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Development of an Egg-Specific Mass Spectrometry Targeted Method for Processed Food Matrices

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DEVELOPMENT OF AN EGG-SPECIFIC MASS SPECTROMETRY TARGETED METHOD FOR PROCESSED FOOD MATRICES

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DEVELOPMENT OF AN EGG-SPECIFIC MASS SPECTROMETRY TARGETED METHOD FOR PROCESSED FOOD MATRICES

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Egg is a major food allergen in the U.S., causing symptoms from mild reactions to severe anaphylaxis. An egg-free diet remains the only management approach for egg allergy. Therefore, individuals and families living with egg allergies rely heavily on allergen statements on packaged food products for informed food choices. Currently, no labeling regulations exist for unintentionally introduced allergens, posing risks to consumers with egg allergies. Therefore, it is critical to have an accurate and reliable detection method in allergen control and management to determine the concentration of total protein from allergenic food sources. Traditional enzyme-linked immunosorbent assays are routinely used to quantify allergen residues but can be hindered by food processing and matrix complexity. In contrast, mass spectrometry (MS)-based methods can potentially enhance sensitivity, specificity, and robustness in detecting and quantifying proteins in processed foods.

This study developed a parallel reaction monitoring MS method to detect and quantify total egg proteins in processed food matrices through three key steps: 1) select target peptides using a discovery-based strategy, 2) optimize sample preparation workflow to improve method's sensitivity, and 3) validate the method in whole egg powder (WEP) incurred food matrices. The developed targeted MS method used a WEP external calibration curve and internal standards to quantify total egg protein in food

samples. Nine egg-specific target peptides were identified to quantify total egg protein in incurred food matrices (cookies, pie crust, pasta, ice cream, and pasta soup). The performance of the MS method was evaluated by quantifying total egg protein from 18 independent WEP calibration curves and WEP-incurred food matrices and comparing with commercial egg ELISA kits. The results demonstrated high sensitivity and reliability, with limits of detection and quantification of 0.47 and 1.18 ppm total egg protein. Protein recovery using the MS method was 57.5% - 96.5% across five food matrices, outperforming four egg ELISA kits. In addition, the developed MS method accurately quantifies the reference dose of 2 mg of total egg protein in food consumption amounts up to 1.69 kg. This method can enhance food allergen control and management, benefiting regulatory agencies, public health authorities, and the food industry.

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LITERATURE REVIEW

1.1 Introduction

Chicken (*Gallus gallus*) eggs are versatile food ingredients due to their high nutritional value (e.g., high-quality protein and minerals) and unique functional properties (e.g., gelation, foaming, and emulsification).[1] Meanwhile, egg is also one of the "Big 9" food allergens in the U.S., affecting 0.9% of children and 0.8% of adults.[2,3] Egg allergy symptoms can range from mild (e.g., hives, swelling, cramps, etc.) to potentially fatal anaphylaxis, and there is currently no standardized treatment available.[2,4] In the U.S., precautionary allergen ('may-contain') labeling (PAL) is voluntary and not specifically regulated. Undeclared eggs in food products pose a threat to individuals with egg allergies.[5] PAL guidance needs to be constructed to improve allergen management in the food industry. In addition, a reliable analytical method of quantifying allergens in finished foods is required.[6]

Commonly used immunoassay methods for egg detection rely on recognizing epitopes of target proteins, but these methods may be affected by food processing and the complexity of the food matrix.[7] Structural changes in proteins caused by food processing can impact the conformation of epitopes on the target proteins.[8] To address these challenges, we developed a novel approach that utilizes mass spectrometry to detect and quantify eggs in processed foods. This method uses peptides as analytes, making it less susceptible to food processing and matrix effects. Additionally, this approach offers significant advantages over existing methods in terms of sensitivity, specificity, and robustness.

1.2 Egg and egg products

Eggs are hard-shelled reproductive bodies produced by poultry birds. They are an affordable source of high-quality proteins found and consumed worldwide in different cultures. Chicken eggs are the most popular, making up 93% of worldwide egg production.[9] However, eggs from other poultry breeds, such as duck, guinea fowl, and quail, are also popular in some Asian, African, and Western countries.[9] According to 2023 FAO data, total global egg production is 86 million tons, with China, the U.S., and India contributing half of the total production.[10] In the U.S., approximately 30% of eggs are separated from their shells and processed into egg products such as liquid, frozen, and dried eggs.[11] Unlike retail shell eggs, egg products are preferred by food manufacturers, bakery suppliers, food processors, and other end users. This is because egg products have advantages such as high food safety, consistent quality, extended shelf life, and convenient handling.[11]

1.2.1 Egg composition

Eggs comprise four main parts: the eggshell, shell membranes, egg white, and egg yolk. According to USDA Food Data, a fresh, whole, large egg contains approximately 34 g of egg white and 17 g of egg yolk, with an overall protein content of 12.4%.[12–14] The egg white, 10.7% protein, contains over 40 different proteins.[1] The most abundant egg white proteins are ovalbumin (54%), ovotransferrin (13%), ovomucoid (11%), and lysozyme (3.4%).[1,14] The egg yolk comprises 16.2% protein, 28.8% lipids, and approximately 50% other solids.[1,13] Upon centrifugation, egg yolks can be separated into plasma and granules. The granules mainly contain high-density lipoprotein and phosvitin, while the plasma is rich in low-density lipoprotein (65%) and livetin (30%).[1]

1.2.2 The functionality of eggs in food

Chicken eggs are versatile and ubiquitous ingredients in the food industry due to their important properties. Egg white powder contains proteins (mainly ovalbumin) and has excellent foaming abilities to trap air and stabilize structures.[1] The presence of ovomucin, along with lysozyme with ovomucoid, can improve the stability of the foam.[15,16] This foam helps achieve the desired light and airy texture in aerated food systems. The great foaming ability of egg white protein can increase the volume and enhance the overall quality of products like ice cream, meringue, and various bakery items (e.g., cakes).[17] Another excellent property of egg white powder is gel-forming. Ovalbumin plays a crucial role in forming a gel-like texture in egg whites.[1] To form a gel structure, the egg proteins are first denatured and unfolded, followed by molecule aggregation of the denatured protein.[1] The gel-forming property of eggs is particularly important in thickening the textural structure of foods (e.g., custard).[17] Recently, egg whites have been used as a substitute for wheat flour in making gluten-free bread primarily due to their excellent gel-forming properties in producing crumb structures.[18] Moreover, egg white powder is one of the traditional fining agents in winemaking.[19] When added to wine, the egg white proteins absorb unwanted tannins and other phenolic compounds aiming to clarify the wine and reduce astringency.[19,20]

Egg yolk proteins, mainly low-density lipoprotein, are crucial emulsifying agents in the food industry.[1] This unique property makes it indispensable in various food products, such as mayonnaise, ice cream, salad dressing, and noodles.[21] Besides improving the flavor of foods, egg yolk is also necessary to create a stable mixture of water- and oil-based ingredients. Moreover, egg yolk contains natural carotenoid

pigments such as lutein, zeaxanthin, and beta-carotene, which give it its characteristic yellow color. [21,22] These pigments enhance the color of foods and provide potential health benefits and antioxidant properties.[23]

1.2.3 Egg product processing

The production of egg products, such as liquid eggs, frozen eggs, and dried eggs, involves several important steps to guarantee safety, quality, and compliance with regulatory standards.[24] The process starts with the reception of shell eggs, which may originate from various chicken farms, followed by processing via in-line or off-line systems. The eggs are washed, sanitized, and candled to ensure cleanliness and quality. In the egg-breaking phase, using breaking machines, sanitized eggs are separated from their shells. The liquid egg is then filtered and cooled for further processing. At this stage, the egg yolk can also be separated from the egg white. The liquid yolk and white can then be processed individually. According to USDA regulations, a liquid egg must be pasteurized within 72 hours of breaking. The pasteurization temperature and duration may vary for whole, yolk, and white products, but often, an HTST (i.e., high-temperature short time) approach is used to deactivate Salmonella and other pathogens. After pasteurization, all subsequent handling and processing of egg products must be handled under hygiene and sanitized protocols. The sanitized liquid egg (e.g., whole egg, egg yolk, egg white, and a blend of liquid egg with added ingredients) can be further processed into frozen or dried egg products. For frozen egg products, the sanitized liquid egg is subjected to blast freezing at temperatures ranging from -10°F to -40°F. For dried egg products, a drying process, such as spray drying or pan drying system, produces the finished product. Spray

drying is the predominant method, where the liquid egg is sprayed into hot steam for rapid evaporation, followed by cooling, sifting, and packaging.

1.3 Food allergy

Food allergy is a hypersensitive immune response to specific protein(s) in food.[25] There is a growing global awareness of food allergies, which have become increasingly prevalent in recent years.[26–28] Millions of people in the U.S., including 5 – 8% of young children and 2 – 11 % of adults, suffer from one or multiple food allergies.[3,25,29] The nine major food allergens in the U.S. (milk, hen's eggs, peanuts, tree nuts, wheat, fish, crustacean, soybean, and sesame) cause nearly 90% of food allergic reactions.[30]

There are three food allergenic pathways, including immunoglobulin E (IgE)-mediated, non-IgE-mediated, and mixed food allergies (characterized by both IgE- and non-IgE-mediated pathways).[31,32] Among these three pathways, IgE-mediated food allergy is the most prevalent and typically associated with acute reactions.[31] The complex of allergenic IgE antibodies and high-affinity Fc receptor (FceRI) on mast cells can recognize and bind to the allergen. Subsequently, it can trigger the release of preformed mediators, such as histamine and arachidonic acid metabolites, which can contribute to various inflammatory responses (e.g., swelling, hives, and diarrhea).[31] Non-IgE-mediated food allergies commonly react to the skin and gastrointestinal tract and may be responsible for chronic inflammation and delayed adverse immune responses.[31] The allergic symptoms can vary between individuals and range from mild (e.g., hives, swelling, rash) to severe reactions.[25] The National Library of Medicine defines anaphylaxis as a systematic allergic reaction in which at least two body systems have adverse reactions.[33] A UK survey found that 30.1% of anaphylaxis admissions were due

to food.[34] The number of hospital admissions for anaphylaxis related to food has been increasing in recent years.[35,36]

The primary method for diagnosing a food allergy is through clinical history and physical examination. If allergenic symptoms resolve upon elimination of a specific food from the diet, that food may be identified as the source of allergens. Laboratory tests, such as measuring allergen-specific serum IgE levels in the blood and conducting skin prick tests (i.e., observing wheal-and-flare reactions,) are used to identify specific allergens and as complementary methods to the clinical history investigation.[29] However, it's important to note that these tests can yield a high rate of false-positive results on foods that the patient tolerates.[37] Advanced diagnostic methods such as component-resolved diagnosis (CRD) and basophil activation tests (BAT) identify allergen-specific molecules and IgE antibodies, respectively.[38,39] These advanced methods can reduce the false-positive rate of allergen testing. Despite their potential, these methods are not yet widely adopted in U.S. clinical practice.[38,39] A double-blind, placebo-controlled food challenge is a gold standard for diagnosing IgE-mediated food allergies.[29] However, this method is costly, time-consuming, and requires professional supervision.

Currently, there are no standardized treatments for food allergies. Food allergy-specific immunotherapy is under development as a potential treatment for IgE-mediated food allergy.[40] It can be classified into three approaches, including oral (i.e., ingesting), sublingual (i.e., under the tongue), or epicutaneous (i.e., skin) immunotherapy. During the immunotherapy treatment, patients undergo repeated administration of gradually increasing the antigen dose for a long period, ultimately leading to clinical outcomes of either tolerance or desensitization to food allergens.[40] Until food allergy-specific

immunotherapy is proven effective and safe by health authorization, strict avoidance of allergenic foods remains the only reliable approach for managing food allergies.[25]

1.4 Egg allergy

Due to its high prevalence, severity (i.e., high anaphylaxis in more than three Codex regions), and medium potency, the egg is recognized as a food allergen on the global priority list.[41] Eggs are the second most common cause of food allergies in infants and young children, following cow's milk, with a prevalence of 0.8% to 0.9% in all children.[2,42] There is a high self-recovery rate among children with egg allergies. A study has shown that 66% of children with egg allergies develop a tolerance to eggs at five years old.[43] When there is a lack of development of tolerance, if the individual has an egg allergy as a child, the allergenic reaction could be severe and life-threatening, affecting 0.1% to 0.8% of adults.[3,44,45] Development of asthmatic symptoms in children and gastrointestinal symptoms are more commonly observed in egg-allergic individuals than in individuals with other food allergies.[2,46] A lifetime elimination diet – avoidance of eggs and egg-derived products is the primary clinical advice for preventing allergic reactions.[25]

The World Health Organization and the International Union of Immunological Societies (WHO/IUIS) have recognized ten allergenic chicken proteins associated with clinically reported allergy incidences.[47] Four major egg white proteins have been recognized, including ovomucoid (Gal d 1, molecular weight of 28 kDa), ovalbumin (Gal d 2, 45 kDa), ovotransferrin (Gal d 3, 78 kDa), and lysozyme (Gal d 4, 14.3 kDa).[48] Additionally, two minor egg yolk proteins, alpha-livetin (Gal d 5, 70 kDa) and yolk glycoprotein 42 (YGP42, Gal d 6, 35 kDa), are also associated with egg-specific allergy.[48,49] Furthermore, four chicken-allergenic proteins (Gal d 7 – Gal d 10) originating

from chicken tissues can trigger allergic reactions.[50,51] Notably, the Gal d 8 (parvalbumin), Gal d 9 (enolase), and Gal d 10 (aldolase) have been found to be cross-reactive to IgE from fish-allergic patients.[51]

It is particularly challenging for individuals with egg allergies to avoid eggs strictly in their daily lives. This is mainly because egg is a ubiquitous food ingredient in food products (such as bakery, sauce/salad dressing, and ice cream) and a major ingredient in its functional properties.[1] In addition, the egg is an auxiliary component in some vaccines and cosmetics products.[21,52] Therefore, egg-allergic individuals and their families must carefully read allergen statements or ingredients on packaged food labels to determine the presence of eggs.

1.5 Management and regulation of food allergens

More than 200 foods have been identified as potential causes of allergic reactions.[53] Notably, the priority food allergens can vary from one region to another due to environmental and regional differences.[28,54] Twenty-five foods or ingredients were recognized as major food allergens across 36 geographic regions.[55] Instead of declaring all potential food allergens on packaged food products, only the major food allergens are regulated following regional food allergen labeling regulations. Despite the differences among the international food allergen regulations, most require the major food allergens to be labeled in plain language on the package.[56] This aims to disclose the presence of allergenic ingredients based on the food product's recipe or manufacturing. This section focuses on the food allergen regulations in the U.S.

1.5.1 Food allergen labeling requirements in the U.S.

The Food Allergen Labeling and Consumer Protection (FALCPA) Act, enforced by the U.S. Food and Drug Administration, has been effective since 2006.[30] This act defines milk, eggs, fish, shellfish, tree nuts, peanuts, wheat, and soybeans, accounting for 90% of food allergies in the U.S., as major food allergens. Effective in early 2023, sesame is added to the list of major food allergens, according to the Food Allergy Safety, Treatment, Education, and Research (FASTER) Act of 2021.[57] Together with the FALCPA and FASTER Acts, any domestically manufactured and imported prepackaged food products containing ingredients derived from any of these "Big Nine" major food allergens must be labeled in plain English in the ingredient statement or a Contains statement. These products, including flavoring, incidental additives, or coloring, are required to be labeled on packages with plain and easy-to-understand allergen statements. For example, when ovalbumin, an egg-derived protein, is used in food products, it should be labeled clearly with "ovalbumin (egg)" on the package. Alternatively, a Contains statement, which follows directly after the ingredient statement, can be used to disclose the ingredients derived from major allergenic food sources (e.g., Contains egg).

1.5.2 Prevention of food allergen cross-contact

The food industry must diligently prevent cross-contact between food product formulations containing allergenic ingredients and non-allergenic ingredients to minimize the unintended introduction of major food allergens. In the U.S., the Food Safety Modernization Act (FSMA) has led to significant enhancements in the food safety system, covering the entire food supply chain from farm to table.[58] The law and rules within FSMA, as well as the modernized Current Good Manufacturing Practices

(cGMPs), now require domestic and foreign food manufacturers to develop comprehensive preventive control plans for human and pet food. This includes allergen management to prevent allergen hazards, such as implementing strict protocols for shared facilities, proper management of rework, and avoiding cross-contact with food allergens. Additionally, the Act mandates clear allergen labeling on supplement ingredients and packaged food products. By adhering to the regulations of FSMA and utilizing cGMPs and preventive controls, food manufacturers are now more aware of the importance of allergen control in minimizing hidden allergens throughout the food supply chain.

1.5.3 Precautionary allergen labeling for allergens

Precautionary allergen statements (PAL), also known as "may contain" allergen statements, are used in the food industry to alert allergic consumers to the potential risk of inadvertent allergen presence. However, these statements are not mandated by law. Due to a lack of clear regulatory requirements, studies have shown that PAL has been overused, and numerous terms/expressions have been utilized worldwide.[6,59] Several surveys have investigated the percentage of packaged food that uses PAL: 65% of all packaged food uses PAL in Australia[60], 33% in Latin American countries[61], and 29% in Malawi[62]. Moreover, studies have revealed that different expressions of PAL were used on food products, such as “May contain,” “allergen X may be present,” “Manufactured on shared equipment,” etc.[6] The different expressions of the PAL can influence the choice of food products by food-allergic consumers, and up to 40% of consumers ignore certain PAL statements.[63] The variation in PAL is not helpful to food-allergic consumers because the different expressions of PAL do not indicate the quantity risk of the allergenic residue that may be present in food products.[64] A Canadian survey[65] has shown that 7% of

samples with PALs contain detectable egg residue with a broad range of concentrations from 0.3 to 777 ppm egg protein. While PAL was initially intended to alert food-allergic consumers of potential risk, its voluntary and ubiquitous use, without proper regulatory guidance, may inadvertently result in a decline in the quality of life of food-allergic individuals as food choices have become increasingly limited.

1.5.4 Risk-based precautionary allergen labeling

1.5.4.1 Estimation of a threshold dose for food allergen

Clinical data from the double-blind, placebo-controlled oral food challenges (DBPCFC) is preferred to determine the threshold dose for individuals with food allergies.[66] During the oral food challenge, the allergic individual ingests progressively increasing amounts of food containing suspected allergens (mg of food protein from the allergenic source of interest). The doses start at very small amounts, are unlikely to induce an adverse immune response, and are escalated until the individual obtains their initial objective allergic symptoms. The goal of the food challenge is to identify the LOAEL (lowest-observed-adverse effect level). The threshold dose for allergic individuals falls between the NOAEL (no observed adverse effect level) and LOAEL determined from the oral food challenge; however, the exact eliciting dose is not titrated.[67] Statistical approaches such as interval-censoring survival analysis can be used to account for the potential eliciting dose between the NOAEL and LOAEL.[66,67]

The Bayesian Stacking statistical model (also referred to as model averaging) consolidates multiple clinical oral food challenge datasets and parametric distributions into a unified predictive distribution for allergen thresholds.[68,69] The model's notable advantage is that it considers the data quality, resulting in a more objective prediction of

the allergen thresholds.[69] Another noteworthy advantage of modeling averaging is that it uses several parametric distributions and a weighted approach to derive an 'average' population dose distribution thereby negating the need to subjectively select one parametric distribution over another when there is no biological rationale to do so.[69]

1.5.4.2 Establish the reference dose of the food allergen

The DBPCFC dataset is similar to the interval-censored data used in survival analysis in medical or health studies, where food allergen doses are recorded against the time until individuals experience initial objective allergic reactions. Wheeler et al.[69] integrated the Bayesian statistical model with the interval-censored statistical concept to develop dose-to-failure distributions for food allergen thresholds. These distributions represent the Eliciting Doses (EDs) of the food allergen relative to the percentage of the allergic population that experiences objective allergic reactions. Specifically, ED_{01} and ED_{05}, representing doses corresponding to 1% and 5% of the allergic population, respectively, are established as the reference doses for food allergens. According to the ED_{01} and ED_{05},[70], risk assessors have systematically reviewed the oral food challenge dataset and used Wheeler et al.'s approach[69] to define the reference doses for 14 allergenic foods.

In 2019, the Allergen Bureau of Australia and New Zealand released the latest version of the Voluntary Incidental Trace Allergen Labeling (VITAL 3.0) program. This update included the adoption of the ED_{01} threshold for establishing reference doses for 14 allergenic foods, as listed in **Table 1.1**.[70] In 2021, the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) expert consultation suggested that the reference doses of food allergens should not only address

the potential severity of allergic response (including potential fatal or severe hazards) among the allergic population but also consider the feasibility and applicability of the current food allergen detection methods to support the utilization of reference doses for risk management decisions.[71] The FAO/WHO expert consultation recommended establishing reference doses for 13 global priority allergic food sources using round-down ED_{05} reference doses (**Table 1.1**).[71] The reference doses recommended by FAO/WHO are 4 to 33 times higher than the VITAL 3.0 reference doses.

Table 1.1 Reference dose for food allergens.

Allergen	VITAL 3.0 (mg protein)	FAO/WHO (mg of total protein from the allergenic source)
Walnut (and Pecan)	0.03	1.0
Cashew (and Pistachio)	0.05	1.0
Almond	-	1.0
Hazelnut	-	3.0
Other tree nuts	0.1[a]	1.0[b,c]
Celery	0.05	1.0[c]
Mustard	0.05	1.0[c]
Sesame	0.1	2.0
Egg	0.2	2.0
Milk	0.2	2.0
Peanut	0.2	2.0
Soy	0.5	10.0[c]
Cereals containing gluten	0.7	5.0
Fish	1.3	5.0
Lupin	2.6	10.0[c]
Crustacea	25	200

[a] In VITAL 3.0, other tree nuts include almonds, hazelnuts, Brazil nuts, and macadamia.
[b] In the FAO/WHO meeting report, other tree nuts include Brazil nuts, macadamia, and pine nuts.
[c] Due to inadequate data or pool data quality, the reference doses of specific allergenic food sources were not included in the FAO/WHO main report. This is a potential value for risk management, as recommended by the FAO/WHO expert consultation.

