with Parallel Reaction Monitoring (PRM). The application of metrology in biology is hindered by several challenges, including the complexity of biological matrices, the high variability of samples, and the limited availability of certified reference materials. Additionally, protein structural heterogeneity and interactions within food matrices complicate the propagation of measurement uncertainty based on standardized criteria. These barriers have so far limited the dissemination of traceable and comparable methods for biomolecule quantification. By integrating concepts such as traceability, absolute quantification, and uncertainty budgets, the method developed in this study overcame the limitations of classical immunological techniques, which are often subject to matrix effects and exhibit poor reproducibility in real-world conditions. Specifically, the protocol included optimization of chromatographic and spectrometric parameters via Flow Injection Analysis (FIA), preparation of calibration curves using certified amino acid standards (NIST), uncertainty evaluation across the analytical chain (from peptide hydrolysis to protein quantification), and selection of specific target peptides for high sensitivity and selectivity. The results demonstrated that the developed method achieved LOQs of 14 μg/L for egg white and 19 μg/L for gelatin, significantly outperforming official methods such as ELISA, which set LOQs at 500 μg/L. Additionally, the absolute protein concentrations measured in real samples ranged from 0.279 to 0.854 mg/mL, with associated uncertainties between 0.018 and 0.126 mg/mL, and coefficient of variation (CV%) below 15%, in accordance with international acceptability criteria (EMA, FDA, OIV). This approach, rarely applied in food analysis, enabled the direct absolute quantification of allergens (in mg/L), ensuring traceability to the International System of Units (SI) and providing a robust benchmark for regulatory or industrial comparisons. The method shows great potential for allergen risk management and for a solid regulatory framework, offering an objective basis to move beyond the precautionary and unstandardized PAL system, which is often used arbitrarily by food producers.

Overall, the thesis proposes an integration between immunological and advanced analytical approaches. The combination of allergen discovery and characterization with precise, objective quantification reflects a comprehensive scientific strategy to address the complexity of food allergy management. Structural and immunological characterization is essential for identifying molecular targets of allergic reactions; metrological quantification is, in turn, fundamental for translating this knowledge into tools for regulation, industrial control, and consumer protection. Mass spectrometry, in particular, has proven critical in overcoming the limitations of conventional techniques, paving the way for future applications in diagnostics, research, and regulation within the food allergy domain.