1.5.4.3 Interpretation of the reference dose to risk-based action level

After the reference dose of a food allergen is defined, it can be translated into Action Levels (AL) to assess the impact of allergen residue in finished food products. The VITAL program introduced the use of ALs to determine whether a PAL should be displayed on a package.[70] That is using ALs considering consumption quantities to indicate potential exposure doses for allergic consumers. The AL is the concentration of total protein from the allergenic source of concern at each eating occasion, which is calculated by dividing the reference dose by the food consumption amount. When the calculated exposure dose (a function of the AL multiplies the portion size or reference amount consumed) is below the reference dose, as in the case of VITAL 3.0 (i.e., ED_{01}), the amount of allergen residue in the food product is considered safe for 99% of the allergic population. In such cases, a PAL is not recommended. Conversely, if the calculated exposure dose exceeds the reference dose, a PAL is recommended to communicate the potential exposure risk to allergic consumers. Because the VITAL 3.0 reference doses are in accordance with the European Union (EU) allergen labeling requirement, most countries in the EU, except Germany, The Netherlands, and Belgium, have considered using the VITAL 3.0 reference doses to determine the use of PAL.[70]

Because the exposure dose of food allergens does not accumulate over a daily period as typically assessed for chemical contaminants, the risk assessment should consider the exposure of allergens on a signal-eating occasion in order to determine the exposure doses and ALs of the food allergens accurately.[72] The reason is that food allergies cause acute allergic reactions, the onset of which is commonly within 2 hours after one eating occasion.[25] It is rare to have a delayed onset of symptoms.[25,32] Care

should be used in selecting the consumption quantity used for calculating the exposure doses and associated AL. Using the serving size on the package can underestimate the actual risk because it is generated based on nutritional value and may not reflect the true quantity of food consumed on a single eating occasion.[72,73]

A food consumption survey that interviews the general public can provide a realistic quantity of food consumed during an eating occasion. An EU project, Integrated Approaches to Food Allergen and Allergy Management (iFAAM), combined food consumption database from the Netherlands[74], France[75], and Denmark[76]. The iFAAM database has been used to conduct a sensitivity analysis to estimate the appropriate consumption quantity for use in food allergy risk assessment.[77] The total food consumption distribution typically follows a slightly skewed pattern, meaning that most people consume a moderate amount of food while a smaller number of individuals consume very small or very large quantities.[77] Bloom et al.[77] examined various percentiles (P50 – P100) to interpret the deterministic risk assessment across various concentrations of allergens (1 to 1000 ppm protein). The study found that using P50 – P75 of food consumption is desired to predict risk adequately. The authors recommend using the P75 value as the ideal food consumption input for risk assessment. It provided a good predictive balance between the outcomes of the deterministic risk assessment approach and the probabilistic (quantitative) risk assessment approach. The FAO/WHO expert consultation recommended the consideration of P50 to P75 as the reference consumption and suggested conducting appropriate sensitivity analyses to calculate the allergen ALs.[71]

In the U.S., the National Health and Nutrition Examination Survey (NHANES), conducted annually by the Centers for Disease Control and Prevention (CDC), interviews 5,000 individuals to gather health and nutrition-related information.[78] NHANES employs detailed dietary intake methods, including 24-hour recalls and food frequency questionnaires, to assess food consumption patterns and nutrient intake among the American population. This extensive data collection aids in identifying dietary habits and potential allergen exposure risks.

Due to cultural and dietary habit differences, food consumption patterns can vary significantly between regions. A systematic study[79] comparing national food consumption surveys from the U.S. and the Netherlands found that these differences can lead to varying risk interpretations for food allergens. This study emphasizes the need to consider regional consumption patterns for food allergen risk assessment. The authors suggest that using the higher of the two food intake quantities for a particular food category in the risk assessment can provide the most conservative risk interpretation, thereby covering the majority of potential scenarios.[79]

In addition, the food allergen risk assessment can also be conducted independently in each country or region with similar consumption habits to ensure accuracy and relevance in risk interpretation and food allergen management. For example, in Canada, data from the 2015 Canadian Community Health Survey (CCHS) were used to conduct a probabilistic risk assessment for the presence of milk in dark chocolate, cookies, and other baked goods with precautionary allergen labeling (PAL).[80] The risk interpretation from this Canadian study may be applicable to other countries and regions with similar dietary habits and consumption patterns.

1.6 Undeclared food allergen major offenders in food recalls

However, food allergen management can be challenging, unlike the preventive control of microbiological hazards – many microorganisms can be inactivated by lethality treatment to achieve “zero” tolerance. Wet cleaning (i.e., using water) is the most effective method to remove allergenic ingredient residue on the processing lines. However, wet cleaning is not always appropriate during food processing because of longer downtime, the potential for microorganism growth, and the accessibility of the equipment. When inadequate cleaning occurs, it can cause the unintentional introduction of allergenic ingredients into products subsequently made using the equipment.

Data from the FDA Recall Enterprise System (RES) from fiscal years 2007 to 2019 indicate that major food allergens were the most frequent cause of recalls.[81,82] Allergen label-related errors accounted for 64.6% of these major food allergen (MFA) recalls, representing 71.1% of MFA recalls with a known root cause.[82] These labeling errors included incorrect packaging, wrong labels on products, and unclear or incorrect terminology about the presence of allergens, which can lead to undeclared allergens in food products, posing serious health risks to allergic consumers.[81,82] From fiscal years 2013 to 2019, the top three major food allergens associated with FDA Class I recall were milk (40.1%), tree nuts (18.4%), and eggs (17.5%).[82] A Class I recall is the most serious type, indicating that the violative product could cause severe adverse health consequences. Specifically, egg allergens accounted for 9.8% of total recalls from FY 2007 to 2019. Recalls due to egg allergens primarily affected bakery products, dough, bakery mixes, and icings, with 33 incidents recorded in this category.[82]

A Chinese study[83] sampled 127 prepackaged snack foods with egg precautionary allergen labeling (PAL) statements from 15 retail points in Nanchang, Jiangxi. Overall, approximately 40% of the sampled products had detectable egg (above the limit of detection of 0.13 ppm whole egg powder) content using the ELISA method; 18.11% of the tested products had egg concentrations above 2.5 ppm whole egg powder (WEP). The top two food categories using egg PAL were puffed and baked foods. Among the puffed foods, 13 out of 22 had detectable levels of egg, with a median concentration of 1.9 ppm WEP. In the baked foods category, 48 out of 77 products contained detectable eggs, with a median concentration of 5.3 ppm WEP. Additionally, individual cases of products with PAL statements showed egg protein levels of over 16,000 ppm WEP in aquatic and confectionery products and over 10,000 ppm WEP in baked food.

The findings from a comprehensive Thai survey[84], which examined prepacked food products imported into Australia, are particularly noteworthy. Out of the 549 products tested, 214 featured a precautionary allergen labeling (PAL) statement. Using the Morinaga ELISA egg test, 47 products were specifically screened for egg content, and 8 of these (17%) were found to contain egg at levels exceeding 20 ppm egg protein. Notably, of the products with a PAL statement, 5 labels indicated the presence of eggs, but only 2 tested positive. These results raise questions about the accuracy of PAL statements. The survey also revealed that wheat, egg, and milk were the most prevalent allergens detected, underscoring their widespread use in Thai processed foods. Furthermore, the survey identified instances where seafood products were found to contain or potentially contain egg, likely due to the use of egg white as an emulsifying agent, foaming agent, and texture improver in these products.

A Canadian survey[65] tested 840 food samples, with 89% of them containing egg precautionary allergen labeling (PAL). Approximately 7% of the tested samples had detectable egg levels using ELISA methods. The food categories with the highest occurrence of detectable egg were baked goods (8%), cookies (13%), and pasta (13%). These categories also showed a broad range of measured egg protein levels, with differences exceeding 120 ppm egg protein. Among these, baked goods had the highest median egg protein concentration at 3.8 ppm, followed by pasta at 2.0 ppm and cookies at 1.6 ppm.

1.7 Allergen detection methods

1.7.1 Polymerase chain reaction method

Polymerase Chain Reaction (PCR) detects target DNA molecules as an indicator of the presence of proteins. PCR is a cyclic process consisting of denaturation to form single-stranded DNA templates), annealing (at a lower temperature to allow primers to bind to the template), and extension of the primers to amplify the quantity of the target DNA in the sample.[85] In 1992, Higuchi et al. developed real-time PCR, also known as quantitative PCR (qPCR), to quantify the DNA template molecules in the initial sample.[86] In qPCR, the fluorescence of dyes or probes is introduced during the PCR amplification cycles. The fluorescence signal is monitored for each cycle simultaneously, and its intensity exponentially increases with the number of cycles.[85] The initial amount of DNA template in the sample can be calculated by interpreting the response curve.

The qPCR method has been developed over the past two decades for detecting and quantifying allergenic ingredients in food. It shows high specificity in differentiating the allergenic ingredient from species within a relative family.[87] Due to its robust

specificity, qPCR has been used as an official analytical method in Japan and Germany for the detection of food allergens (e.g., celery, wheat, peanut, almond, etc.).[87,88] For example, the specific detection of celery from other food ingredients in the family Apiaceae, such as carrot and parsley, where those foods are difficult to differentiate at the protein level.[89,90]

However, the qPCR method is not suitable for identifying milk, eggs, and their derived products. Only a limited number of target genes are specific to milk and egg proteins. This limitation hinders the development of qPCR methods for detecting low levels of their residue in food products.[87] The target gene for milk and eggs can also indicate the presence of parts from beef and chicken, such as meat products. Koppel et al.,[91] have developed a qPCR method for simultaneously detecting milk, eggs, almonds, sesame, pistachios, and walnuts in food products. This multiplex qPCR method uses target genes from chickens to indicate the presence of eggs in food products.[91] The authors analyzed various market samples using this multiplex qPCR method, finding that 32% tested positive for eggs. However, some positive signals were obtained from chicken meats in the sample investigated (e.g., chicken sandwich). This may lead to incorrect detections in foods without declared egg ingredients.

1.7.2 Enzyme-linked immunosorbent assays

Immunoassay methods, particularly the widely used enzyme-linked immunosorbent assay (ELISA), play a crucial role in determining the presence of allergenic ingredients in foods. ELISA, a rapid and commercially available method, is routinely used in the food industry to quantify allergen residues in food products and environmental samples.[92,93] It's also a standard analytical method in food allergen-related

research. The effectiveness of the ELISA method relies on its ability to recognize epitopes on target proteins from food commodities. The epitopes are highly specific to the antibodies used in the ELISA, which enables the detection and quantification of the target proteins, such as allergens, in samples.

The two main types of ELISA methods are sandwich ELISA and competitive ELISA. Sandwich ELISA is named for the process by which two layers of antibodies recognize the target antigen.[94] This method requires at least two epitopes for the antigens to bind to the capture and detection antibodies. A substrate for the enzyme, attached to the detection antibody, is added to form an enzyme-catalyzed reaction, typically resulting in a color change. The color intensity is directly proportional to the target antigen concentrations present in the sample. As for the competitive ELISA, this method measures the concentration of target antigens by observing the competition between the antigen in the sample and a known concentration of labeled antigen for a limited number of antibody binding sites.[94] A substrate for the enzyme attached to the labeled antigen is added to produce a color-changed signal. Therefore, in the competitive ELISA method, the intensity of the color is inversely correlated to the concentration of the target antigen present in the sample. Competitive ELISA requires only one epitope on the target antigen for detection.

Current commercial egg ELISA kits primarily are sandwich ELISAs. The target analyte used in egg ELISA kits varies from manufacturer to manufacturer, but it is typically whole egg protein, egg white protein, ovalbumin, and/or ovomucoid. Besides the differences in the target analytes, the sample extraction protocol, including extraction buffers and antibody conjugate, can be different. For example, a surfactant agent (e.g.,

SDS) and a reducing agent (e.g., 2-mercaptoethanol) can be added to the protein extraction buffer, which improves protein quantification in processed foods.[95] Some egg ELISA kits use additives such as wheat flour and non-fat dry milk powder in the extraction buffer to improve extraction efficiency and protein stability.

Smits et al.[96] tested seven commercial ELISA kits for detecting eggs in nine food matrices (e.g., cookies, pasta, ice cream, chocolate, etc.) that were spiked with whole egg powder. According to the VITAL 3.0 reference dose, egg protein is detectable at a concentration relative to the consumption size of nine different food matrices, ranging from 0.71 to 10 ppm total egg protein. However, as for processed food products (e.g., cookies), egg proteins undergo physical and chemical interactions with other ingredients during food processing. Food processing with heat, pressure, irradiation, fermentation, etc., can alter and modify protein structures by denaturing, aggregating, hydrolyzing, and precipitating them. These structural changes can destroy or mask the conformation of epitopes, potentially hindering the detection of the ELISA kits.[97,98]

Several studies have shown that egg ELISA kits face challenges in detecting egg proteins in different food matrices with incurred egg proteins. When whole egg powder is heated in the presence of water to above 100°C or dry heated to above 176°C, the detected egg proteins are substantially underestimated.[99] A study found that dark chocolate, incurred with whole egg powder and tempered at 46°C for 4 hours, had little impact on detecting eggs using different ELISA kits.[100] The tested ELISA kits detect egg protein in unprocessed and tempered chocolate matrices at the limit of quantification (LOQ) of the kits.[100] After boiling pasta, which was incurred with 1000 ppm egg protein, for 15 minutes, eggs become undetectable by ELISA.[101] In another study,[102] ELISA kits

tend to overestimate egg detection in unprocessed cookie dough. However, after baking for 25 minutes, the protein recovery decreases to below 50%, and none of the ELISA kits can detect egg protein in the baked cookies at the kit's LOQ. Moreover, the use of different ELISA kits for the detection of eggs in food products can result in inconsistent results. Khuda et al.[5] used two egg ELISA kits to detect egg protein in food products with PAL. These two ELISA kits differ in the target antibodies, extraction protocol, and calibration standards, leading to a disagreement on the presence of eggs in 5.2% of the bakery samples tested.

1.7.3 Mass spectrometry method

Mass spectrometry is a powerful and widely used analytical technique that can identify and precisely quantify thousands of proteins from complex samples.[103] Prior to MS analysis, proteins from foods are extracted, followed by reduction, alkylation, and enzymatic digestion into peptides. Instead of intact proteins, peptides are used as the analyte for MS analysis, showing many advantages over ELISA. Mass spectrometers consist of three main components: ionization, mass analysis, and detection.

1.7.3.1 Ionization

The rapid development of mass spectrometry in proteomics can be attributed to the advancements in electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), which are the two major types of ionization used for peptides and proteins.[104,105] ESI and MALDI allow the ionization and volatilization of peptides or proteins without causing significant fragmentation, which is crucial for analyzing these large biomolecules. ESI operates by applying a high voltage to the liquid samples to form a spray of charged droplets and nebulizing gas (usually nitrogen) to

produce charged gas-phase ions.[104,106] This method typically analyzes complex samples and couples them with liquid chromatography. For MALDI, the ionization takes place on the crystallized and dry matrix samples by applying a laser pulse and then irradiating the matrix. This method is often used in combination with time-of-flight mass spectrometry.[105,106] The mass-to-charge ratio (m/z) of the generated ions is then analyzed and detected using tandem mass spectrometry.

1.7.3.2 Tandem mass spectrometry

Tandem mass spectrometry (MS/MS) typically involves mass analysis of precursor and fragmented ions to provide detailed information about peptides or proteins.[107] To achieve the two stages of analysis, at least two mass analyzers are assembled and work in tandem. In the first mass analyzer, all ions are screened based on their m/z to select ions of interest, also known as precursor ions. The spectrum of the precursor ions is named the MS1 spectrum.

Only the selected precursor ions are fragmented into smaller ions, also known as product ions. Ion fragmentation is achieved by collision-induced dissociation (CID), higher-energy collisional dissociation (HCD), or other means.[108–110] At sufficient energy, the whole peptide sequence is cleaved at its peptide backbone.[111] In CID fragmentation, the peptide can be broken at the specific peptide bond containing three possible charged ions at each amino (a, b, and c) and carboxyl-terminal (x, y, and z).[112,113] Therefore, depending on the peptide length, product ions can be generated during fragmentation. Notably, the fragmentation of b and y ions (i.e., the cleavage of the CO-NH bond) is the most common occurrence.[111] Therefore, the fragmentation pattern of b-y ion pairs from

the experimental spectra can be compared with the theoretical spectra to determine peptide sequences.[111]

Then, the m/z of product ions is measured in the second mass analyzer. The spectrum of the product ions is named the MS2 spectrum. In proteomic research, there are many combinations of mass analyzers, such as time-of-flight, ion trap, and quadrupole.[106] Among these tandem MS/MS systems, triple quadrupole mass spectrometers and quadrupole-orbitrap mass spectrometers have been developed and used in proteomics research.[114]

1.7.3.3 Triple quadrupole mass spectrometer

A triple quadrupole mass spectrometer (QqQ) comprises three quadrupole mass analyzers arranged in a series. The first and third quadrupoles are used to select precursor ions and analyze fragment ions, while the second quadrupole is responsible for ion fragmentation. It is noted that only the pre-determined product ions are fragmented and can be detected.[107] The pair of the precursor and one fragment ion is called a transition. One precursor ion can have multiple transitions that are paired with different relative fragment ions. Multiple Reaction Monitoring mass spectrometry (MRM) (sometimes called Selected Reaction Monitoring, or SRM), performed on QqQ, is a targeted quantitative analysis. In the MRM method, predefined transitions are analyzed for each peptide of interest within the scheduling window. This way can enhance specificity and confidence in peptide detection and quantification. Typically, two or more transitions are monitored for each peptide of interest. The determination of optimal transitions of the peptide depends on empirical evidence by applying various collision energies and instrument parameters.[115]

1.7.3.4 Quadrupole-Orbitrap mass spectrometer

The quadrupole-orbitrap mass spectrometer for MS/MS analysis consists of three main stages: the selection of precursor ions in the quadrupole, fragmentation in the collision cell, and detection of all relevant product ions in orbitrap (producing MS2 spectra).[116,117] The orbitrap mass analyzer measures the m/z ratios of ions based on their movement frequency around a spindle-shaped inner electrode and outer barrel-like electrodes. The movement frequency is an orbital-like motion back and forth along the inner electrode, which is directly related to the m/z ratio.[117] Noteworthy, the orbitrap method can be employed for both untargeted and targeted analysis in proteomics research.

1.7.3.5 Untargeted analysis

Untargeted analysis, also known as discovery proteomics, comprehensively and objectively characterizes the presence of proteins/peptides in samples. One primary purpose of the untargeted analysis is to discover potential biomarkers that signify the presence of proteins of interest.[118] This is crucial because protein expression levels often signal various biological conditions, such as diseases, responses to drug treatments, etc. Biomarker identification relies on assessing the relative quantitative differences among samples under different conditions (e.g., healthy vs. diseased).[119]

Two main methods are employed for untargeted analysis: data-dependent acquisition (DDA) and data-independent acquisition (DIA).[120] In the DDA method, the most abundant precursor ions, typically referred to as the "Top N ions," detected during one MS1 scan are further fragmented and subsequent MS/MS analysis.[121] The DDA method is preferred in studies focusing on high-abundance ions, which can identify

thousands of proteins.[121] In the DIA method, all precursor ions within a predefined m/z window are fragmented simultaneously. The m/z window typically spans a small mass range (10 – 25 m/z) and is systematically moved across the entire mass range of interest.[122] Fragmentation is triggered for all ions within each small m/z window. The DIA method allows for unbiased analysis of all ions and facilitates the detection of low-abundance compounds.[121] The DDA and DIA methods enable the generation of MS1 and MS2 spectra in samples. The spectra are compared with the theoretical spectra from the protein database to identify the protein.

1.7.3.6 Targeted parallel reaction monitoring method

The targeted MS method aims to identify and quantify a limited number of predefined precursor ions m/z within a retention time window. Parallel reaction monitoring (PRM) is a novel, high-resolution targeted method that can be employed on the quadrupole-orbitrap mass spectrometer.[123,124] The PRM method allows the simultaneous scanning of all product ions of a given precursor in one MS scan. The MS2 spectra are monitored to ensure the identification and quantification of the peptide, and the areas under the curve of product ions are measured for quantification.[124] The PRM method has advantages over the traditional targeted MRM method using QqQ. First, the discovery analysis and the targeted PRM method can be operated on one orbitrap mass spectrometer. Therefore, converting the discovery DDA method to a targeted PRM method is relatively easy. In addition, the discovery results can contribute to building a spectral library for targeted MS analysis. This library provides reference spectra for comparing experimental spectra obtained from the targeted analysis. Second, compared to the MRM method, there is no need to predefine transitions of the target peptides.[124]

Only the m/z values of the precursor ions (e.g., peptides) are required for the PRM method. Third, the PRM is highly specific due to the high-accuracy measurement of fragment ions and is particularly desirable for detecting low-abundance peptides to avoid false positives.[124] This is because numerous product ions could serve as identifiers of the presence of the peptide in the sample. Last, since the PRM method examines all possible product ions simultaneously, it became easier to identify and rule out those ions that are affected by interferences in the sample.[124] These advantages allow the development of a specific, accurate, and reliable PRM method, especially in a complex matrix.

1.8 The development of a targeted MS method for egg analysis

In recent decades, there has been increasing attention to applying the MS-based method for food allergen analysis. In addition to basic MS-specific factors, the complexity of food matrices and the effects of food processing must be considered to develop an MS-based food allergen detection method.[125] This section discusses the considerations for selecting a target peptide and the quantification strategies for developing a food allergen MS method.

1.8.1 Selection of the target peptides

The selection of target peptides is the first and critical step in MS method development. The target peptides for quantification, named quantotypic peptides, must be unique to the protein of interest and reproducible in MS analysis. The abundance of the peptide should be correlated to the parent protein.[124] In general, to identify suitable target peptides, a set of parameters must be considered, including peptide length, miscleavage, post-translational modification, precursor charge, chromatographic peak, and signal intensity.[124]

Instrumental, in silico (computational), and discovery-driven approaches can be used to select target peptides.[126,127] The instrumental approach is achieved by analyzing food allergens on high-resolution mass spectrometry (HRMS), such as the time-of-flight mass analyzer. This approach matches observed MS2 spectra against theoretical spectra from the protein database using bioinformatics software (e.g., Mascot, Sequest).[126,127] Several studies have successfully identified target peptides for tree nuts[128], peanuts (raw and roasted)[129], and soy[130] using the time-of-flight analyzer. In contrast, the in silico approach relies on protein sequence databases to select target peptides. Computational algorithms predict the possible digested peptides and identify target peptides and transitions based on predefined criteria, such as excluding peptides containing methionine residues.[124,126] This method works well for food allergens with a complete proteome database, such as milk and eggs. However, it is less effective for food allergens with incomplete protein databases (e.g., tree nuts) or those with numerous related species (e.g., fish).[125]

The discovery-driven approach selects target peptide based on the empirical evidence. This approach has been used in allergen method development, where the selection of target peptides is determined by their performance in purified protein(s), protein fractions, or allergenic food sources.[125] In the discovery-based approach, a set of criteria based on specificity and robustness is important to select optimal target peptides.[131–133] In the development of a method to detect egg, most MS methods identify target peptides from the discovery analysis of either whole egg powder[134], egg white powder[135], or purified egg protein(s)[136–138] (e.g., ovalbumin and lysozyme). For example, analyzing by a linear ion trap mass spectrometer with DDA, Monaci et al.[135] used whole

egg powder as a reference sample to determine the target peptide. Instead of identifying the peptides from any of the proteins from soy, egg, and milk, the authors focused on a few allergenic proteins (12 soy proteins, two egg proteins, and two milk proteins). As a result, two peptides specific to ovalbumin for the detection of eggs were identified as target peptides in the study. However, the target peptides originating from one white protein may not be reliable for detecting eggs in various processed foods, such as food products that only contain egg yolk.

The complexity of the food matrix and the effects of food processing can reduce the extractability and digestion efficiency of proteins.[7] Therefore, the detection of the target peptides must be validated in egg-incurred food matrices.[125,139] Validation is essential to ensure accurate detection of the peptides in the tested food matrices in regard to potential interferences or variations caused by the food matrix. The egg-incurred food matrix is produced by incorporating eggs before food processing. This allows egg proteins to undergo both physical and chemical interactions with other ingredients/proteins during food processing. In addition, using the incurred sample to validate the detection of target peptides considered the variables and interferences caused during sample preparation for MS analysis. The processed egg proteins had reduced protein extractability compared to the native egg proteins, as seen in Fu et al.'s study[99].

Notably, Planque et al.[140] used in silico and discovery-based approaches to identify target peptides for milk, egg, soy, and peanut. First, the authors narrowed down the number of proteins for each food allergen. Then, they used the in silico approach to select the peptides and transitions for the proteins of interest to build an MRM method. These predefined peptides were further analyzed in the raw food allergen and baked

cookies incurred with 5000 ppm protein sequentially. This approach yields eight peptides from four egg proteins to develop the MS method to detect eggs in cookies.

Gavage et al.[132] and Pilolli et al.[141] identified target peptides directly by analyzing egg-incurred food matrices. Gavage et al.[132] developed a data-driven approach to select target peptides across various food matrix models, including raw and heated egg powder, high-fat matrix (i.e., chocolate and mayonnaise), and low-pH matrix (i.e., mayonnaise with vinegar). Similarly, Pilolli et al.[141] successfully used the discovery-based approach to identify egg target peptides in incurred chocolate and broth powder. However, these tested food matrices underwent a moderated thermal process (≤ 100°C), which may not reflect the worst-case scenario. Studies have demonstrated a massive reduction in the detection of egg proteins after thermal processing above 175°C.[99,102] The tempering (46°C) used to make chocolate has little effect on egg protein detection using ELISA methods.[100] Moreover, baked goods and cookies, which are processed at a high temperature, are the primary offenders found in undeclared eggs.[5,65,82] Therefore, it is rational to identify target peptides in highly processed food matrices, such as cookies or retorted soup.

1.8.2 Quantification of egg in foods

As mentioned, an MS method provides high specificity for detecting the food allergen by pairing the target precursor and their product ions. Additionally, the indispensable advantage of an MS-based method is its ability to provide absolute quantification of allergens in processed foods. Reliable and accurate quantification of allergens in finished food products is fundamental to implementing risk-based allergen

labeling. This is a crucial factor in ensuring food safety and effective allergen management.

Stable isotope labeling is widely used in MS-based methods for quantifying allergens in food.[142] Stable isotope labeling can be applied to peptides, proteins, and concatemers (i.e., a chimeric protein consists of all labeled peptides).[126] This technique alters the isotopes (i.e., an atom) within amino acids or amino acid groups to create a small mass difference.[143] The stable isotope-labeled peptides (heavy) and endogenous peptides (light) exhibit similar physical and chemical characteristics, allowing them to behave nearly identically during the MS analysis.[126] This mass difference is crucial for distinguishing between the two.

This similarity between heavy and light peptides is beneficial for quantitative MS methods.[126,144] First, the similar proportion of the product ions and co-elution at the same retention time ensures the specificity of the target peptide. Second, the intensity of the labeled peptides directly correlates with that of the light peptide. This correlation can build a calibration curve by comparing the signal from light peptides to the signal from the labeled peptide (light-to-heavy ratio) with known concentrations. Third, using heavy peptides as internal standards (IS) can potentially correct for the variations or losses that occur during the sample preparation and MS analysis. For example, the constant amount of the IS was added to the sample before MS analysis and after sample resuspension. Therefore, the peak area of the IS obtained from the samples can reflect the run-to-run variability introduced during the MS analysis. As for the heavy-labeled protein and the concatemer standards, they are often added to the sample before enzymic digestion. Comparing the measured concentration of the standard in the sample with their known

added concentration helps to correct for such losses and variability during sample handling and the MS analysis. The percentage loss of standards can then be extended to correct the measured concentration of the light peptides. However, the use of stable isotope-labeled proteins and concatemer standards are more expensive than stable isotope-labeled peptides, which makes them difficult to promote in routine application in allergen detection.[126]

Eventually, the allergen detection method should be able to be routinely used by the food industry or regulatory authorities. In ELISA methods, the range of quantification concentrations, the interpretation from the calibration curve, and the reporting units are all described in detail in the instruction manual. This detailed information allows end users, such as the food industry or regulatory authorities, to easily implement and interpret the ELISA methods. When implementing an MS method for allergen quantification, there are three main considerations, including 1) the definition of a positive detection, 2) quantification using calibration, and 3) the determination of the reported results.[144] These considerations ensure that the MS methods provide clear and understandable information from the quantitative analysis.

A positive detection of peptides should be determined based on the selectivity and specificity of the obtained signal. To achieve a degree of confidence, each of the target peptides should be monitored with at least three product ions.[145,146] In addition, to ensure unbiased comparisons in the diversity of the food matrix, the positive detection criteria should be predefined, allowing consistent application across different types of food metrics. The positive detection criteria can be established by comparing the quality of the obtained signal to that of the stable-isotope-labeled internal standard.[146] In addition, a

statistical-based approach, such as the probability of detection, can be employed to further validate and ensure the accuracy of the detection limits.[147]

Calibration is a crucial step in accurately quantifying target peptides, especially in allergen quantification using stable isotope-labeled peptides. It establishes a relationship between the obtained signal and the quantity of the peptide present. Internal calibration and external calibration with ISs can be used in the quantitative allergen MS methods.[144] Using internal calibration, the target peptide in the sample is quantified by interpreting a calibration curve generated from known molar quantities of the IS (e.g., heavy peptides).[148] The measured molar amount of the peptide is then converted into the amount of target protein based on the molecular weight. When interpreting the amount of total protein in the allergenic food source, external conversions are required. These conversions are based on assumptions regarding the proportion of target protein in the whole ingredients and the proportion of the allergic ingredient in the food product. External calibration with ISs involves using an allergenic food product or the incurred food matrix as a calibrant.[144] The fixed amount of IS is added to the calibrant and the unknown samples. The target peptide measurement is determined by calculating the light-to-heavy ratio, which can be directly interpreted into the calibrant concentrations.

Unlike ELISA methods, MS-based methods quantify each target peptide individually, which can provide multiple interpretations of the protein content in the samples. However, food allergen risk assessment typically requires a single reported concentration of the total protein from allergenic food sources.[144] While the MS-based methods can provide quantification of individual peptides, it is essential to integrate these signals into a single measurement representing the total protein content in a food sample.

Johnson and Downs[144] describe the integration of multiple peptide signals using either the sum of the multiple peptides or a single fixed quantifier peptide for interpreting percentage recovery in three theoretical scenarios. They also note that these two integration approaches can potentially underestimate the percentage recovery from the samples depending on different food matrices. Chen et al.[149] describe the two reporting approaches averaging among all monitoring peptides and the selected maximum measurement from any of the monitoring peptides. They found that these two reporting methods obtained a similar percentage of recovery from the soy MS detection method.

In the following sections, a couple of the MS methods for quantifying eggs in food will be reviewed, focusing on the detection criteria, quantification strategies, and reporting approaches.

In the study of Pilolli et al.,[150] the authors published a quantitative MRM method for six food allergens (milk, egg, peanut, soybean, hazelnut, and almond) in the chocolate matrix. This study didn't specify positive detection criteria but confirmed no interferences from the blank chocolate matrix. They employed a matrix-matched external calibration method incorporating ISs to determine the concentration of the target peptide. The external calibration curve was created by adding known concentrations (in fmol/μL) of synthetic light target peptides to a blank chocolate digest. A fixed amount of heavy-labeled target peptide mixture was introduced to all calibration standards and samples. The concentration was calculated from the ratio of light-to-heavy peak areas. To quantify the total allergenic food protein, a two-step conversion process was used: converting fmol of the peptide to mg of the original protein, then to mg of total allergenic food protein in chocolate. For egg quantification, one target peptide originating from each egg white and

yolk protein were used as quantifiers, interpreting the total egg protein from a single peptide. This method achieves a limit of quantification (LOQ) of 0.8 mg of total protein, with recovery rates of 100% for the egg white peptide and 97% for the egg yolk peptide in chocolate.

Other studies[151–153] also used matrix-matched calibration curves with heavy-labeled internal standards for quantifying multiple food allergens (including eggs) in food matrices (cereal bars, muffins, cookies, bread, salad dressing, and red wine). Some methods[151,152] consistently achieved over > 60% recovery of egg protein in the tested incurred food matrices. In Parker et al.'s study[151], the authors outlined the positive detection criteria involving the retention time alignment, product ions ratio, and peak points assessment. They found no significant differences in protein recoveries between the maximum (the maximum concentration obtained from any of three egg peptides) and the average reported value (the mean concentration from the egg peptides), which have no significant differences when applying their method to muffins and cereal bars.

Planque et al.[154] published an MRM method for detecting milk, egg, peanut, and soy in foods. In this study, the authors outlined a set of positive detection criteria involving retention time alignment, product ions ratios, and the signal-to-noise ratio. This method achieves the LOQ for egg protein at 3 – 15 ppm for egg white peptides and 60 ppm for egg yolk peptides. As for the calibration strategy, the authors assessed three approaches involving 1) using stable isotope labeled-target peptides as internal standard (short IS), 2) using stable isotope labeled-concatemers as internal standard (long IS, chimeric protein containing two target peptides), and 3) using standard addition with either of IS (i.e., a desired amount of target allergen was added to the calibration curve).

These three calibration approaches were employed to calculate the concentrations of milk, egg, soy, and peanut spiked in eight food products. Both short and long IS were added before the protein extraction step, which was used to correct the variability introduced during sample preparation to MS analysis. The author found that corrections by the IS, regardless of the type, improved the precision of the quantification. With the addition of the target allergen in the calibrant coupled with ISs, this approach efficiently reduces the standard deviation of the protein recovery, particularly at the LOQ. Using the standard addition and ISs to the calibration, the protein recovery of total egg protein was between 60 – 120% in most spiked food products.

1.9 Conclusions and future directions

This literature review demonstrated the need to develop a reliable and accurate analytical method for quantifying eggs in processed food. MS-based methods show advantages over current allergen detection methods (e.g., ELISA) due to their high specificity, sensitivity, and robustness.

The current egg MS methods have shown effective detection and quantification of egg in various incurred food matrices (e.g., chocolate, muffins, cereal bars, cookies).[150–152] However, they may have a few limitations. First, few studies have used discovery-based approaches to identify egg-specific target peptides in cookies or other bakery products. Including bakery goods in selecting the target peptides is crucial because this food category is the major offender that causes undeclared eggs.[82] Second, most egg detection methods are designed to simultaneously detect multiple food allergens, known as a multi-allergen detection method. The multiplex MS method typically restricts the number of egg peptides (usually 2 – 5 peptides), thereby limiting the detection of one to

three egg proteins from samples.[150–152,154] Egg is a versatile ingredient and can be used as whole, white, yolk, or egg isolate (lysozyme in winemaking[155]) in food products. Therefore, an MS method that monitors peptides originating from proteins found in these fractions/isolates of egg is preferred. Monitoring egg peptides from multiple egg proteins may enhance the application of the egg MS method for detecting egg in a wide array of food products. Last, matrix-match calibration in these egg MS methods may not be suitable for routine application. For example, it is challenging to find a blank food matrix to prepare the calibration curve with unknown food matrices.

Taken together, to address these limitations, we proposed using a discovery-based approach to select target peptides in various incurred food matrices and developing an egg-specific PRM method for quantifying total egg protein in processed foods.

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CHAPTER 2

EGG-SPECIFIC TARGET PEPTIDE IDENTIFICATION FROM PROCESSED FOOD MATRICES

2.1 Abstract

Egg is recognized as a primary allergenic ingredient that affects approximately 1 in 100 children globally. Current egg detection methods that are routinely used in food industries are intact protein-based. However, structural changes occur in egg proteins during food processing that can hinder the accuracy of currently used methods. The limitations of the current method highlight the urgent need for reliable and sensitive egg detection methods. Mass spectrometry (MS) based methods can be an alternative egg detection method in processed foods because the MS methods use peptides as analytes that are less prone to be influenced by the food matrix effects. Therefore, this study aimed to use a data-driven and discovery-based approach to identify egg-specific target peptides, which is the first and most critical step in developing an MS-based method. Considering the complexity of food matrix effects, four processed food matrices (cookies, pie crust, pasta, and ice cream) that were incurred with whole egg powder (WEP) were used for the target peptide identification. First, a discovery-based approach was applied to select egg-specific candidate peptides for each food matrix, resulting in 88 candidate target peptides. Second, these candidate target peptides were screened using the targeted MS method. A set of criteria was developed to evaluate peptides' performances and to identify the target peptides from each WEP-incurred food matrix. As a result, we identified 11 egg-specific and robust peptides originating from five egg proteins across the WEP-incurred food matrices. These 11 target peptides can develop a reliable targeted

MS method for the detection and quantification of egg proteins in four processed food matrices. The discovery-based approach is capable of being applied across different food matrices and can be easily adapted for the development of MS-based methods for other food allergens.

2.2 Introduction

Chicken (*Gallus gallus*) eggs are recognized as one of the top eight priority food allergens by FAO/WHO.[1] Egg allergy is commonly known as a childhood food allergy, with a prevalence of approximately 0.9% among children in the U.S.[2] Even though most young children outgrow egg allergy with age[2], over 2 million U.S. adults suffer from egg allergies.[3] The symptoms of egg allergy can be mild to life-threatening. Currently, no proven treatment for egg allergies is available. Therefore, the primary approach to managing egg allergies is avoiding egg and egg-derived ingredients in the diet.[4] To practice food avoidance in daily life, individuals with egg allergies and their caregivers rely on knowing the allergenic ingredients in the food products by carefully reading the allergen statements on the packages.

Unfortunately, over the past decade, undeclared food allergens have been a primary reason for food recalls, and these issues continue to diminish the quality of life of individuals with food allergies.[5,6] To protect individuals with food allergies from undeclared allergens, the Food Allergen Labeling and Consumer Protection Act of 2024 (FALCPA) and the Food Safety Modernization Act (FSMA) regulate the intentional and unintentional introduction of major food allergens into food products during manufacturing, respectively.[7,8] The food industry is required to label all the major food allergens that were intentionally introduced as ingredients with a clear and

understandable allergen statement on the packaged foods.[7] Additionally, good manufacturing practices, such as proper cleaning and hygiene training, are required to prevent unintentional allergenic ingredient cross-contact during manufacturing.[8] However, egg is a versatile ingredient used in many food products and can be unintentionally introduced into no-egg food products produced on shared processing lines or utensils. A Canadian survey has revealed that about 7% of the tested food products that have no eggs on the ingredient list but with precautionary allergen statements or no statements contain detectable egg with a broad range of concentrations.[9] The quantification of low levels of eggs in processed foods is a critical challenge for the food industry.

In the food industry, the most widely used methods to determine the presence of allergenic ingredients in foods are the immunoassay methods, such as enzyme-linked immunosorbent assay (ELISA) and lateral flow devices. The immunoassay methods rely on antibody-antigen recognition and work well in most cases but show challenges in processed foods. Food processing treatments and the complexity of food matrices can hinder the detection accuracy of these methods due to the modification or masking of target protein epitopes. For example, studies have shown that egg ELISA kits lose sensitivity after baking[10], and different ELISA kits give inconsistent results[11]. The limitations of ELISA demonstrate the urgent need for complementary and alternative analytical methods to identify and quantify egg in processed foods. Mass spectrometry (MS)-based methods have potential advantages over current allergen detection. MS-based methods may be less prone to the influence of the food matrix effect because the methods use peptides instead of intact proteins. In addition, the detection of peptide analytes,

which are the surrogate of protein, increases the specificity and reduces the chance of false detection. Moreover, MS methods can use rigorous extraction buffers to increase the solubility of the protein from processed foods. MS methods promise to serve as robust and sensitive methods for food allergen analysis of processed foods.

Identifying target peptides as protein surrogates is the first and critical step for developing a targeted MS method. Despite the benefits of MS-based methods, they traditionally identify target peptides only from selected proteins of interest *in silico*.[12] Most current mass spectrometry (MS)-based egg detection methods identify target peptides depending upon the MS analysis of either egg white powder[13], egg powder[14], or purified egg protein (i.e., ovalbumin and lysozyme)[15–17]. However, the effect of food matrices can reduce protein extractability, which can further influence the detectability of the analytical method.[18] Therefore, the selection of the target peptides must take into account various food matrix effects.

This study aimed to utilize a discovery-based and data-driven strategy to identify target peptides for a targeted MS method for detecting and quantifying total egg proteins in various processed foods. Specifically, four common processed foods were chosen as the matrix models, including sugar cookies, pie crust, dry pasta, and ice cream, to reflect the different processing treatments, such as heat vs. no heat treatments and wheat or milk-based foods. Whole egg powder (WEP) was incurred to make food matrices before food processing treatments. This study identified target peptides from the MS analysis of each of the four whole egg powder (WEP)-incurred food matrices, independently. A discovery-based approach and performance criteria were developed to select WEP-specific and robust peptides for the development of quantitative MS methods.

2.3 Materials and Methods

2.3.1 Preparation of whole egg powder incurred food matrices

2.3.1.1 Ingredients

Four common processed foods, including sugar cookies, pie crust, pasta, and ice cream, were selected as the food matrix models. To prepare a food matrix, a known amount of whole egg powder (WEP, Michael Food Inc. Omaha, NE) with 47% of total protein determined by Dumas analysis was incorporated before food processing. Other ingredients, including all-purpose flour (flour), granular cane sugar (sugar), butter, whole milk (milk), and heavy cream for the food matrices were purchased from local stores (Lincoln, NE, USA). All food matrices were prepared in a food-grade kitchen and Good Manufacturing Practices (GMP) pilot plant at the University of Nebraska-Lincoln. To prevent cross-contact during preparation, each batch of food matrix was individually produced from blank to WEP-incurred foods. All utensils were cleaned using hot soapy water, rinsed with distilled water, and air-dried.

Before the preparation of incurred food matrices, the ingredients were analyzed using Veratox® Egg ELISA kits (Catalog No. 8450) to verify the absence of egg (i.e., concentration lower than the limit of quantification of 2.5 ppm WEP). The ingredients were sampled and extracted at 1:25 (w/v) in triplicate using 5.0 g each and analyzed in duplicate wells following the instructions from the ELISA kit.

2.3.1.2 Preparation of concentrated WEP-spiked carrier materials

To ensure the even distribution of WEP in the food matrices, carrier material, such as flour or sugar, was used. Flour was used as the carrier material for making cookies, pie crusts, and pasta matrices. The WEP was spiked and mixed with flour to achieve the

concentration of 40,000 ppm WEP by mixing 30 g of WEP and 720 g of flour using a KitchenAid stand mixer with a 6-wire whip for 5 minutes. Sugar was used as carrier material for making ice cream. A concentrated WEP-sugar carrier material with 57,691 ppm WEP was prepared by pulsing 17.3 g of WEP and 282.7 g of sugar in a food processor for 1 minute.

The homogeneity of the carrier material was assessed using Neogen Veratox® Egg ELISA kits. The carrier material was spread on a flat surface (33 cm x 46 cm tray). Eight 1 ± 0.1 g subsamples were taken from the sides, corners, and center, followed by extraction and ELISA analysis. The homogeneity of the carrier materials was indicated by having below 25% coefficient of variation among eight subsamples (**Table 2.1**).

Table 2.1 The coefficient of variation observed with subsamples of concentrated carrier material analyzed by ELISA.

Food matrix	40,000 ppm WEP spike	10 ppm WEP-flour spike
Cookie	5.8%	9.5%
Pie crust	8.3%	5.5%
Pasta	6.3%	13.2%
Ice cream	23.4%[a]	7.1%

[a] One of the eight subsamples showed extreme results (outliner). When the outliner was excluded from the calculation, the percent coefficient of variation among seven subsamples was 19.30%CV with a recovery of 107.08%.

2.3.1.3 Preparation of WEP-incurred sugar cookies

The sugar cookies incurred with no WEP (blank cookies) and 10,000 ppm WEP were prepared following the American Association of Cereal Chemists (AACC) International Method 10-50.05 (**Table 2.2**).[19] To make 10,000 ppm WEP-incurred

cookies, a portion of the blank flour (500 g) was replaced with the WEP-flour carrier material (40,000 ppm WEP in flour) to achieve a final concentration of 10,000 ppm WEP in cookie dough. Flour, salt, and baking soda were mixed in a KitchenAid with a 6-wire whip for 5 minutes, stopping to scrape the sides and bottom each minute. Butter, sugar, and water were blended in a Hobart mixer with a flat beater at medium speed for 4 minutes. Dry mixed ingredients were added to the wet ingredients and mixed for 4 minutes in the Hobart mixer. The dough was rolled out using a rolling pin to an even thickness of approximately 0.6 cm and cut into 2 x 2 cm squares using a square cookie cutter. Cookie squares were baked at 205°C for 10 minutes in the Reed Revolving Reel Oven and weighed before and after baking to calculate water loss.

Table 2.2 Formulation of cookie.

Ingredients	Weight (g)
All-purpose wheat flour	952.18
Butter	270.84
Sugar	550.15
Salt	8.89
Sodium bicarbonate	10.58
Dextrose solution (5.9%, w/v)	139.65
Water	67.71
Total	2000

The formulation of the cookie was adapted from AACC International Method 10-50.05.[19]

2.3.1.4 Preparation of WEP-incurred pie crust

The formulation for pie crust incurred with no WEP and 10,000 ppm WEP was modified from the recipe used in previous work (**Table 2.3**).[20] The WEP-incurred pie crust was prepared by replacing the blank flour with 500 g of WEP-flour carrier material with 40,000 ppm WEP. Flour and salt were mixed in a KitchenAid mixer with a 6-wire whip for 5 minutes, stopping each minute to scrape the sides and bottom. The remaining dry ingredients were added to the butter and mixed in a Hobart mixer with a flat beater for 6 minutes. Water was incorporated for 2 minutes, mixing at low speed and another 2 minutes at medium speed. The pie crust dough was shaped into a long rectangle and rested at 4°C for 2 hours. The dough was rolled out using a pasta maker (Model: B001CGMKA4 Pinzon^{TM}) at position 1 to produce a pie crust sheet with an approximately 0.3 cm thickness. The sheet was then cut into 2 x 2 cm squares using a square cookie cutter. The squares were placed in a plastic bag at -20°C overnight. Before baking, the frozen squares were placed on a baking tray lined with parchment paper to thaw. The squares were baked at 190°C for 20 minutes in the Reed Revolving Reel Oven, with the tray rotated mode for 10 minutes. The water loss was calculated from the weight of the pie crust before and after baking.

Table 2.3 Formulation of pie crust

Ingredients	Weight (g)
All-purpose wheat flour	1142
Butter	390
Salt	30
Water	438
Total	2000

The formulation of the pie crust was adapted from the recipe used in previous work.[20]

2.3.1.5 Preparation of WEP-incurred pasta

Dry pasta (pasta) was prepared using method 66-41.01 from AACC International, including 1200 g wheat flour and 544 g water (**Table 2.4**).[21] The dry pasta with 10,000 ppm WEP was made by mixing 300 g of the flour carrier material with 40,000 ppm WEP and 900 g of blank flour in a KitchenAid mixer. To ensure even distribution of WEP in flour, the ingredients were mixed with a 6-wire whip for 5 minutes, stopping each minute to scrape the sides and bottom. The mixture of WEP and flour was transferred into a Hobart mixer. Then, water was cooperated and mixed at low speed for 2 minutes, followed by kneading at medium speed for 4 minutes. The pasta dough was wrapped in plastic and rested overnight at 4°C. The dough was rolled and flattened using a pasta maker on position 7 (approximately 0.15 cm thickness). The pasta sheet was cut into 20 cm fettuccine using a cutter attachment. Blank flour was spread on the pasta sheet to prevent pasta from sticking together on the cutter. Before drying, the pasta was arranged on perforated sheet pans to provide air circulation and evenly dry the pasta. The pasta was then dried in an environmental chamber (Model: I-36VL Percival Scientific) using a two-stage drying cycle beginning at 70°C for 10 hours and then at 40°C for 12 hours in a constant relative humidity of 65%. The pasta was weighed before and after drying to calculate water loss.

Table 2.4 Formulation of pasta

Ingredients	Weight (g)
All-purpose wheat flour	1200
Water	544
Total	1744

The formulation of the pasta was adapted from AACC International Method 66-41.01.[21]

2.3.1.6 Preparation of WEP-incurred vanilla ice cream

Vanilla iced cream was prepared following the instruction manual from the Ice Cream Maker (ICE-21 Cuisinart®)(**Table 2.5**) using the concentrated WEP-sugar carrier material with 57,691 ppm WEP used to make incurred ice cream with 10,000 ppm WEP. Before preparing the ice cream, the freezer bowl of the ice cream maker was frozen at -20°C overnight. Whole milk, sugar, and salt were blended in a KitchenAid mixer with a 6-wire whip at low speed for 5 minutes, and the sides and bottom were scraped each minute. Heavy whipping cream and vanilla were incorporated and mixed for one minute. The mixture was cooled at 4°C for two hours before operating in the ice maker for 20 minutes. The finished ice cream was dispensed into 50 mL tubes and stored in plastic bags at -20°C.

Table 2.5 Formulation of ice cream

Ingredients	Weight (g)
Heavy cream	462
Whole milk	240
Sugar	150
Pure vanilla extract	13
Salt	0.36
Total	865.36

The formulation of the ice cream is adapted from the instruction manual from the Ice Cream Maker (ICE-21 Cuisinart®).

2.3.1.7 Sample homogenization for cookies, pie crust, and pasta

Approximately 100 g of baked cookies and pie crust were ground into a fine powder using a food processor. Dried pasta was first broken into smaller pieces and then ground into a fine powder by operating a coffee grinder. All ground processed foods were carefully dispensed into 50 mL Falcon tubes. As well as the unprocessed dough of cookies, pie crust, and pasta after cutting into smaller portions. All four food matrices were stored at -20°C until the sample preparation for MS analysis.

2.3.2 Sample preparation for proteomics analysis

2.3.2.1 Reagents and chemicals for MS analysis

Urea was purchased from Invitrogen (Carlsbad, CA). Dithiothreitol (DTT) was purchased from Acros Organics. Thiourea, Trizma hydrochloride (Tri-HCl), ammonium bicarbonate (ABC), and iodoacetamide (IAA) were purchased from Sigma-Aldrich. MS-grade trypsin, water, methanol, acetonitrile (ACN), and formic acid (FA) were purchased from Thermo-Fisher Scientific.

2.3.2.2 Protein extraction

Proteins from WEP (0.04 g), as well as unprocessed doughs for cookies, pie crust, and pasta, finished foods for all food matrices (1.0 g) were extracted in 20 mL of protein extraction buffer (1:20 w/v, 2 mol/L Thiourea, 6 mol/L Urea, 50 mmol/L Tris-HCL at pH 8.9, and 20 mmol/L DTT) in triplicate. The protein extraction consisted of incubation in a 60°C shaking water bath for 10 minutes, resuspension by vortex for 1 minute, sonication at room temperature for 10 minutes, and repeated incubation at 60°C for 10 minutes. Subsequently, two sequential centrifugations were performed after the incubation. First, two 1000 µL of the sample extract were collected after the centrifugation at 3000 g for 10 minutes at 4°C. Second, two 700 µL of the previous supernatant were centrifugated at 17,000 g for 10 minutes at room temperature. Afterward, the supernatants were pooled together as final sample extracts. Particularly, the WEP protein extract (40,000 ppm WEP) was then diluted with protein extraction buffer to make the concentration 0.5 mg WEP/mL, equivalent to the WEP concentration in the WEP-incurred food sample extract (10,000 ppm WEP in 1 g of food sample). The soluble protein content in the sample extracts was estimated using a Cytiva 2-D Quant kit following the manufacturer's instructions.

2.3.2.3 Enzymatic digestion and desalting

The methodology of enzymatic digestion and desalting was designed according to the theoretical protein content of finished foods and the maximum peptide binding capacity of the Pierce® C18 spin columns (≤ 30 µg of total peptide, catalog no. 89870). Sample extracts were subjected to in-solution tryptic digestion. Before digestion, 12 µL of the extract was reduced at 95°C for 5 minutes with the addition of 4.5 µL of 100 mM

DTT, 45 µL of 50 mM of ABC, and 19.5 µL of MS-grade water. The reduced protein was then alkylated with 9 µL of 50 mmol/L IAA and incubated in the dark at room temperature for 20 minutes. Afterward, the alkylated protein was digested with 3 µL of trypsin solution (100 ng/µL) at 37°C for two hours and an additional 3 µL of trypsin solution at 30°C for overnight (at least 14 hours). The completion of digestion was confirmed by the side-by-side comparison of digested and undigested samples (sample extracts) at equal amounts of protein content using SDS-PAGE.

The resultant peptides were cleaned up with C18 spin columns following the manufacturer's instructions with one modification, where the sample peptides were eluted in 40 µL of elution buffer 55% (v/v) ACN in water and frozen at - 80°C for 30 minutes. Lastly, the frozen elution was dried in a SpeedVac vacuum evaporator (Model: SPD120). The lyophilized peptides were stored at - 20°C until further analysis. The resulting peptides were resuspended with 40 µL of 5% (v/v) ACN and 0.1% (v/v) FA in MS-grade water. The MassPREP^{TM} enolase digestion standards (UniProt P00924) were used as internal standards in untargeted MS analysis. Each sample was made of 36 µL of the resuspended peptides and 5 µL of 100 fmol/µL enolase standard peptides.

2.3.3 Discovery-based proteomics analysis

2.3.3.1 Parameters for ultra-high performance liquid chromatography

Separation of peptides was performed on a Thermo Fisher Dionex UltiMate 3000 RS ultra-high performance liquid chromatography (UHPLC) system with 4 µL of injection volume at 0.06 mL/min flow rate. The temperature-controlled autosampler was kept at 5°C, and the column compartment was kept at 35°C. The column was a Hypersil GOLD C18 Selectivity LC Column (100 mm × 1 mm, 1.9 µm, 175 Å). A UHPLC filter

cartridge (1 mm ID, 0.2 μm) was attached to the column. The mobile phase consisted of solvent A: 0.1% (v/v) FA in water, solvent B: 0.1% (v/v) FA in ACN, and solvent D: 100% (v/v) methanol. The following gradient program was conducted: 0 – 3 min: 2% solvent B; 3 – 70.05 min: 2 – 40% solvent B; 70.05 – 76 min: 40% solvent B; 76.01 – 81.01 min: 98% solvent B. After sample analysis, the column was washed with 100% methanol for 6 minutes at 0.15 mL/min before re-equilibration to the initial condition with 2% solvent B for 28 minutes. The total run time was 115 minutes.

2.3.3.2 Untargeted mass spectrometry method

Detection of peptides was performed on a Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer operated with the data-dependent acquisition (DDA) in positive ionization mode. Source parameters were set as follows: sheath gas flow rate of 15 arbitrary units (au), sweep gas flow rate of 1 au, positive ion spray voltage of 4000 V, heated electrospray ionization (HESI) capillary temperature of 320 °C, S-lens RF of 60 au. The top 20 abundant peptides were selected for fragmentation with a 20-second dynamic exclusion window. The normalized collision energy was 27. For the MS1 scan, the parameters were as follows: a resolution of 70,000, a scan range of 200 to 2000 m/z, Automatic Gain Control (AGC) of 3×10^6, and a maximum Injection Time (IT) of 100 milliseconds. The parameters of the MS2 for DDA were as follows: a resolution of 70,000, a scan range of 200 mass-to-charge ratio (m/z) to 2000 m/z, AGC of 1×10^5, and a maximum IT of 240 milliseconds. The intensity threshold was set at 6.3×10^4. The mass isolation width for the precursor of interest was 2.0 m/z with a 0.4 m/z offset.

2.3.3.3 Targeted mass spectrometry method

Following the selection of candidate peptides, a targeted MS method was employed to screen all candidate peptides with scheduled inclusion lists. The preliminary DDA data provided information about selected peptides, such as precursor ion m/z, charge state, and isolation window, to create scheduled inclusion lists for parallel reaction monitoring (PRM) acquisition. Initially, candidate peptides that were pre-determined by the discovery-based approach needed to be screened using the targeted MS method. The candidate peptides were sorted according to their retention time and were separated into three inclusion lists to ensure the concurrent targets were limited to 10 peptides. The targeted MS using PRM in positive ionization mode was conducted on the same instrument with the same source and MS2 parameters.

2.3.4 Software data processing for peptide identification and label-free quantification

DDA results were analyzed using the commercial software PEAKS Studio version 8.5 (Bioinformatics Solutions Inc®). The identification of peptides was conducted by searching against a combined database from chicken (*Gallus gallus*; proteome ID UP000000539) and wheat (*Triticum aestivum*; proteome ID UP000019116) or bovine (*Bos taurus*; proteome ID: UP000009136) UniProt proteome databases, associating with the food matrices. Global Proteome Machine common contaminant database was included after excluding those entries for chicken proteins. Mass error tolerance for precursors was up to 10.0 ppm; for fragment ion, 0.05 Da was tolerated. Trypsin was selected as the protease with the allowance of non-specific cleavage at both ends of the peptide, with a maximum of 2 missed cleavages per peptide. Carbamidomethylation of

cysteine was set as fixed post-translation modification (PTM). In addition, oxidation of methionine and carbamylation of N-termini were selected as variable PTMs. The false discovery rate was 1.0% for both protein and peptide levels.

After peptide identification, the data proceeded to label-free quantification (LFQ) using PEAKS Studio version 8.5. Each sample, with triplicate injections, was arranged into one group for LFQ comparison. The mass shift between different runs matched up to 5 ppm, and the retention shift between the runs within the 6-minute range was tolerated. The internal standards (enolase standard proteins) were used to normalize the intensity of peptides across samples. In the peptide filters, parameters were set: feature quality and average intensity ≥ 0, peptide ID count ≥ 1, charge between 1 and 10, and having ≥ 1 confident sample. The parameters for protein filters were set with fold change ≥ 1 and unique peptides ≥ 1.

2.3.5 Data processing for target peptide refinement

Skyline Software (University of Washinton, MacCoss Lab) was used to evaluate peptides' performances. To build the spectral library, the DDA results from PEAKS were exported and imported to Skyline to match the desired metrics with the four product ions, including the m/z of the product ions greater than the m/z of its precursor and the most intense ions from the spectral library.

2.4 Results and Discussion

2.4.1 Preparation of WEP-incurred food matrices

Four popular processed egg-containing foods (cookies, pie crust, pasta, and ice cream) with different matrix bases (wheat or milk) and food processing conditions (thermal or nonthermal) were selected as food matrices with a known amount of WEP. To

examine the protein extraction efficiency, we used the 2-D quant kits. The 21 – 40% extraction efficiency for wheat-based food matrices was lower than the 94% extraction efficiency of the ice cream and 72% extraction efficiency of WEP (**Table 2.6**). These results indicate that wheat protein in cookies, pie crust, and pasta may have reduced the extractability of the total proteins into the protein extraction buffer. The low extraction efficiency of cookies, pie crust, and pasta matrices could be explained by the use of all-purpose wheat flour, containing 8 – 11% gluten, in the preparation of food matrices, which are insoluble in the aqueous phase extraction buffer.[22] Moreover, heat processing can cause insoluble protein aggregation.[23]

Water loss after baking or drying varied from matrix to matrix. The weight differences between the unprocessed dough and processed food were measured to calculate the water loss of cookies, pie crust, and pasta (**Table 2.6**). The loss of water reduced the total weight of the processed food and changed the concentration of WEP (mg WEP/kg food) in cookies, pie crust, and pasta. If proteins were extracted from a constant sample size (i.e., 1 g) across the unprocessed dough and processed foods samples, the amount of egg protein extracted and analyzed by the MS method could vary considerably. In the later candidate peptide selection, label-free quantification (LFQ), a semi-quantification method, was used to compare the relative abundance of peptides across samples with different experimental conditions.[24] A peptide with relatively high abundance is a key indication for the inclusion as a candidate peptide. Therefore, the sample size was adjusted according to the calculated water loss to allow 0.5 mg WEP/mL to be extracted for discovery-based MS analysis (**Table 2.6**). By doing so, the abundance of the egg peptides reflects the detectability in different food matrices. The relative

abundance of peptides can be compared across the three sample categories and four food matrices.

Table 2.6 Extraction efficiency and adjusted sample size of extraction of four food matrices used in the study.

Food samples	Water loss (%)	Extraction efficiency[b] (mean + SD)	Calculated WEP concentrations[c] (ppm)	Adjusted sample size for extraction[d] (g)
Cookie dough	N/A	33.36 ± 0.58	10003	1.000 ± 0.01
Cookie	13.77[a]	20.78 ± 1.47	11601	0.862 ± 0.01
Pie crust dough	N/A	33.97 ± 0.29	10007	0.999 ± 0.01
Pie crust	29.8[a]	36.79 ± 0.41	14255	0.702 ± 0.01
Pasta dough	N/A	39.97 ± 0.65	6745	1.483 ± 0.01
Pasta	31.32[a]	30.35 ± 0.52	9816	1.019 ± 0.01
Ice Cream	N/A	93.53 ± 0.30	10006	0.999 ± 0.01

[a] The dough and processed samples were weighed before and after food processing (baking or drying). The difference in weight is divided by the dough to calculate the water loss.
[b] Soluble protein concentration is determined by using a Cytiva 2D-quant kit (n = 6, triplicate extracts in duplicate wells). Extraction efficiency was calculated by dividing the measured protein content by the theoretical protein content in the sample, then multiplying by 100.
[c] The WEP concentration is calculated by the amount of WEP incurred divided by the amount of total added ingredients.
[d] Adjusted sample is used to ensure an equal amount of WEP was analyzed on the mass spectrometer.
Whole egg powder: WEP
N/A: water loss is inapplicable.

2.4.2 Selection of candidate peptides using discovery-based strategy

WEP alone and four food matrices incurred with 0 or 10,000 ppm WEP were analyzed using the data-dependent acquisition (DDA-MS) method to select candidate peptides that were influenced less by food matrix effects. The relative abundance of peptides was compared across three sample categories: unprocessed dough (cookies, pie crust, and pasta), processed foods of all food matrices, and WEP alone. A developed three-step approach was then applied to select candidate peptides based on specificity and robustness from each food matrix.

First, chicken-specific peptides unique to the chicken (*Gallus gallus*) proteomic database were selected. Through the PEAKS peptide identification, there were 4525 peptides identified in the cookie, 4170 in pie crust, 6660 in pasta, and 3519 in ice cream. Those peptides were annotated with UniProt protein accession numbers in chicken and wheat or bovine proteome databases. By sorting the source of species, more than 50% of the peptides were denoted as from the wheat database, and 36% of the peptides were denoted as from the bovine database (**Figure 2.1A**). Approximately 1800 peptides were identified from the chicken database across four food matrices in wheat-based food matrices (cookies, pie crust, and pasta), while a slightly higher number of peptides (2169) were identified from the chicken database in ice cream. Those chicken-specific peptides were from approximately 70 different egg protein groups across four food matrices (**Figure 2.1B**). More chicken-specific peptides were detected in ice cream compared to the wheat-based matrices (cookies, pie crust, and pasta). The gluten from wheat flour during kneading and cooking forms a gluten network that plays a vital role in dough-making.[25] The gluten network can interact with egg proteins and reduce the extractability

of egg proteins into the extraction buffer.[26] Interestingly, the results showed that the amount of gluten in the food matrix had little impact on identifying egg proteins/peptides. Even though the theoretical amount of wheat proteins and gluten varies in cookies, pie crust, and pasta, a similar number of egg peptides and proteins were identified from the LFQ results.

Second, WEP-specific peptides were selected based on empirical evidence from the discovery analysis of the blank unprocessed dough and processed food samples. The peptides that were detected in the blank dough and food samples (0 ppm WEP) were excluded from the candidate peptide list. The chicken-specific peptides that were undetectable in any of the WEP-incurred dough and processed food samples and WEP were excluded from the candidate peptide list. After this step, approximately 18% of chicken-specific peptides from the cookie experiment had passed WEP-specific criteria and remained in the candidate peptide list, 16% in pie crust, 18% in pasta, and 48% in ice cream (**Figure 2.2**). The peptides that were detected in the blank food had a precursor m/z similar to chicken peptides. However, many of these peptides from these blank samples had no associated spectra (MS2), although spectra were determined from WEP-positive samples. The semi-quantitative LFQ assumes that low abundant precursor m/z without MS2 spectra are the same as abundant precursor m/z with MS2 spectra found in WEP-positive samples, eluting at a similar RT. Thus, false positive detection in the blank samples. The exclusion of these peptides is preferred as similar precursor m/z in the matrix could interfere with targeted MS quantitation.

Last, a third layer of the selection criteria in terms of the robustness of the peptide was employed. The abundance of the peptide can indicate its detectability, where the

peptides with the highest abundance are high-quality potential candidate target peptides. In addition, a peptide that resists the food matrix effects plays a key element in selecting candidate target peptides. Therefore, the best-performance peptides with high peak area (abundance in the top 20% of peptides for cookies and pie crust, top 50 most abundant peptides for pasta and ice cream) and comparable peak area to the WEP sample > 0.1 (the ratio of the mean abundance of peptide in WEP-incurred dough or processed food matrix to the mean abundance of the same peptide in WEP) were selected. The overlap peptides that met the robustness criteria in both the WEP-incurred dough and processed food matrix were considered as candidate peptides for the food matrix. Based on the robustness criteria, 49 peptides were selected in cookies, 43 in pie crust, 50 in pasta, and 50 in ice cream, originating from five egg proteins (ovomucoid, ovalbumin, lysozyme C, ovotransferrin, vitellogenin-2, and apovitellenin-1) (**Figure 2.2**).

The high-abundance cutoff for the top 20% and top 50 peptides varied based on the food matrix (**Figure 2.3**), where the cutoff in ice cream had a higher abundance cutoff, followed by pasta, cookie, and pie crust. The ice cream has simple process treatments (mainly mixing and freezing at -20°C) and the fewest heat-induced effects among these four food matrices. If the robustness criteria with the top 20% abundance were applied to select candidate peptides in ice cream, more than 200 peptides would have passed these criteria, which is undesirable for further target peptide refinement because it is too much. Therefore, a top-50 peptide abundance cutoff was introduced and applied for both ice cream and pasta.

High-temperature food processing (i.e., baking) can reduce relative recovery and abundance. Most of the selected candidate peptides in cookies and pie crust had a relative

recovery of less than 0.4. For pasta and ice cream with moderate heat or nonthermal food processing, all selected candidate peptides had a relative recovery of greater than 0.4. By comparing unprocessed dough and processed cookie and pie crust samples, the relative recovery of the peptide had an average of 2.6-fold and 2.2-fold reduction in cookies and pie crust, respectively. Using ovalbumin as an example, which is the most abundant protein in egg[27], the distribution of the candidate ovalbumin peptides in cookies and pie crust showed that the ovalbumin peptides in processed foods clustered in the lower left of peptides in the dough samples (**Figures 2.3A and B**). This suggested that the relative recovery and abundance of the peptides were reduced after baking. In contrast, the relative recovery and abundance of the peptides slightly increased in pasta compared to the peptides in pasta dough (**Figure 2.3C**). Compared to the intensity and the relative recovery obtained from the unprocessed dough and processed foods, the results indicated that heat treatment was the major factor in reducing the extractability of egg protein. The effect of the baking process (i.e., cookies and pie crust baked at > 100°C) reduced the extractability of egg proteins greater than the effect of the moderate heat process (i.e., pasta dried at 70°C and 40°C) and nonthermal process (i.e., ice cream). Studies have shown that the percent protein solubility of dried egg powder is below 80% at 70°C, and the percent protein solubility decreases to around 40% at 100°C.[28] Moreover, the egg white proteins interact with wheat protein to foam protein network during dough making, which decreases the solubility of the egg proteins.[29]

This discovery-based method was applied to four food matrices independently, which can narrow down the candidate peptide list. A total of 88 peptides were selected as candidate target peptides across four food matrices. Without a pre-determined protein of

interest, the identified candidate peptides originated from twelve different egg proteins, with 32 candidate peptides from ovalbumin and 25 from vitellogenin-2. Ovalbumin is the major protein in egg whites and vitellogenin-2 is the major protein in egg yolks.[30] The inclusion of both egg white peptides and egg yolk peptides is crucial because the egg is a versatile ingredient that can be used as whole egg, egg white, egg yolk, or individual egg proteins in a food product.

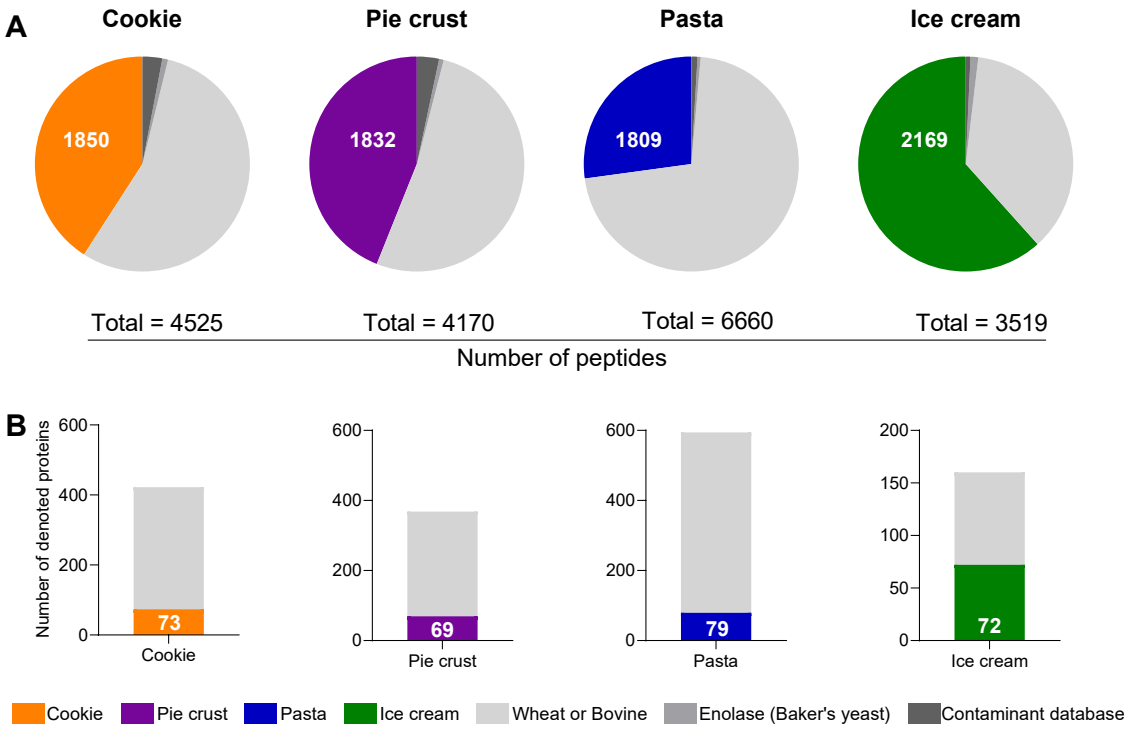

Figure 2.1 The total number of peptides (A) and proteins (B) identified in chicken (*Gallus gallus*), wheat (*Triticum aestivum*), and bovine (*Bos taurus*) UniProt databases. The color of the area represents the proportion of peptide or protein denoted by the chicken database in cookies (orange), pie crust (purple), pasta (blue), and ice cream (green). The light gray area represents the proportion of peptide or protein denoted by the wheat or bovine database. The number of peptides identified by enolase from baker's yeast (P00924) and the contaminant database were shown in medium gray and dark gray, respectively.

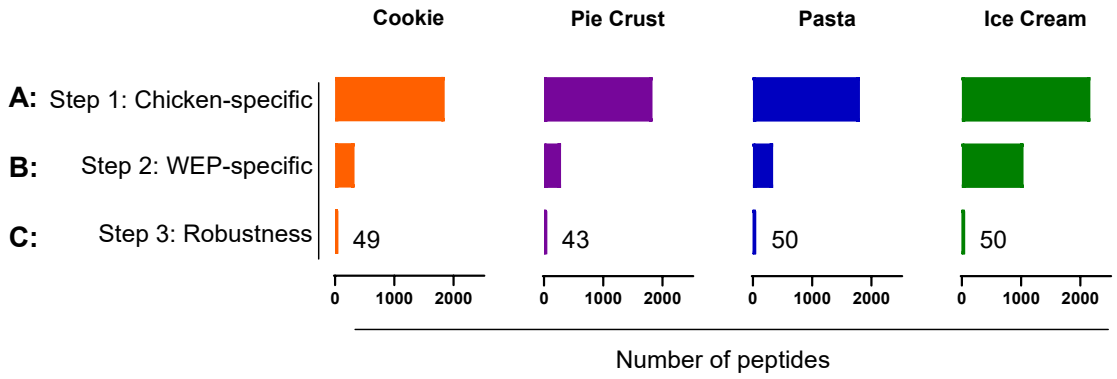

Figure 2.2 A three-step discovery-based strategy to select candidate target peptides for each of four food matrices. Step 1, Chicken-specific peptides unique to the *Gallus gallus*, proteomic database (UniProt) were selected. Step 2, Matrix insensitive peptides, passing specificity criteria and present in whole egg powder (WEP)-incurred samples and WEP were selected. Step 3, the best-performance peptides, passing the first two criteria with high intensities (peak areas in the top > 20% or top 50 peptides) and comparable intensities to the WEP sample (matrix incurred/WEP > 0.1) were selected as candidate peptides.

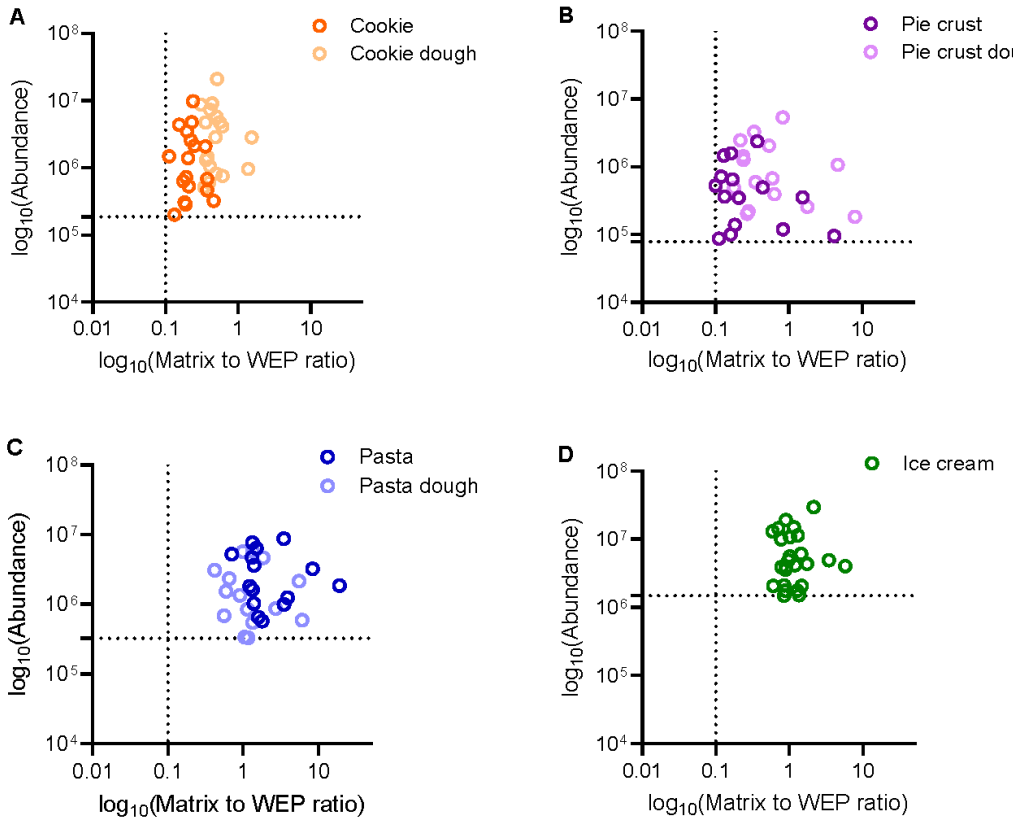

Figure 2.3 Example of the WEP-specific and robust peptides were identified as candidate target peptides in each incurred food matrix. Individual circles indicate ovalbumin peptides detected by label-free quantification. The color of the circle indicates different food matrices with cookie (A, orange), pie crust (B, purple), pasta (C, blue), and ice cream (D, green). The horizontal dotted lines indicate the peak area of the top 20% for A and B or the top 50 for C and D. The vertical dotted lines indicate a relative recovery greater than 0.1 for all four matrices.

2.4.3 Target peptide refinement

The sensitivity of a PRM-targeted MS method is highly related to the number of precursor ions monitored in the schedule window.[31] With more scans within a scheduled acquisition window, the mass spectrometer will lose the sensitivity to detect target peptides. Therefore, an iterative refinement process was conducted to shorten the target peptide list. The 88 candidate peptides from previous discovery analysis were screened from serially diluted WEP and incurred food samples using PRM-MS with scheduled windows. A set of criteria was developed to select optimal target peptides for developing a targeted MS method for detecting eggs in processed foods (**Table 2.7**).

The peptides without variable post-translation modification (PTM) (i.e., oxidation of methionine and carbamylation), ragged ends (sequential lysine and arginine at both ends), and missed cleavage sites are the primary candidate peptides for the development of the targeted MS analysis. The formation of variable PTM, ragged ends, and missed cleavage to the peptides can occur and often are unpredictable. The presence of those modified peptides can reduce the sensitivity of their tryptic peptides (**Figure 2.4**).

Table 2.7 Description of major criteria for target peptide refinement

Criteria	Description
Tryptic peptide	No missed cleavage and no post-translational modification of the peptides. No sequential K and/or R (KR, KK, RK, RR) on both ends of the peptide sequence.
Peptide length	6 – 28 amino acids.
Peak quality	The dot product value of the peptide is ≥ 0.75 in each measurement. The peptide contains at least three product ions of the peptide with a mass-to-charge ratio greater than its precursor ions.
Replicability	Detectable (dot product value ≥ 0.75) in at least two of four replicates from WEP-incurred food matrices and WEP alone at the concentration of 500 ppm WEP.
Linearity	Linear dilution curve of the peptides from various concentrations of WEP at 250, 500, 750, and 1000 ppm WEP.

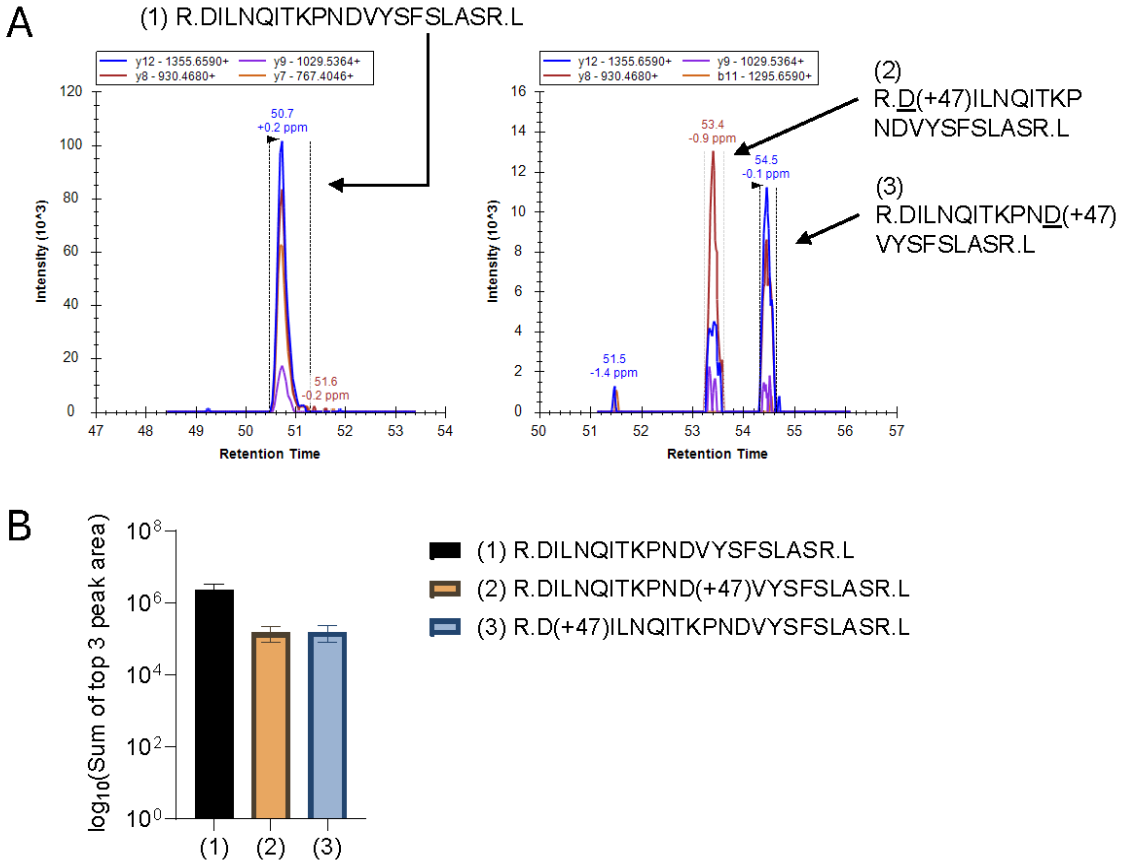

Figure 2.4 Examples of a peptide with variable post-translation modification. (A) The detection of peptide R.DILNQITKPNDVYSFSLASR.L and its two modified peptides with carbamylation of aspartic acid (an addition of 47 Da to the original formula weight) in pie crust at 5000 ppm whole egg powder. (B) Comparison of the abundance among the original and its two modified peptides.

Variable modifications of oxidation and carbamylation can occur during MS sample preparation. The sulfhydryl-containing methionine is readily oxidized to form methionine sulfoxide (+16 Da). Because the oxidation of methionine is reversible, the proportion of the unmodified peptides and modified peptides containing methionine sulfoxide is unpredictable.[32,33] Therefore, rejecting the peptide containing methionine residues is a common exclusion criterion for target peptide selection.[16,32,34,35] In this study, peptides with oxidized methionine were rejected, but not peptides with methionine residues. Interestingly, no peptides containing oxidized methionine were selected for the candidate peptides after the process of candidate peptide selection. From the DDA results, approximately 5 – 7% of chicken peptides containing methionine sulfoxide were observed across four food matrices. The peptides containing unmodified methionine residues (32 of 88 candidate peptides) were included for the further peptide refinement process. The inclusion or exclusion of the carbamylation peptides was assessed case by case. Carbamylation (+43 Da) can be formed at the N-termini of the protein/peptide and some amino acid residues (e.g., lysine, arginine, and cysteine). This formation likely occurs due to a high concentration of urea used at high temperatures during sample preparation. Among the 88 candidate peptides, 17 peptides were modified by carbamylation. Most of the peptides modified by carbamylation were selected together with their unmodified peptides in the candidate peptide list. Therefore, it was decided to reject peptides modified by carbamylation with no unmodified peptides in the candidate peptide list. For example, the modified GGL peptide (G[+43]GL) was selected as a candidate peptide from all four food matrices, whereas the unmodified GGL peptide was selected as a candidate peptide only from the cookie matrix. In this case, the G[+43]GL

peptides were excluded from the candidate peptide list for pie crust, pasta, and ice cream for further investigation. Both unmodified and modified GGL peptides were advanced to further target the peptide refinement process. Notably, the GGL peptide is the most selected target peptide by other MS methods for detecting eggs.[36] To consider the potential impact of the carbamylation, the modified G[+43]GL peptide was monitored and included in the inclusion list for the PRM-MS method.

The formation of ragged ends and missed cleavage relies upon the trypsin cleavage rule, which states that trypsin-specific cleaves are at lysine and arginine except in the presence of proline.[37] In this study, peptides with ragged ends and missed cleavage sites were removed with one exception of the K.LTEWTSSNVMEER.K peptide (LTE). The LTE peptides with sequential arginine and lysine at the C-terminus were kept for monitoring because the peptide shows potential in other studies.[14,17,38] By removing the peptide with modification, ragged ends, and missed cleavage, the candidate peptides list was shortened to 43 in a cookie, 33 in pie crust, 43 in pasta, and 36 in ice cream.

The primary quality metric from the selection criteria is the library dot product value (dotp), which Skyline generates to indicate the score matching the proportion of selected product ions to the MS2 spectral library (dotp = 1 indicates perfect matching to the spectral library).[39] Notably, the number of monitored product ions can influence the value of the dotp. Therefore, for fair comparisons across all peptides, the top four most abundant product ions with a m/z greater than the precursor m/z were monitored for each peptide. Then, we used a dotp ≥ 0.75 to determine the peptide detected in the samples. The detectability and consistency of peptides were examined from serially diluted WEP alone and WEP-incurred food samples (5000, 1000, 750, 500, and 200 ppm WEP). The

results of using the dotp values for peptide removal from the diluted WEP and food samples are shown in **Tables 2.8 – 2.11**. A peptide that was undetectable (dotp < 0.75) in more than 2 of 4 replicates was excluded from the candidate peptide list. We set the 500 ppm WEP as the standard for deciding the potential target peptide list for each food matrix.

Peptides with a linear dilution curve from the WEP and food samples, indicating the sensitivity and robustness of the peptide, are promising potential targets in a targeted MS method. Therefore, the linearity of the dilution curve from the WEP and food samples was evaluated by visual observation of the peak areas of the peptide at 250, 500, 750, and 1000 ppm WEP. An example of linear and non-linear dilution curves is shown in **Figure 2.5**. After applying the major metrics to remove peptides, the potential target peptides list was shortened to 12 in a cookie, 5 in pie crust, 11 in pasta, and 10 in ice cream.

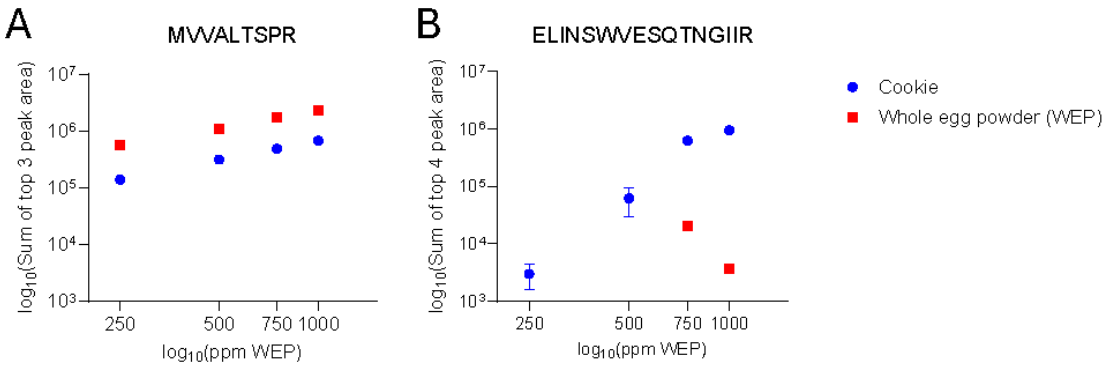

Figure 2.5 Examples of linear and non-linear dilution curves from WEP and WEP-incurred cookie matrix. The linear dilution curve of (A) MVVALSPR and non-linear dilution curve of (B) ELINSWVESQTNGIIR peptides from whole egg powder (WEP) alone and cookie at 250, 500, 750, and 1000 ppm WEP (n = 4).

Table 2.8 Library dot product values are used to determine the detection of peptides in diluted WEP and WEP-incurred cookies.

	250 ppm		500 ppm		750 ppm		1000 ppm	
Peptides	CK	WEP	CK	WEP	CK	WEP	CK	WEP
HIATNAVLFFGR‡	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
GGLEPINFQTAADQAR‡	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √
G[+43]GLEPINFQTAADQAR‡	√		√ √ √ √	√ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √
LTEWTSSNVMEER‡	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √
MVVALTSPR‡	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
FESNFNTQATNR‡	√ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
NTDGSTDYGILQINSR‡	√ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
SAGWNIPIGTLLHR‡	√ √ √		√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
IANADNLESIWR‡	√	√ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √
VGATGEIFVVNSPR‡	√ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
ADHPFLFC[+57]IK‡		√	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √
YLLDLLPAAASHR‡	√	√	√ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
YNPTNAILFFGR			√ √		√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
ELINSWVESQTNGIIR			√ √ √		√ √ √ √		√ √ √ √	
E[+43]LINSWVESQTNGIIR					√√		√ √ √ √	
DILNQITKPNDVYSFSLASR			√ √		√ √ √ √		√ √ √ √	
D[+43]ILNQITKPNDVYSFSLASR								
DILNQITK[+43]PNDVYSFSLASR							√	

AVPATEFVTTAVLPEER					√ √ √ √	√ √ √	√ √	√
NVLQPSSVDSQTAMVLVNAIVFK					√ √ √ √		√ √ √ √	
N[+43]VLQPSSVDSQTAMVLVNAIVFK								
NVLQPSSVDSQTAMVLVNAIVFK[+43]GLWEK								

‡ Peptide was selected as candidate target peptides based on the detection at 500 ppm WEP.

√ indicates the peptide had the library dot product value greater or equal to 0.75.

Cookies: CK

Whole egg powder: WEP

C[+57]: the presence of carbamidomethylation of cysteines with an addition of 57 Da to the original formula weight.

[+43]: the presence of carbamylation at the N-termini with an addition of 43 Da to the original formula weight.

Table 2.9 Library dot product values are used to determine the detection of peptides in diluted WEP and WEP-incurred pie crust.

	250 ppm		500 ppm		750 ppm		1000 ppm	
Peptides	PC	WEP	PC	WEP	PC	WEP	PC	WEP
HIATNAVLFFGR‡	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
MVVALTSPR‡	√ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
VMVLC[+57]NR‡	√ √ √	√ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
VGATGEIFVVNSPR‡	√ √		√ √ √	√ √ √ √	√ √ √	√ √ √ √	√ √ √ √	√ √ √ √
NTDGSTDYGILQINSR‡			√ √	√ √ √ √	√ √ √ √	√	√ √ √ √	√ √
SAGWNIPIGTLIHR			√ √ √ √		√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
YLLDLLPAAASHR			√ √		√ √ √	√ √	√ √ √ √	√ √ √
DILNQITKPNDVYSFSLASR					√		√ √ √ √	
D[+43]ILNQITKPNDVYSFSLASR							√	
ELINSWVESQTNGIIR					√		√ √ √ √	
E[+43]LINSWVESQTNGIIR							√	

‡ Peptide was selected as candidate target peptides based on the detection at 500 ppm WEP.
√ indicates the peptide had the library dot product value greater or equal to 0.75.
Pie crust: PC
Whole egg powder: WEP
C[+57]: the presence of carbamidomethylation of cysteines with an addition of 57 Da to the original formula weight.
[+43]: the presence of carbamylation at the N-termini with an addition of 43 Da to the original formula weight.

Table 2.10 Library dot product values are used to determine the detection of peptides in diluted WEP and WEP-incurred pasta.

	250 ppm		500 ppm		750 ppm		1000 ppm	
Peptides	PA	WEP	PA	WEP	PA	WEP	PA	WEP
LPLSLPVGPR‡	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
HIATNAVLFFGR‡	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
VMVLC[+57]NR‡	√ √ √ √	√ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
MVVALTSPR‡	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
SAGWNIPIGTLI (L) HR‡	√ √ √ √	√ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
NTDGSTDYGILQINS R‡	√ √ √ √		√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
QQLTLVEVR‡	√ √	√ √ √ √	√ √ √ √	√ √	√ √ √ √	√ √ √	√ √ √ √	√ √ √ √
GSAPDVPMQNYGSL R‡	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
FESNFNTQATNR‡	√	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
YLLDLLPAAASHR‡		√	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
GTDVQAWIR‡			√ √ √	√ √	√ √ √ √	√ √ √	√ √ √ √	√ √ √ √
ADTYFDNYR	√ √		√ √	√	√ √	√	√ √	√
YNPTNAILFFGR			√ √	√	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √
EALQPIHDLADEAIS R			√ √ √		√ √ √ √	√ √ √ √	√ √ √ √	√ √ √
NSIAGQWTQPVWM GELR			√ √ √ √		√ √ √ √		√ √ √ √	
ELINSWVESQTNGIIR	√ √		√ √ √ √		√ √ √ √		√ √ √ √	
E[+43]LINSWVESQT NGIIR			√ √ √		√ √ √ √		√ √ √ √	
TGGLQLVVYADTDS VRPR	√		√ √ √ √		√ √ √ √		√ √ √ √	

NVLQPSSVDSQTAMVLVNAIVFK	√ √		√ √ √ √		√ √ √ √		√ √ √ √	
NVLQPSSVDSQTAMVLVNAIVFK[+43]GLWEK								
DILNQITKPNDVYSFSLASR	√ √		√ √ √ √		√ √ √ √		√ √ √ √	
D[+43]ILNQITKPNDVYSFSLASR							√ √	
DILNQITK[+43]PNDVYSFSLASR					√			
VGATGEIFVVNSPR	√ √	√	√ √ √		√ √ √ √	√ √	√ √ √ √	√ √ √ √
HGLDNYR			√		√ √ √	√ √	√ √ √ √	√ √ √
YVPGVALVLGFSEAHQR			√		√ √		√ √ √ √	
WLLSAVSASGTTETLK					√ √ √ √		√ √ √ √	
AVPATEFVTTAVLPEER					√		√	
QVYQISPFNEPTGVAVMEAR					√			

‡ Peptide was selected as candidate target peptides based on the detection at 500 ppm WEP.
√ indicates the peptide had the library dot product value greater or equal to 0.75.
Pasta: PA
Whole egg powder: WPE
C[+57]: the presence of carbamidomethylation of cysteines with an addition of 57 Da to the original formula weight.
[+43]: the presence of carbamylation at the N-termini with an addition of 43 Da to the original formula weight.

Table 2.11 Library dot product values are used to determine the detection of peptides in diluted WEP and WEP-incurred ice cream.

	250 ppm		500 ppm		750 ppm		1000 ppm	
Peptides	IC	WEP	IC	WEP	IC	WEP	IC	WEP
HIATNAVLFFGR‡	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
H[+43]IATNAVLFFGR‡								
NFLINETAR‡	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
MVVALTSPR‡	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
VMVLC[+57]NR‡	√ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
QQLTLVEVR‡	√ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
NTDGSTDYGILQINSR‡	√	√ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
SAGWNIPIGTLI (L)HR‡	√	√	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
FESNFNTQATNR‡		√	√ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
VGATGEIFVVNSPR‡	√ √	√ √ √ √	√ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
LSQLLESTMQIR‡		√	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
IANADNLESIWR		√	√	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
GYSLGNWVC[+57]AAK			√	√ √	√ √ √ √	√ √ √ √	√ √ √ √	√ √ √ √
ADHPFLFC[+57]IK			√	√ √	√ √ √	√ √ √	√ √ √ √	√ √ √ √
EALQPIHDLADEAISR			√	√ √	√ √ √	√ √ √ √	√ √ √ √	√ √ √ √
TGGLQLVVYADTDSVRPR			√		√ √ √ √		√ √ √ √	√
NAVSFGHSWILEEAPC[+57]R					√	√ √	√ √ √ √	√ √ √ √
VTEQESKPVQMMYQIGLFR					√ √ √ √		√ √ √ √	√ √ √ √

V[+43]TEQESKPVQM MYQIGLFR							√ √ √ √	
VTEQESK[+43]PVQM MYQIGLFR							√ √ √ √	
NVLQPSSVDSQTAM VLVNAIVFK						√ √ √ √	√ √ √ √	√ √ √ √
NVLQPSSVDSQTAM VLVNAIVFK[+43]GL WEK								
DILNQITKPNDVYSF SLASR					√ √ √ √		√ √ √ √	√ √ √ √
DILNQITK[+43]PNDV YSFSLASR							√ √	
D[+43]ILNQITKPNDV YSFSLASR							√ √	
ELINSWVESQTNGIIR					√ √ √ √		√ √ √ √	√
E[+43]LINSWVESQT NGIIR							√ √	
NSIAGQWTQPVWM GELR					√ √ √		√ √ √ √	√ √
AVPATEFVTTAVLPEE R							√	√ √ √ √

‡ Peptide was selected as candidate target peptides based on the detection at 500 ppm WEP.
√ indicates the peptide had the library dot product value greater or equal to 0.75.
Ice cream: IC
Whole egg powder: WEP
C[+57]: the presence of carbamidomethylation of cysteines with an addition of 57 Da to the original formula weight.
[+43]: the presence of carbamylation at the N-termini with an addition of 43 Da to the original formula weight.

Further peptide performance metrics were applied to refine the target peptide list by visually investigating the signal of the peptides in the samples. In a targeted MS method, three product ions of each peptide will be monitored. The peak area ratio of these three product ions varies among peptides. The least abundant product ion limits the sensitivity of the peptide in the targeted MS method. Therefore, we included the criteria of assessing the least abundant product ion as the additional performance metric to refine the target peptide list. Furthermore, the peak quality and the abundance of the peptides were assessed to remove peptides from the target peptide list. Examples of the poor quality of the peptides are shown in **Figure 2.6**.

The developed discovery-based and target peptide refinement strategies can be applied across four food matrices. After applying the performance criteria, we identified a total of 11 egg-specific target peptides (**Table 2.12**), three peptides were found common across four food matrices (**Figure 2.7**). These 11 peptides originated from four egg white proteins, one egg yolk proteins, and apovitellenin-1. Notably, all identified egg white proteins ovomucoid (Gal d 1), ovalbumin (Gal d 2), ovotransferrin (Gal d 3), and lysozyme C (Gal d 4) are recognized as major allergenic egg white proteins, associated with reported clinical allergenic responses, by the World Health Organization International Union of Immunological Societies. Even though the yolk-allergenic proteins, serum albumin (Gal d 5) and YGP 42 (Gal d 6), were not included in the target peptide list, four peptides from vitellogenin-2, the abundant yolk protein, were included.

The uniqueness of the target peptides was validated using a Basic Local Alignment Search Tool (BLAST). The target peptide sequences were searched against the non-redundant protein sequence database (nr, NCBI). The peptide sequences of VMV,

GGL, HIA, LTE, QQL, VGA, LPL, and NFL peptides have been found to align with protein sequences from bacteria and/or exotic birds with 100% query coverage and identity. However, bacteria and exotic birds were less likely to be present in food products. Moreover, the MVV, NTD, and SAG peptides aligned with the proteins from common birds that can produce edible eggs, such as turkey, pheasant, duck, quail, and guinea fowl. These peptides showed promise for developing an egg-specific targeted MS method.

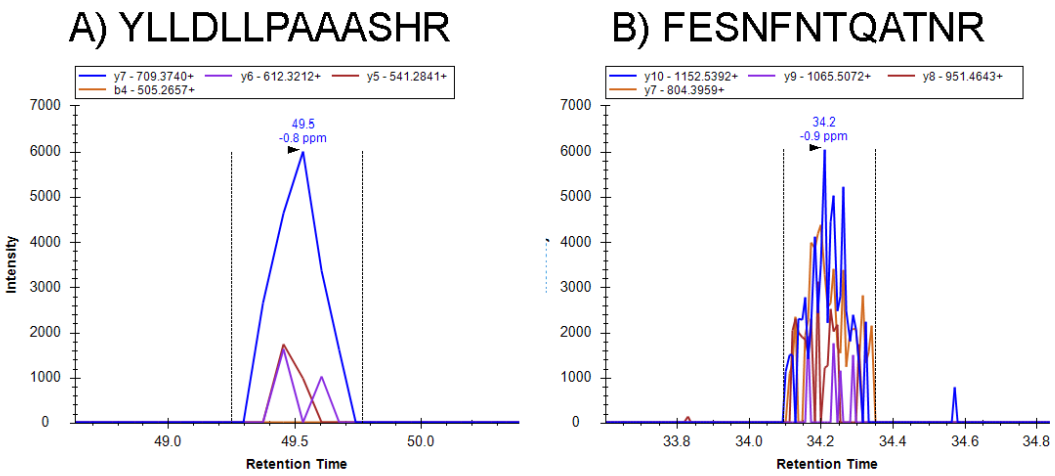

Figure 2.6 Examples of poor signal quality of peptides. The detection of the peptides in cookie at 500 ppm whole egg powder. A) the least abundant product ions of the YLLDLLPAAASHP peptide were low. B) the FESNFNTQATNR peptide had a poor peak shape.

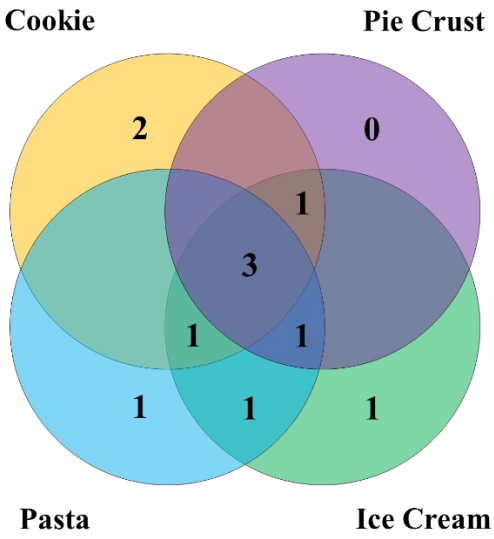

Figure 2.7 Venn diagram of the whole egg powder-specific candidate target peptides in incurred food matrices.

Table 2.12 Eleven target peptides for detection of total egg protein.

Symbol	Sequence[a]	m/z	Charge state (z)	UniProt Accession	Protein	No. ref[b]
VMV	K.VMVL[C]NR.A	446.2306	2	P01005	Ovomucoid	
GGL	R.GGLEPINFQTAADQAR.E	844.4235	2	P01012	Ovalbumin	19
HIA	K.HIATNAVLFFGR.C	449.2507	3	P01012	Ovalbumin	7
LTE	K.LTEWTSSNVMEER.K	791.3643	2	P01012	Ovalbumin	13
QQL	R.QQLTLVEVR.S	543.3193	2	P02845	Vitellogenin-2	
VGA	R.VGATGEIFVVNSPR.T	723.3910	2	P02845	Vitellogenin-2	
MVV	R.MVVALTSPR.T	487.2786	2	P02845	Vitellogenin-2	
LPL	R.LPLSLPVGPR.I	524.8293	2	P02845	Vitellogenin-2	1
NFL	R.NFLINETAR.L	539.2880	2	P02659	Apovitellenin-1	1
NTD	R.NTDGSTDYGILQINSR.W	877.4212	2	P00698	Lysozyme C	8
SAG	R.SAGWNIPIGTLLHR.G	512.2878	3	P02789	Ovotransferrin	3

[a] [C]: the presence of carbamidomethylation of cysteines with an addition of 57 Da to the original formula weight.
[b] The number of references was from a critical review by Pilolli et al.[36]

Most current mass spectrometry (MS)-based methods identify target peptides using an in silico-based approach, depending upon the MS analysis of either egg white powder[13], egg powder[14], or purified egg protein (i.e., ovalbumin and lysozyme)[15–17]. Notably, to develop a quantitative MS method, those pre-identified egg peptides from the *in silico* approach must be examined and validated in the WEP-incurred foods.[36]

Egg target peptides identified by the discovery-based approach show promise for developing a quantitative MS method for foods because these peptides have been examined using the incurred food matrices. Gavage et al.[32] and Pilolli et al.[34] used a discovery-based approach to identify egg peptides from different WEP-incurred foods, including mayonnaise, chocolate, and broth powder. These food matrices represent sample types with high in fat, high in polyphenols, and protein background, respectively.[32,34] However, bakery products cannot be neglected because they are the biggest offenders of undeclared food allergens.[5,6] In addition, the temperature and the length of baking can reduce the detection of eggs using current ELISA kits.[10] Therefore, it is necessary to include bakery products as food matrices to identify egg target peptides. In this study, four food matrices (i.e., cookies, pie crust, pasta, and ice cream) were involved to identify egg target peptides, which reflects the detection of egg peptides in the real-case scenario. Five egg target peptides (GGL, LPL, HIA, SAG, and NFL) identified from our four WEP-incurred food matrices were also identified by Gavage et al.[32] and Pilolli et al.[34] from incurred chocolate and/or incurred broth powder.

Compared with the critical review by Pilolli et al.[36], four of 11 peptides, including QQL, VGA, and MVV peptides from vitellogenin-2 and the VMV peptide from ovomucoid, were first identified as target peptides for egg-specific MS methods (**Table**

2.12). This may be because most current MS-based egg detection methods focus on detecting allergenic egg white proteins. The VMV peptide contains a fixed PTM with carbamidomethylation of cysteine. This peptide would be rejected by Gavage et al.[32] and Pilolli et al.[34]'s selection criteria. Seven target peptides were previously identified as target peptides in other MS methods (**Table 2.12**).

To develop an accurate and reliable PRM-MS method for quantifying low levels of egg proteins in processed food, the sensitivity of the PRM-MS method with these 11 target peptides will be examined in WEP-incurred foods with various concentrations of WEP. The performance of the quantitative MS method will be compared with commercial egg ELISA kits by quantifying egg proteins from the WEP-incurred food matrices.

2.5 Conclusion

This study described a discovery-based approach for selecting WEP-specific candidate peptides using a DDA-MS method. A set of criteria was developed to evaluate the peptide performance using the PRM-MS method to refine target peptides. This approach can be applied to select peptides for other allergenic food ingredients. Our approach has successfully identified 11 WEP-specificity and robust target peptides capable of developing the sensitive PRM-MS method for various processed foods. Our discovery-based approach for identifying target peptides from incurred food matrices used numerical metrics informing the determination of the inclusion and exclusion of the peptides. Using clear and numerical metrics as selection criteria allows the selection of target peptides without prior knowledge and unbiasedly. Our discovery-based approach can be adapted and applied to identify target peptides for other food allergens.

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CHAPTER 3

OPTIMIZATION OF SAMPLE PREPARATION WORKFLOW TO ENHANCE DETECTION OF EGG-SPECIFIC PEPTIDES USING TARGETED MASS SPECTROMETRY

3.1 Abstract

In allergen detection, the sensitivity of mass spectrometry (MS) methods is crucial to accurately measure the target concentration at relevant reference doses. This study aimed to optimize a targeted parallel reaction monitoring MS method for egg detection. Initial assessments indicated that the sensitivity of the detection method, which was used for target peptide selection, did not meet the necessary requirements. To address this, several specific optimization strategies were implemented. The sample preparation workflow was optimized, with a focus on key variables such as the type of trypsin used, the inclusion of carrier proteins in both the protein extraction buffer and diluent, the use of desalting columns to remove unwanted salts, and adjustments to the digestion volume to ensure optimal peptide recovery. These modifications were evaluated to enhance the efficiency and sensitivity of peptide detection. Following these optimizations, the MS method was finalized, resulting in improved sensitivity suitable for allergen detection. This enhanced method demonstrates improvement in the reliable quantitation of egg protein in processed foods.

3.2 Introduction

Recently, the FAO/WHO expert consultant has recommended a requirement for an effective allergen detection method for foods, and the method's limit of quantification (LOQ) should be three times lower than the action levels set for the respective food

groups. Regarding egg allergen, 2 mg of total egg protein was specified as the reference dose by FAO/WHO expert consultant.[1] Based on food challenge data, 5% of the allergic population may experience objective allergic reactions at this dose. In order to calculate a concentration as a goal for the method development, the reference dose and the consumption quantity must be considered. Cookies, pie crust, pasta, ice cream, and retorted pasta soup are the target food matrices for developing the egg-specific quantitative MS method. The reference food consumption for these five food matrices ranges from 50 – 400 g. Specifically, the food consumption for cookies, pasta, ice cream, and soup provided in the report from the FAO/WHO expert consultation used the rounded-up quantity of the 75th percentile of the food consumption survey.[2] As for the pie crust, the quantity was estimated based on the serving size of a toaster pastry product (96 g). In order to meet the reference dose for eggs, the lowest required LOQ of the allergen analytical method among these five food matrices is 1.6 ppm total egg protein for 400 g of food consumption.

The sensitivity and accuracy of the MS method are impacted by the quality of the target peptide, sample preparation, and instrument parameters. To enhance method performance, it is important to first investigate the optimization of the food sample preparation workflow. Sample preparation for MS analysis involves four main procedures: protein extraction, enzyme digestion, desalting, and further dilution.

Protein extraction is fundamental to solubilizing proteins from samples and potentially unfolding them for subsequent procedures. Several factors that affect protein extractability, such as the composition of the extraction buffer, sample-to-buffer ratio, and extraction temperature, can be optimized. To enhance the extractability of proteins from

food samples, denaturing agents (e.g., urea) and reducing agents (e.g., dithiothreitol) can be used in the extraction buffer.[3–6] Henrottin et al.[6] have compared two concentrations of urea (2M vs. 5M) in the protein extraction buffer for extracting proteins from a chocolate matrix. The authors found that protein using 2M urea significantly improved the detection of certain egg peptides compared to 5M urea. In contrast, extraction with 5M urea significantly enhanced the detection of peanut peptides.[6]

In-solution digestion, where proteins were reduced, alkylated, and digested directly in the sample extracts, can be employed.[7] In this process, trypsin, a common protease in proteomic research, is added during the sample extraction.[8] Trypsin specifically cleaves at the carboxyl side of lysine (K) and arginine (R) residues in the extracted proteins, generating peptides suitable for MS analysis.[9] To improve trypsin digestion efficiency, the quality of trypsin, digestion times, and conditions (e.g., temperature and pH) can be optimized.[10–12] Zheng et al.[10] have found that the digestion profiles of peptides can vary, including slow-forming, fast-forming, decaying, and oscillating profiles. Among these, the fast-forming profile, where peptides are formed in the first period and remain stable thereafter, is desirable.[10,13]

Desalting removes salt and contaminants from the digested peptides and is typically achieved by using desalting resins or filters, often in column format. Various types of desalting columns are commercially available, which can effectively remove salts and contaminants through gravity flow or centrifugation. Two types of desalting column polymeric phase Strata-X[6,14–16] and reverse phase C18 desalting columns[17–21] have been used in the development of MS methods for detecting food allergens. Both types of desalting columns have demonstrated effective performance in purifying target

peptides for food allergens (milk, egg, soy, peanut, almond, and hazelnut) from a chocolate matrix.[6]

Proper dilution of the samples is crucial for achieving the desired concentrations for MS analysis, especially for preparing the calibration curve. Non-specific absorption of peptides to plasticware has been found to be a potential factor responsible for the loss of peptides from the sample, particularly at low concentrations.[19,21,22] To minimize the non-specific absorption of peptides during sample preparation, effective strategies include selecting appropriate plasticware and using carrier proteins or blockers (e.g., casein, bovine serum albumin, egg white proteins, etc.) in solution.[19,21–23]

The present chapter focused on optimizing the sample preparation workflow to improve the sensitivity of the MS method. The detection and performance of the 11 egg-specific peptides identified in the previous chapter were evaluated using different sample preparation conditions.

3.3 Method and Materials

3.3.1 Reagents, chemicals, and materials

Urea was purchased from Invitrogen (Carlsbad, CA), and dithiothreitol (DTT) from Acros Organics. Thiourea, Trizma hydrochloride (Tri-HCl), ammonium bicarbonate (ABC), and iodoacetamide (IAA) were purchased from Sigma-Aldrich. MS-grade trypsin, water, methanol, acetonitrile (ACN), and formic acid (FA) were purchased from Thermo Fisher Scientific. MS-grade water was used to prepare all chemical solutions in the study.

The whole egg powder (WEP) used as incurred material was sourced from Michael Food Inc. (Omaha, NE). Dumas analysis was used to determine that its total

protein content was 47%. Sugar cookies were incurred with 10,000 ppm WEP and prepared in a food-graded kitchen and GMP processing pilot plant at the University of Nebraska-Lincoln, following the detailed preparation methods outlined in Chapter 2, Materials and Methods.

Stabel isotope-labeled target peptides (use heavy peptides forward) were custom synthesized by Thermo Fisher Scientific (AQUA™ Basic grade, with > 95% purity). To prepare the stock solution, each of the heavy peptides was resuspended in 50% (v/v) ACN water to make a heavy peptide cocktail, achieving a final concentration of 500 fmol of each heavy peptide per µL in 50% (v/v) ACN water.

3.3.2 Optimization for sample preparation

3.3.2.1 The original sample preparation workflow

WEP (0.04 g) was extracted in 20 mL of protein extraction buffer (2 M Thiourea, 6 M Urea, 50 mM Tris-HCL at pH 8.9, and 20 mM DTT) in triplicate. Protein was extracted by incubation in a 60°C shaking water bath for 10 minutes, vigorous resuspension by vortex for 1 minute, sonication at room temperature for 10 minutes, and repeated incubation at 60°C for 10 minutes. Subsequently, 1000 µL of the sample extract was centrifuged at 17,000 g for 10 minutes at room temperature. The supernatants were then used as the sample extract. The WEP protein extract (2 µg of WEP/µL) was further diluted with protein extraction buffer to achieve a concentration of 0.5 µg of WEP/µL (i.e., 10,000 ppm WEP extract). Sample preparation after protein extraction was performed in Eppendorf LoBind® microcentrifuge tubes.

The 10,000 WEP sample extract, following the original digestion protocol (i.e., 12 µL of sample extract, see **Table 3.1**), was reduced in a hot block at 95°C for 5 minutes

with the addition of reducing buffer, digestion buffer, and water. The reduced protein was then alkylated and incubated in the dark at room temperature for 20 minutes. Alkylated protein was digested with the first addition of the trypsin solution (100 ng/µL) at 37°C for two hours, followed by an addition of the trypsin solution and incubation at 30°C overnight (14 hours). Following the trypsin digestion, the digested peptides were desalted using Pierce® C18 spin columns (catalog no. 89870, 30 µg of peptide maximum capacity) following the manufacturer's instructions with one modification, where the desalted peptides were eluted in 55% (v/v) ACN in water. The eluted peptides were then frozen and lyophilized using a SpeedVac vacuum evaporator (Model SPD120). The lyophilized peptides were stored at -20°C until MS analysis.

The resultant 10,000 ppm WEP peptides were resuspended with 50 µL of 0.1% FA/5% ACN in water (v/v). A serial dilution was performed to make a dilution curve with either 0.1% FA/5% ACN in water (v/v) or the addition of 0.1% non-fat dry milk powder (NFDM) peptide in the diluent. The NFDM peptides were prepared along with the WEP samples. The resultant NFDM peptides were resuspended in 0.1% FA/5% ACN water (v/v) to match the concentration of 0.1% NFDM in the protein extraction buffer.

A heavy peptide working solution was freshly prepared before MS analysis. Upon injection for MS analysis, the heavy peptide working solution was mixed with the samples in the HPLC vial to achieve 5 fmol of each heavy peptide. Heavy peptides were analyzed together with the sample peptides on the HPLC column. The injection volume was 5 µL for samples that prepared from 12 µL of sample extract, and the injection volume was 13 µL for samples that prepared from 60 µL of sample extract.

3.3.2.2 Scale-up sample extract for reduction, alkylation, and digestion

The original digestion protocol using 12 µL of sample extract was used for discovery-based MS analysis. The scale-up digestion protocols increased the volume of sample extract used for digestion by 5-fold (60 µL of sample extract) and 10-fold (120 µL of sample extract). The volume used for the reduction, alkylation, and digestion was increased proportionally following the in-solution trypsin digestion protocol (**Table 3.1**). Apart from the volumes used for the sample preparation, other procedures, such as microcentrifuge speed, temperature, and time, were operated in the same manner as the original sample preparation workflow, as described in section 3.2.2.1. The original digestion protocol was used in the experiments to compare two MS-grade trypsin (section 3.2.2.3) and evaluate the inclusion of carrier protein (section 3.2.2.5). The 5-fold scale-up digestion protocol was used in the time-course trypsin digestion experiment (section 3.2.2.4). The 10-fold scale-up digestion protocol was used in the experiment to compare three types of desalting columns (section 3.2.2.7).

Table 3.1 The volume of reagent for enzymatic reduction, alkylation, and digestion in original and scale-up digestion protocol

	Original digestion (µL)	5-fold Scale-up digestion (µL)	10-fold Scale-up digestion (µL)
Sample extract	12	60	120
Reducing buffer (100 mM DTT)	4.5	22.5	45
Digesting buffer (50 mM ABC)	45	225	450
MS-grade water	19.5	97.5	195
Alkylation buffer (50 mM IAA)	9	45	90
First addition of trypsin solution (100 ng/µL)	3	15	30
Second addition of trypsin solution (100 ng/µL)	3	15	30

DTT: MS-grade dithiothreitol
ABC: MS-grade ammonium bicarbonate
IAA: MS-grade iodoacetamide

3.3.2.3 Comparison of two MS-grade trypsin

The efficiency of the two MS-grade trypsin (Pierce Trypsin vs. Promega Trypsin) was compared. WEP was extracted with the chaotropic buffer without the addition of NFDM (0.5 µg of WEP/µL). Six individual 12 µL of WEP extracts were reduced, alkylated, desalted, and digested by each two MS-grade trypsin (original sample preparation workflow, section 3.2.2.1). The two MS-grade trypsin were freshly prepared before digestion following the manufacturer's instructions. In brief, to make the Pierce trypsin solution, the lyophilized trypsin was resuspended to 1 µg/µL with 50 mM acetic acid and diluted to 100 ng/µL with MS-grade water. To make the Promega trypsin solution, the lyophilized trypsin was resuspended to 0.5 µg/µL with the resuspension

buffer provided by the manufacturer (composition of 50 mM acetic acid) and diluted to 100 ng/µL with MS-grade water. A total volume of 6 µL of the trypsin solution (100 ng/µL) was used for the 16-hour digestion. The resultant WEP peptides (10000 ppm WEP) were resuspended and diluted with 0.1% FA/5% ACN in water (v/v). Eleven target peptides were analyzed with duplicate injections at 250 and 500 ppm WEP (n = 12).

3.3.2.4 Trypsin time-course experiment

A trypsin time-course experiment was conducted to compare the effect of one-addition trypsin and two-addition trypsin on the digestion efficiency and to investigate the trypsin digestion efficiency over time. Cookies incurred with 10,000 ppm WEP and WEP alone samples were extracted with the chaotropic extraction buffer with the addition of 0.1% NFDM. Using the 5-fold scale-up reduction and alkylation protocol (**Table 3.1**), 60 µL of the sample extracts from the cookies and WEP were reduced and alkylated in a LoBind tube with 16 replicates. Eight of 16 replicates were digested with two-addition trypsin, where 15 µL of the trypsin solution was added to the alkylated sample and incubated at 37°C for two hours, and an additional 15 µL of the trypsin solution was added to the sample and incubated on a 30°C hot block for 18 hours (**Figure 3.1A**). Another eight of 16 replicates were digested with one-addition trypsin, where following the alkylation, 30 µL of the trypsin solution (100 ng/µL, Promega trypsin) was added and incubated at 37°C for a total of 20 hours, as illustrated in **Figure 3.1B**. Two of each one-addition and two addition trypsin digested samples were collected at different digestion durations (4-, 8-, 18-, and 20-hour time points). The collected digested samples were stored at -20°C to stop the trypsin digestion. Before desalting, the collected digested samples were fully thawed at room temperature. Afterward, the 600 µL of digested

samples were desalted with the Pierce® peptide desalting spin column (catalog no. 89851, 5 mg of peptide maximum capacity) and lyophilized for MS analysis.

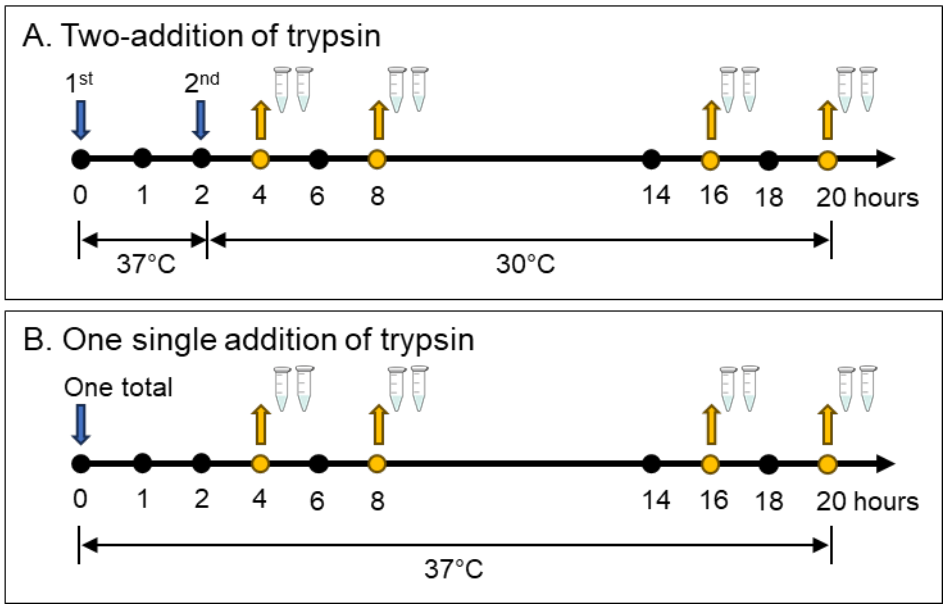

Figure 3.1 The experimental design of the trypsin time-course experiment. A) Two-addition of trypsin treatment: the samples were digested with trypsin by adding 15 µL of the trypsin solution (100 ng/µL) at 0 hours and the second 15 µL of the trypsin at 2 hours. B) One single addition of trypsin treatment: a total of 30 µL of the trypsin solution (100 ng/µL) was added to the samples. Two independent digests were collected at 4, 8, 16, and 20 hours-digestion duration. The digestion was stopped by moving the samples in -20°C.

3.3.2.5 The inclusion of carrier protein

Proteins from WEP (0.04 g) were extracted in 20 mL of protein extraction buffer in triplicate in the presence or absence of 0.1% NFDM. The 10,000 ppm WEP sample extracts were reduced, alkylated, and desalted following the original sample preparation workflow (section 3.2.2.1). The resultant 10,000 ppm WEP peptides were resuspended with 50 µL of 0.1% FA/5% ACN in water (v/v). To prepare the diluent, NFDM (0.12 g) was extracted in 20 mL of the protein extraction buffer in the absence of 0.1% NFDM. The NFDM extract was digested in the same manner as the WEP. The resultant NFDM peptides were resuspended with 300 µL of 0.1% FA/5% ACN in water (v/v) to match the concentration of 0.1% NFDM in the protein extraction buffer, named NFDM peptide diluent. A serial dilution was performed to make 250 and 500 ppm WEP with either 0.1% FA/5% ACN in water (v/v) or the addition of NFDM peptide diluent. The diluted WEP samples were injected with 4 µL (no addition of HP) for MS analysis. The peak areas of the 11 target peptides were evaluated across the sample preparation conditions with or without NFDM.

3.2.2.6 The detection of heavy peptides in various concentrations of NFDM background

The effect of NFDM peptide in the diluent on detecting heavy peptides was evaluated. NFDM (0.14 g) was extracted in 20 mL of the protein extraction buffer in the absence of 0.1% NFDM, following Promega trypsin digestion and Strata-X column desalting. The eluted NFDM peptides were aliquoted to achieve approximately 180 ug of NFDM peptide in LoBind tubes. The resultant NFDM peptides were resuspended with 750 µL of 0.1% FA/5% ACN in water (v/v) to make a final concentration of 400 ng

NFDM peptide/µL. The resuspended NFDM was then serially diluted with 0.1% FA/5% ACN in water (v/v) to make concentrations of 0, 6.25, 12.5, 25, 50, 100, and 200 ng NFDM peptide/µL. The HP peak areas (5 fmoles of each HP on column) were detected with the presence of various concentrations of carrier material by mixing 3 µL of the heavy peptide mixture (5 fmoles of each heavy peptide per µL) with 36 µL of NFDM diluents with various concentrations.

3.3.2.7 Comparison of three different desalting columns

Three different brands of desalting columns, including the Piece® C18 spin columns (catalog no. 89870), Piece® peptide desalting spin columns (catalog no. 89852), and Phenomenex® Strata-X 33 µm polymeric reversed phase columns (catalog no. 8B-S100-AAK), were compared. Twelve 120 µL of the WEP at 10,000 ppm extracts were digested with Promega trypsin. The volume used for the reduction, alkylation, and digestion was increased ten times with the original digestion protocol (**Table 3.1**). In order to rule out potential variability among individual digests, these twelve sample digests were prepared individually and pooled into a glass vial before desalting. To assess the effectiveness of the three different desalting columns, 100 fmoles of each heavy peptide was added before the desalting step (pre-HP) or before the MS analysis (post-HP).

After the trypsin digestion, the pooled digested sample was dispended into six glass vials for six different treatments (three different desalting columns with or without the addition of the heavy peptide mixture). An equal amount of WEP (29.8 µg) with or without the presence of heavy peptide was desalted using three different desalting

columns following the manufacturer's instructions with a few modifications, as described in **Figure 3.2**.

To achieve an equal amount of WEP (7.7 ug) and heavy peptides (100 fmoles of each heavy peptide) in 13 µL of injection volume, the pre-HP samples were resuspended with 50 µL of 0.1% FA/5% ACN in water (v/v) and the post-HP samples was resuspended with 40 µL of 0.1% FA/5% ACN in water (v/v) and 10 µL of heavy peptide mixture (38.46 fmoles of each heavy peptide per µL). Each of the six treatments was conducted in triplicate with duplicate injections of MS analysis (n = 6).

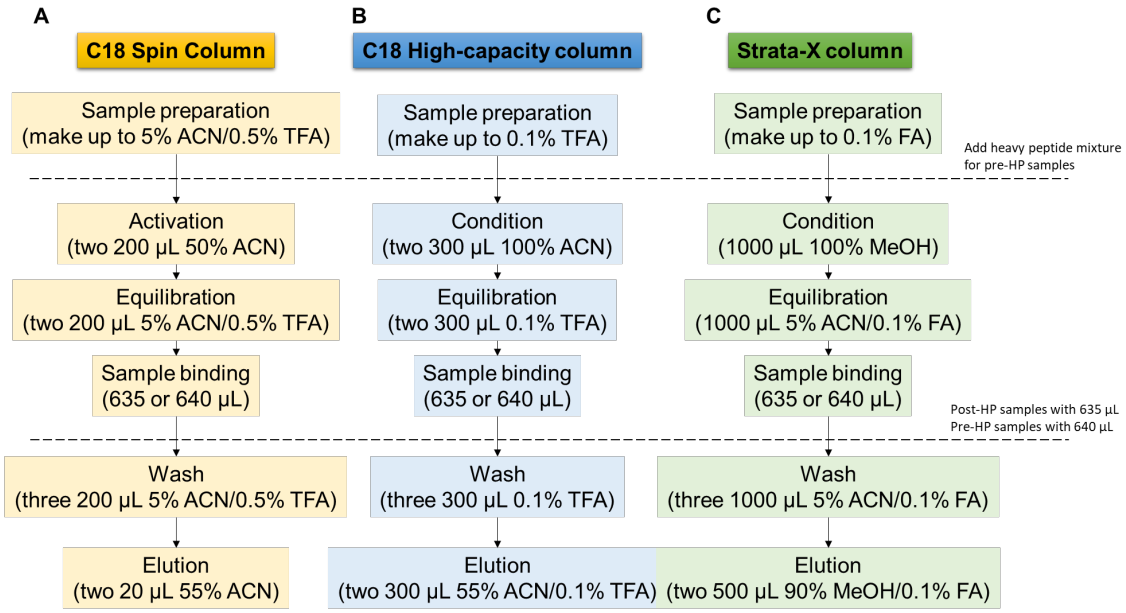

Figure 3.2 Schematic protocol for three different desalting columns (A. C18 spin column; B, C18 high-capacity desalting column; and C, Strata-X desalting column).

3.3.3 UHPLC-MS/MS parameters for discovery proteomics analysis

The parallel reaction monitoring (PRM) MS analysis was employed on a Thermo Fisher Dionex UltiMate 3000 RS ultrahigh performance liquid chromatography (UHPLC) system coupled with Thermo Scientific™ Q Exactive™ Plus hybrid quadrupole-Orbitrap™ mass spectrometer in positive ionization mode.

Two elution gradients (115 minutes vs. 35 minutes) were conducted to separate the target peptides in UHPLC (Figure 3.3). In the 115-minute elution gradient, a Hypersil GOLD C18 Selectivity LC Column (100 mm × 1 mm, 1.9 µm, 175 Å) was used with a flow rate of 0.06 mL/min (**Figure 3.3A**). In the 35-minute elution gradient chromatogram method, the Hypersil GOLD C18 Selectivity LC Column with a larger diameter (100 mm × 2 mm) was used with a flow rate of 0.3 mL/min (**Figure 3.3B**).

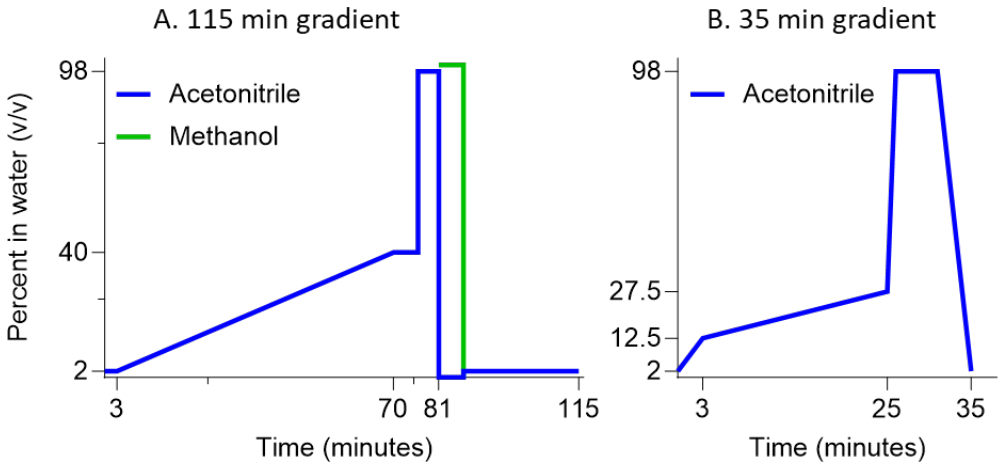

Figure 3.3 Two elution gradients of acetonitrile (blue line) and methanol (green line) on C18 reversed-phase columns in (A) 115 minutes or B) 35 minutes.

The source parameters were set as follows: sheath gas flow rate of 50 arbitrary units (au), aux gas flow rate of 10 au, positive ion spray voltage of 3500 V, heated electrospray ionization (HESI) capillary temperature of 320°C, S-lens RF of 60 au. For PRM-MS, the parameters were a resolution of 70,000, automatic gain control (AGC) of 1×106, and maximum injection time (IT) of 240 milliseconds. The mass isolation width for the precursor of interest was 0.8 mass-to-charge ratio (m/z) with no offset. The normalized collision energy was 27.

3.3.4 Data analysis

The PRM data was carried out using Skyline software to calculate peak area and evaluate peptide performances.[24] Eleven egg-specific target peptides, identified in Chapter 2, were monitored with fixed three product ions. The m/z of the product ions are greater than the m/z of its precursor and are the most intense ions from the spectral library (generated by previous discovery work). Library dot product values (dotp) and reference dot product (rdotp) values were used to evaluate the performance of the peptides. They are automatically generated by Skyline software, indicating the quality of the peptide signal compared to the spectral library and heavy peptide, respectively. The dotp and rdotp values ≥ 0.8 indicate the acceptable quality of the peptide signal. GraphPad Prism (version 10.0) was used to perform one-way ANOVA, followed by Tukey's comparison.

3.4 Results and Discussions

3.4.1 Comparison of two MS-grade trypsin

Trypsin is most widely used in MS proteomics and is critical in protein digestion.[8] Two commonly used commercial MS-grade trypsin (A, Pierce Trypsin and B, Promega Trypsin) were used for protein digestion. To find the optimal trypsin for WEP-specific peptides, we evaluated the performance of trypsin A and B by the relative abundance of 11 peptides and the precision of the peak areas in six replicates and duplicate injections (n = 12) of WEP digests. Trypsin A was used in the original digestion procedures and prepared using the in-house resuspension buffer, while trypsin B was prepared using the resuspension buffer provided by the manufacturer. Both the in-house and provided resuspension buffers are composed of 50 mM acetic acid. The peak area obtained from trypsin A and B, prepared by either the in-house resuspension buffer or the provided resuspension buffer, were compared. One-way ANOVA, followed by Tukey's comparisons, was conducted. When comparing the effectiveness of in-house and provided resuspension buffers, it was found that the activity of Trypsin B was likely affected by the resuspension buffer used. Specifically, when trypsin B was coupled with different resuspension buffers, five peptides (GGL, LPL, NFL, QQL, and VMV) showed significant differences ($p < 0.05$). In contrast, only VMV exhibited a significant difference when coupled with different resuspension buffers using trypsin A ($p < 0.05$).

To simplify the comparison, trypsin A with the in-house resuspension buffer and trypsin B with the provided resuspension buffer are shown in **Figure 3.4**. Regarding the variability within six replicates, A and B trypsin show no distinctive difference. However, there are significant differences in terms of the observed peak areas of all peptides using

trypsin A and B ($p<0.05$). Moreover, higher peak areas were seen with peptides generated by B trypsin. Several factors, such as pH, temperature, and urea concentration, can impact trypsin efficiency in digestion.[11,12] The digestion protocol remained identical apart from the trypsin brand. Thus, these factors may not explain this observation. The difference in the obtained peak areas may be attributed to varying trypsin-specific activity (i.e., the active unit per milligram of total protein) from different manufacturers. The B trypsin was prepared using the manufacturer-provided resuspension buffer as the preferred digestion for further work.

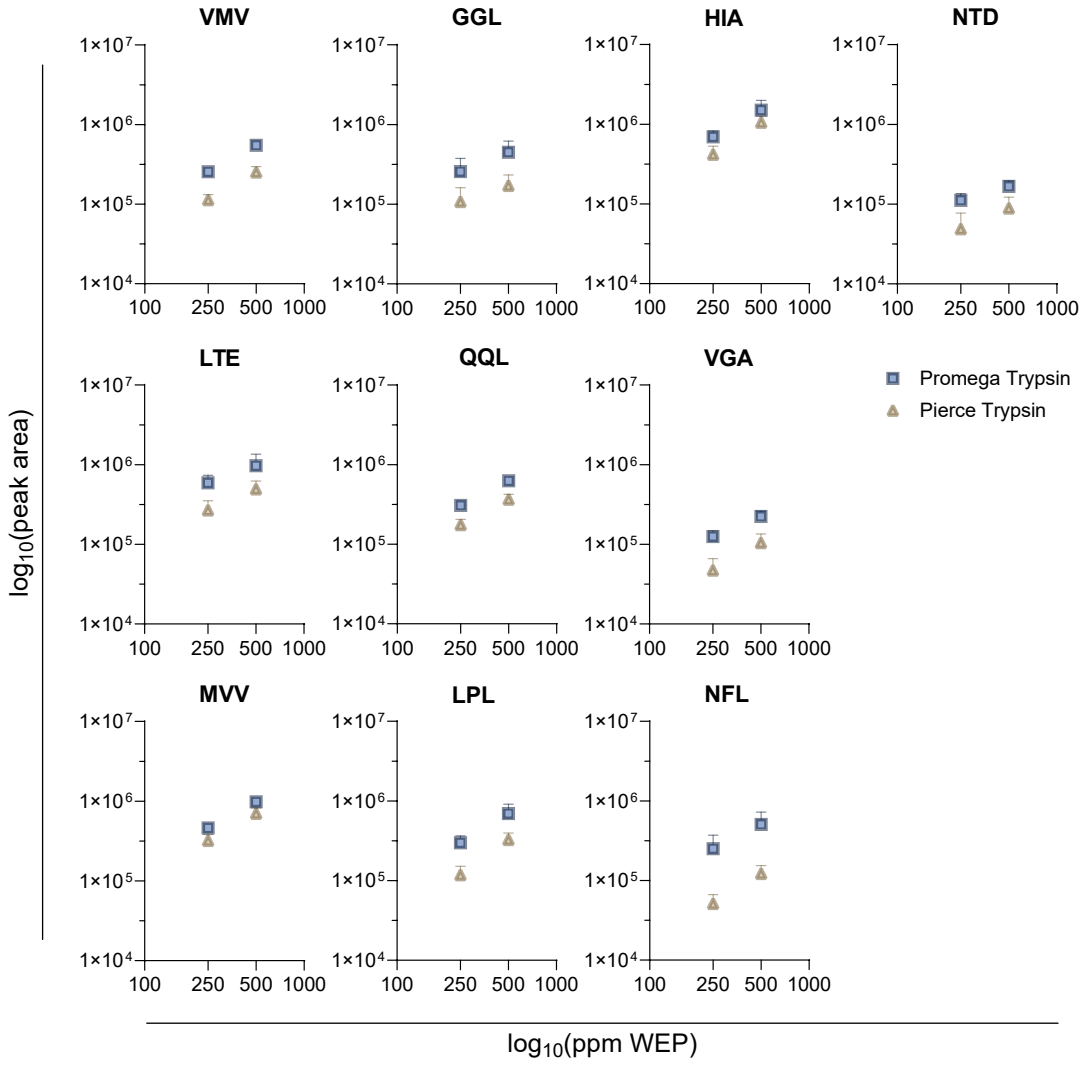

Figure 3.4 Comparison of the peptide peak areas of peptides from two MS-grade trypsin in digestion. The blue box indicates that the peptides were digested by Promega trypsin, while the beige triangle indicates that the peptides were digested by Pierce trypsin. The y-axis indicates $\log_{10}$ (sum of top 3 product ion peak areas). Data reported as mean ± standard deviation (n = 12, 6 digestions with duplicate injections on MS). Peptide SAG is not shown because it is not detectable in this study. The target peptides are listed with their first three amino acid letters. Using Promega trypsin in digestion significantly

increased the mean peak area ($p < 0.05$, one-way ANOVA followed by Tukey's comparison).

3.4.2 Digestion time

A time-course trypsin experiment was designed to evaluate the digestion durations (4, 8, 16, and 20 hours) and the additional load of trypsin (single addition vs. two-addition) in trypsin's efficiency in generating WEP-specific peptides. The peak areas of the target peptides obtained from each of the four digestion durations were analyzed using the targeted MS method. The efficiency of trypsin digestion was evaluated by relative comparison among the obtained peak areas across four digestion durations. Interestingly, the results showed that the digest profiles were stable in cookies compared to the WEP samples (**Figure 3.5**). Therefore, digestion profiles were investigated in the WEP samples to determine the optimal digestion duration and addition of trypsin (**Figure 3.5**). When using a single addition of trypsin solution, most peptides reached their highest light-to-heavy ratio after 4 hours of digestion. As time progressed, the light-to-heavy ratio decreased. With two additions of trypsin solution, most peptides reached their highest light-to-heavy ratio after 8 hours of digestion, and the ratio decreased with further digestion time. One-way ANOVA and Tukey's comparison were employed to compare four digestion durations with two additions of trypsin. The results indicated that the light-to-heavy ratios of HIA, GGL, LPL, NTD, VMV, and MVV were significantly higher at 8 hours compared to 16 hours (data not shown). However, overnight digestion is preferred, considering the practical aspects of the sample preparation workflow (extraction, digestion, and desalting). The results showed that most peptides that were digested with

two additions of trypsin had greater peak areas than those that were digested with a single addition of trypsin. Taken together the length of digestion and obtained peak area, 16-hour digestion with two additions of trypsin solution at the 0 and 2-hour time points was used in the final method.

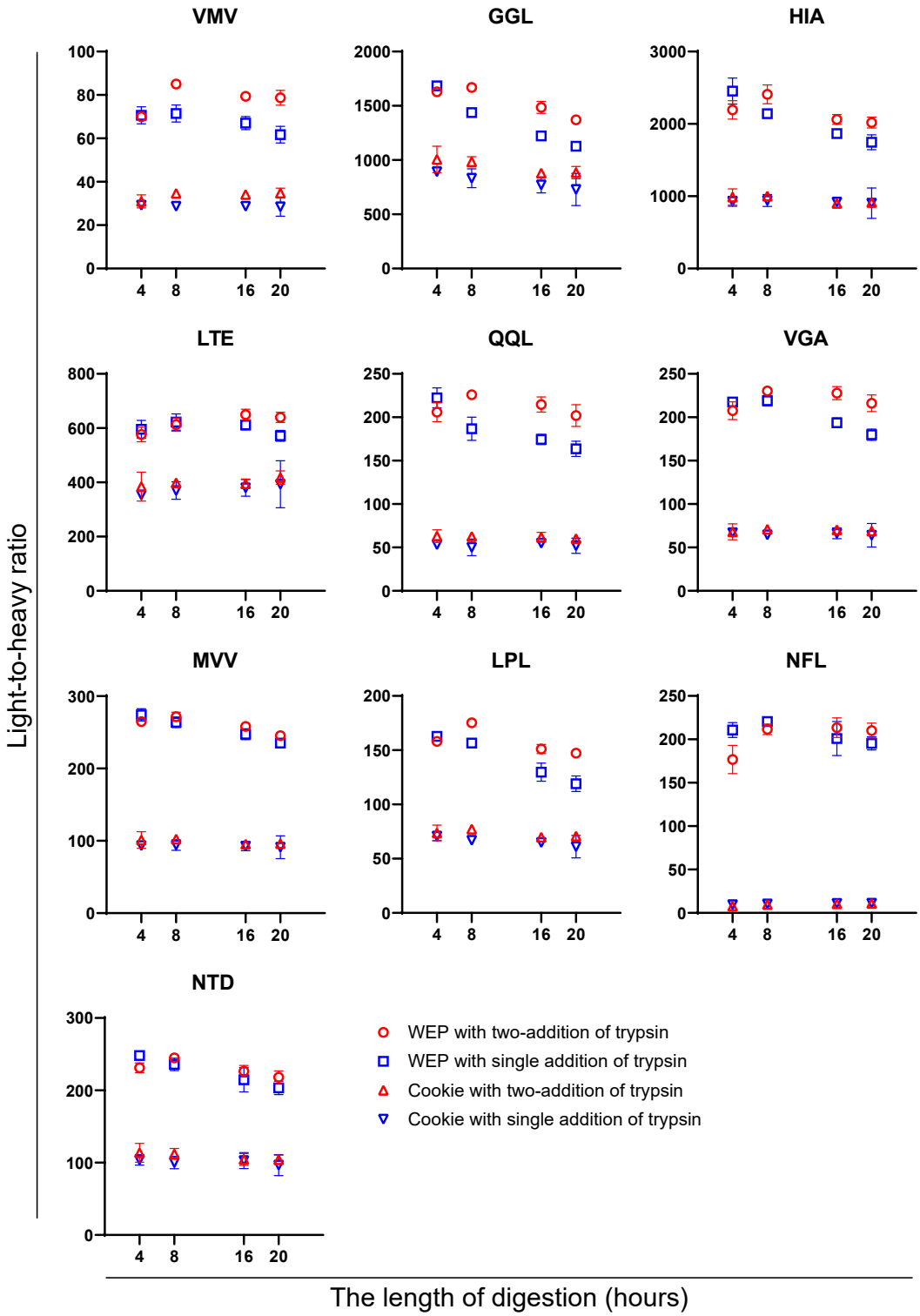

Figure 3.5 Comparison of the peak areas of target peptides with four trypsin digestion treatments. Whole egg powder (10000 ppm WEP, ○ and □) and cookies incurred with

10000 ppm WEP (△ and ▽) were digested for 4, 8, 16, and 20 hours. Symbols in red indicate that the samples were digested with two additions of trypsin. Symbols in blue indicate that the samples were digested with a single addition of trypsin. The target peptides are listed with their first three amino acid letters. Data reported as mean ± standard deviation (n = 4, 2 digestions with duplicate injections on MS).

3.4.3 Inclusion of carrier material in extraction buffer and diluent

According to initial observations, the recovery rate of peak areas of target peptides decreased when diluted in regular microcentrifuge tubes compared to LoBind® microcentrifuge tubes. This led us to suspect that the reduction of peak areas was due to the non-specific adsorption of peptides to plastic and glass containers during the sample preparation. The inclusion of carrier proteins during sample preparation, which act as competitive binding agents, has proven effective in reducing non-specific absorption.[25] The present study used non-fat dry milk powder (NFDM) as the carrier proteins. The effect of the carrier proteins was assessed by introducing an additional 0.1% (w/v) of NFDM in the protein extraction buffer, 0.1% of digested NFDM peptides in the diluent, and both in combination. As shown in **Figure 3.6**, most of the WEP-specific peptides obtained increased peak areas with the inclusion of NFDM peptide in the diluent and the combination of extraction buffer and diluent. For example, in the 250 ppm WEP diluted sample, the SAG peptides were undetectable without carrier proteins (**Figure 3.6A**). However, in the 500 ppm WEP diluted samples, the presence of NFDM peptides in the diluent dramatically increased the peak areas of the SAG peptides **(Figure 3.6B)**. By further investigating the variables across triplicate samples at 500 ppm WEP, the

inclusion of NFDM in the protein extraction buffer improved the precision of all target peptides except the NFL peptide. The range of the percent coefficient of variation (%CV) was 8.59 – 56.3% without the use of carrier proteins and 1.20 – 13.20% with the combination use of NFDM. The results showed that using a carrier protein in the extraction buffer and carrier peptides in the diluent increased the peak areas of 8 specific peptides by up to 21-fold ($p < 0.05$). Other studies have shown that carrier proteins improve the detection of peptides or proteins using MS analysis.[19,21,22] Specifically, Chen et al.[21] prepared an external calibration curve in the NFDM background to develop a soy quantitative MS method. The authors found the calibration curve displayed linearity, and the peak area of the soy target peptide detection increased with the presence of NFDM in the background.

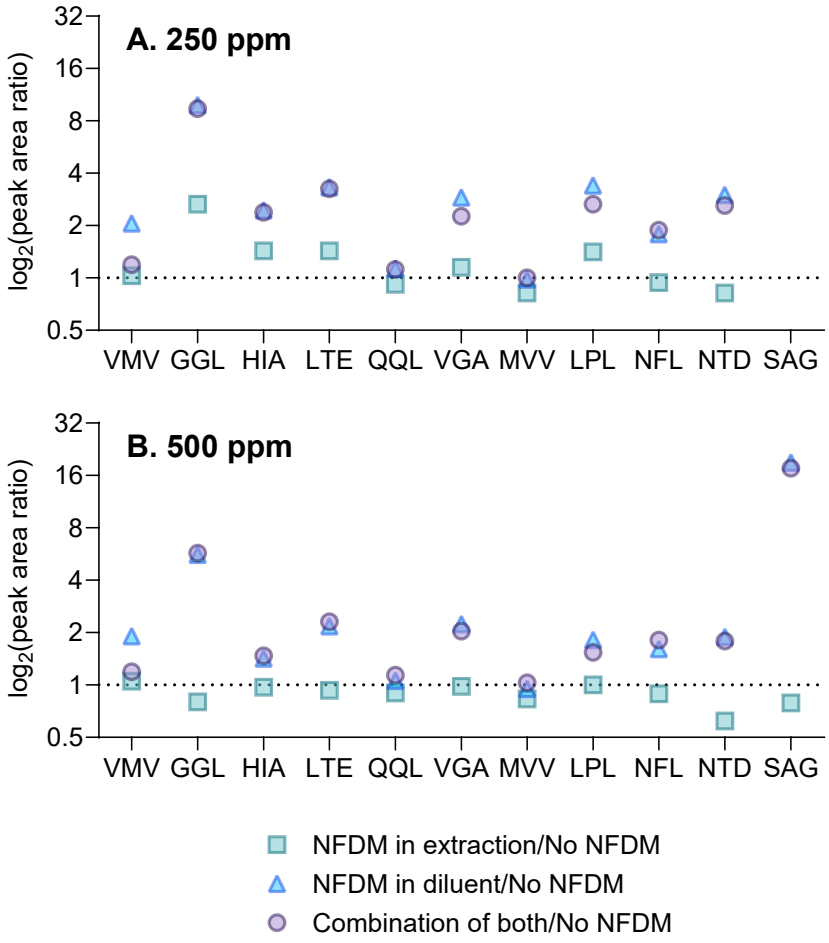

Figure 3.6 Comparison of the relative abundance of target peptides from the experiments with the inclusion of NFDM in extraction buffer (mint square), the inclusion of NFDM in diluent (artic blue triangle), and the combination of both (violet dot). The y-axis indicates log 2 (mean peak area of the peptide with the inclusion of NFDM in sample preparation divided by the mean peak area of the same peptide without the inclusion of NFDM). The x-axis lists all eleven target peptides with their first three amino acid letters.

3.4.4 Evaluation of detection of heavy peptides in the presence of NFDM in diluent

An experiment using WEP-specific heavy peptides (HP) was conducted to optimize peptide detection by determining the optimal amount of the NFDM peptide in the diluent. The peak areas of HP (5 fmoles of each HP on column) were measured in diluent with different concentrations of NFDM peptide ranging from 0 – 200 ng/µL. The results revealed that the concentrations of NFDM peptide in the diluent had a greater impact on the peak areas of some HP peptides than others (**Figure 3.7**). For example, the GGL peptide obtained the lowest peak area when no NFDM peptide was present in the diluent. As the concentration of NFDM peptide increased from 0 to 50 ng NFDM peptide/µL, the peak area of GGL also increased. However, within the 50 – 200 ng NFDM peptide/µL range, the peak area of GGL slightly decreased. However, the various concentrations of NFDM in diluent had less effect on detecting VMV, QQL, MVV, and NFL. Compared to other peptides, those peptides had relatively greater peak areas in the diluent absence of NFDM, and their peak areas were moderately stable with the presence of NFDM (**Figure 3.7**). All WEP-specific HP obtained maximum and stable detection with a carrier protein background of 50 - 100 ng NFDM protein/µL (**Figure 3.7**). The results suggest that the WEP-specific peptides are likely to bind non-specifically to surfaces and materials during dilution. The adopted method used 0.1% (w/v) NFDM in protein extraction buffer and 80 ng NFDM protein/µL diluent.

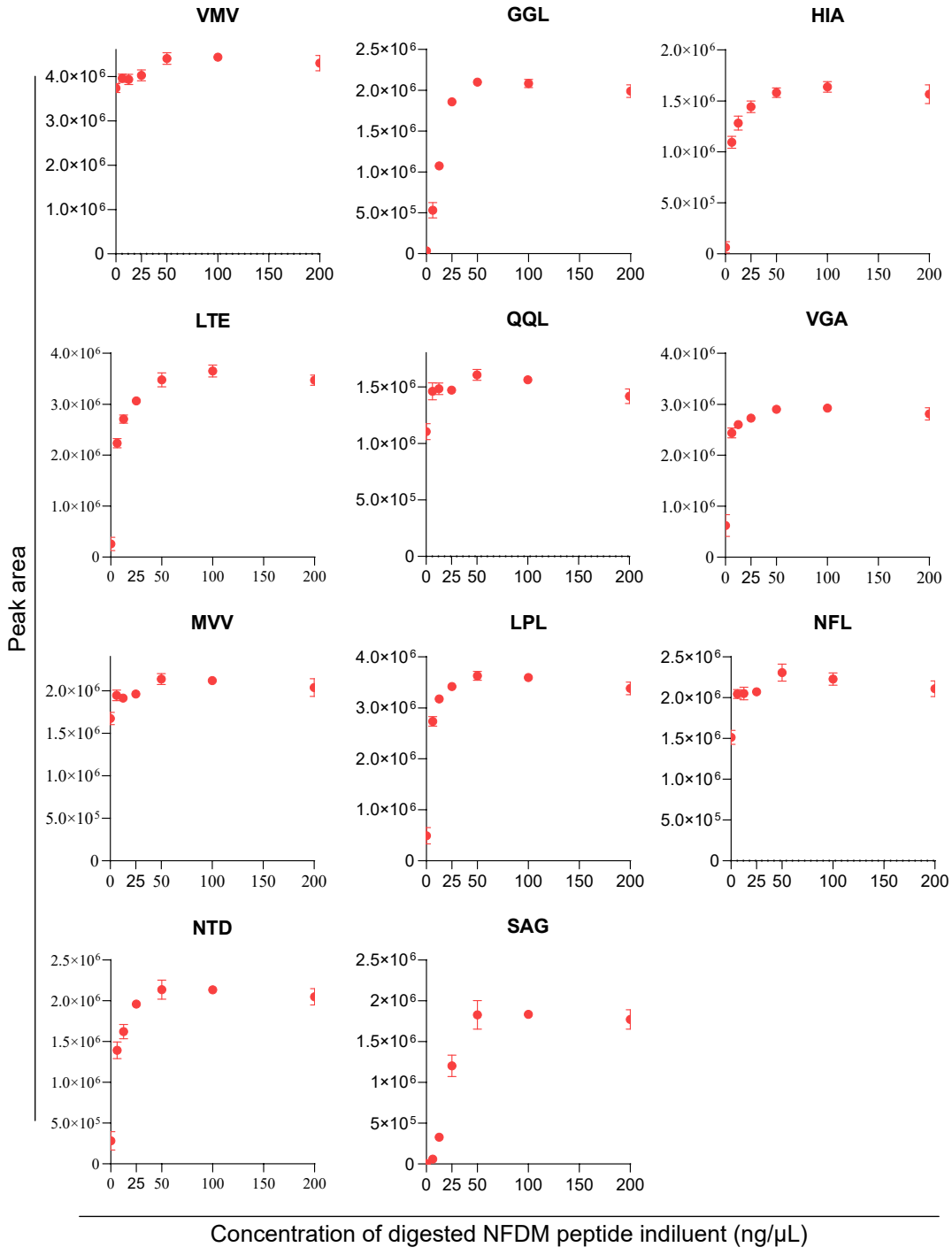

Figure 3.7 The detection of isotope-labeled peptides with different concentrations of NFDM peptide in the diluent. All eleven target peptides with their first three amino acid

letters were displayed. Data reported as mean ± standard deviation (n = 4, duplicate samples with duplicate injections on MS).

3.4.5 Comparison of three desalting columns

Peptide desalting columns often contain C18 resin or polymeric sorbent, which are used in reversed-phase chromatography to capture peptides and remove excess salts and other contaminants from samples. The desalting process is crucial in MS analysis because the presence of salts and contaminants can influence ionization, leading to reduced sensitivity of MS analysis.[26,27] In this study, three different commercial desalting columns, including the C18 spin column (C18), C18 High-capacity columns (C18-HC), and Strata-X columns (Strata), were compared. The heavy peptide (HP) peak areas were measured before (pre-HP) and after (post-HP) the desalting process. The percentage of HP recovery after desalting was calculated by dividing the peak area obtained from post-HP samples by the peak area obtained from pre-HP samples, then multiplying by 100. By averaging the percentage of HP recoveries across the eleven target peptides, the C18 columns had an average HP recovery of 42.8 ± 7.2%, C18-HC of 76.5 ± 4.3%, and Strata of 66.8 ± 9.0% (**Figure 3.8**). The use of Strata columns has advantages, such as simple processing and protein-binding capacity. The protocol can be easily adapted for desalting a larger digest volume (up to 1000 µL). A 12-well vacuum manifold was used for Strata columns, which may potentially cause variability during the desalting process because the sealing conditions vary from the operating wells. In contrast, despite the limitation of processing volume, the C18 and C18-HP columns were operated using a centrifuge. This way, they may control the speed and desalting procedures better, leading to reduced

batch-to-batch variations. Therefore, the C18-HP columns were used in the adopted method for peptide desalting.

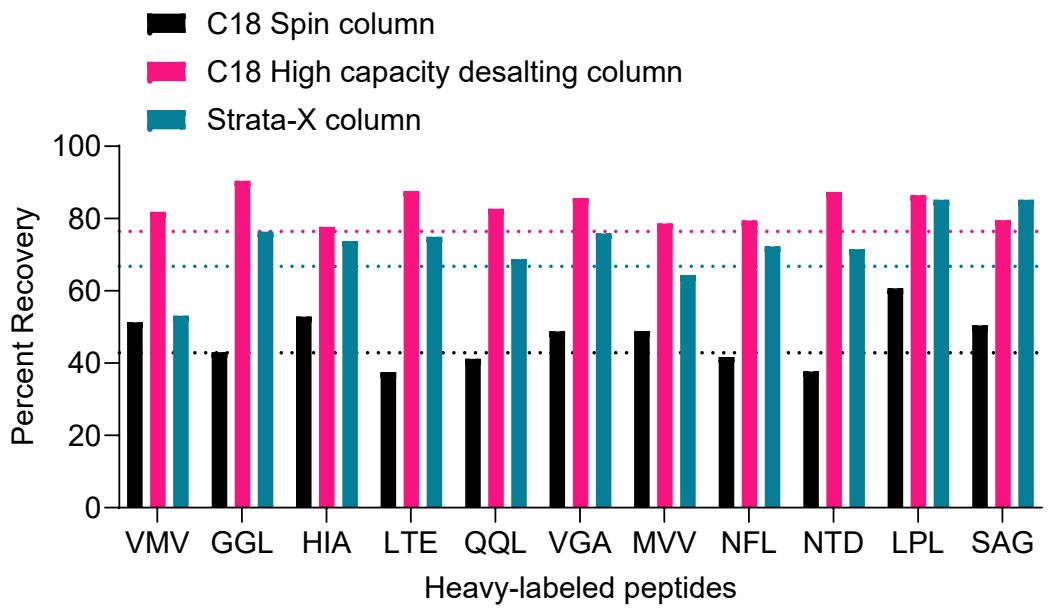

Figure 3.8 Comparisons of the percentage of heavy peptide recovery through three desalting columns. Black columns indicate the use of C18 spin columns, the pink columns indicate the use of C18 high-capacity desalting columns, and teal columns indicate Strata-X desalting columns. Percentage recovery was calculated by dividing the peak area of heavy peptide added before desalting by the peak area of heavy peptide added before MS analysis. The x-axis lists all eleven target peptides with their first three amino acid letters.

3.5 Summary

This chapter focuses on the optimization strategies for the sample preparation workflow to enhance the detection of 11 WEP-specific targets using the targeted MS method. The study examined the efficacy of two commercial MS-grade trypsin preparations, the inclusion of carrier material in both the protein extraction buffer and diluent, and three different commercial desalting columns. The selected condition for sample preparation employed 0.1% NFDM powder (w/v) in the protein extraction buffer. The 60 µL of the protein extract was digested with Promega trypsin solution for 16 hours, then desalted using the C18 high-capacity desalting columns. The desalted resultant peptides were then lyophilized and resuspended in 0.1% (v/v) FA and 5% (v/v) ACN water. For further sample or calibration dilution, the resuspended sample was diluted with a diluent containing 80 ng NFDM peptide/µL. All food samples and WEP calibrations for subsequent MS analyses were prepared using this optimized workflow.

The final method used an increased sample volume for MS analysis due to the MS method's sensitivity limiting the number of peptides analyzed per injection. An increased amount of sample extract was used for trypsin digestion fivefold to increase the concentration of peptides in the sample. To adapt to the increased total peptide to be analyzed, the LC column with a larger diameter was opted to use that can separate peptides in a shorter time (35 minutes) at a higher flow rate.

The enhanced sensitivity of the MS method, achieved through these optimizations, is assessed in both longitudinal WEP calibration curves and incurred food matrices, as discussed in the next chapter.

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CHAPTER 4

METHOD VALIDATION OF AN EGG-SPECIFIC TARGETED MASS SPECTROMETRY METHOD FOR QUANTIFICATION OF TOTAL EGG PROTEIN IN PROCESSED FOOD MATRICES

4.1 Abstract

Eggs are among the most common food allergens. They are ubiquitous ingredients that can be used in whole, white, and yolk forms in the food industry. Accurately quantifying total egg proteins in processed foods is critical for food safety and allergen management. This study aimed to develop and validate a mass spectrometry (MS) method using parallel reaction monitoring (PRM) for the quantification of low levels of total egg protein in diverse processed food matrices. Quantification was achieved using an external calibration curve of whole egg powder with stable-isotope-labeled targeted peptides as internal standards, ranging from 0.12 to 47 ppm total egg protein. Method performance was assessed through the consistency of detection across 18 independent WEP calibration curves and the sensitivity of target peptides in five food matrices (cookie, pie crust, pasta, ice cream, and retorted pasta soup) incurred with various concentrations of total egg protein. Results demonstrated that nine egg-specific target peptides reliably quantified total egg protein in processed food matrices, with limits of quantification between 1.18 and 4.7 ppm. The MS method effectively quantified total egg protein in all tested food matrices at concentrations relevant to allergen risk-based evaluation. Compared to four commercial egg ELISA kits, the MS method significantly enhanced protein recovery in cookies, pie crust, and retorted pasta soup. Protein recovery using the MS method ranged from 57.5% to 96.5% across various food matrix models,

including baking, drying, freezing, and retorting. Overall, the results demonstrated that this targeted MS method is egg-specific, robust, and sensitive. The method shows potential to be a reliable tool to improve allergen management and ensure food safety.

4.2 Introduction

Food allergy is an IgE-mediated hypersensitivity immune response to one or more specific proteins,[1] affecting approximately 4.3% of the overall population worldwide.[2–4] The awareness of food allergies has been growing with an increased prevalence of food allergies in recent years. FAO/WHO expert consultant recently confirmed that eggs and egg products are one of the global priority food allergens due to their high prevalence in preschool children, the high proportion of anaphylaxis, and median potency.[5,6] Egg can cause mild (e.g., hives, swelling, cramps, etc.) to severe allergic reactions (e.g., anaphylaxis). Egg allergy can develop asthmatic symptoms in children and cause more gastrointestinal symptoms than other food allergens.[7,8] Once diagnosed, lifelong avoidance of eggs and egg-derived products is the primary clinical advice for preventing allergenic reactions because there is no cure for food allergy.[1] Therefore, patients with egg allergies rely on reading allergen statements or ingredient lists on packaged food labels to understand the presence of eggs. A clear and reliable allergen statement on packaged food is the first guard to protect individuals with food allergies.

In the U.S., eggs are one of the “Big-nine” major food allergens, according to the U.S. Food Labeling and Consumer Protection Act (FALCPA).[9] As a major food allergen, packaged foods containing eggs or egg-derived ingredients must follow the food allergen labeling regulations and be labeled in clear language on the package. However, no labeling regulation exists to cover the unintentional introduction of food allergens due to

cross-contact (e.g., inadequate cleaning, use of shared facilities and equipment, and rework of allergen-containing material). Nevertheless, food industry uses voluntary Precautionary Allergen Labeling (PAL), also known as "may contain" labeling, to communicate potential allergenic ingredients in food products.[10] However, PAL has been overused worldwide for 30% - 64% of food products, and the expressions of PAL are unhelpful to food allergy consumers.[10–14] Studies have revealed that there is no relationship between expressions of PAL and the amount of food allergen present in food.[15,16] Taken together, there is an urgent need to use standardized PAL.

Risk-based approaches have been established to provide informative guidance for the usage of PAL. The Allergen Bureau in Australia first introduced Voluntary Incidental Trace Allergen Labeling (VITAL) using the Reference Dose (RfD) to determine possible levels of allergen in processed foods.[17,18] The food allergen RfD was established based on the Eliciting Doses (ED) of food-allergic patients in clinical food challenges.[18,19] In 2019, VITAL version 3 (VITAL 3.0) updated the RfD of 14 major food allergens to comply with the ED_{01}, meaning it is safe for 99% of individuals with food allergies when the exposure dose of the allergenic protein is below the RfD,[18] Three years later, the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO) expert consultant recommended a higher RfD based on the ED_{05}, which is safe for 95% of individuals with food allergies.[20] This higher RfD was determined considering the balance between the severe risk of fatality and the feasibility of the current allergen detection methods.[20] An action level (AL) is calculated by knowing the RfDs of food allergens and food consumption, indicating the total protein concentration from the allergenic source per kg of food consumed. Accurately measuring the protein

concentration in food products within estimated consumption quantities can guide the food industry and regulatory agencies in quantifying the risk of food products containing unintended allergenic ingredients.[19]

Reliable and accurate allergen detection tools are crucial to implement the risk-based PAL system. Different research groups have developed several mass spectrometry (MS)-based methods to detect and quantify eggs in processed food.[21–29] Studies have shown that these MS-based methods enhance the detection and improve quantification of egg protein in complex food matrices such as cookies, chocolate, and mayonnaise.[21–27] However, all current egg MS-based methods, except one[27], are developed for multi-allergen detection. In addition, only two to five egg peptides were analyzed using the multi-allergen MS method. These peptides originate mainly from egg white proteins, specifically ovalbumin and/or lysozyme, with fewer peptides originating from egg yolk proteins being evaluated in the incurred food samples. It should be noted that eggs are widely used food ingredients that can be incorporated into recipes in various forms, such as whole eggs, egg whites, and egg yolks. Moreover, purified proteins derived from eggs are also used in food products, such as lysozyme in wine. Two MS-based methods have been developed specifically for milk and egg white protein(s) in wine.[28,29] Therefore, it is essential to have an MS-based method specific to eggs that can be applied to a broad range of food products.

In a previous study, we used a discovery-based approach to identify ten target peptides from whole egg powder (WEP)-incurred food matrices. We optimized the sample preparation and MS parameters to improve the sensitivity of the MS method. Eventually, we developed an egg-specific Parallel Reaction Monitoring MS method

(PRM-MS) for quantifying egg protein in processed food matrices. The present study aims to evaluate the performance of the PRM-MS method in foods. First, the stability of the PRM-MS method was evaluated by analyzing 18 independent WEP calibration curves that were analyzed across eight months. Second, the sensitivity of each peptide was evaluated in five food matrices (cookie, pie crust, pasta, ice cream, and pasta soup) incurred with various concentrations of egg protein (0.4 – 67.3 ppm). Finally, the performance of the PRM-MS method was compared with four commercial egg ELISA kits by the average protein recoveries in the incurred food matrices.

4.3 Materials and Methods

4.3.1 Preparation of whole egg powder incurred food matrix

4.3.1.1 Preparation of concentrated whole egg powder carrier materials

Flour and sugar from the food formulation were used as carrier materials to introduce WEP into the food matrix. The preparation of WEP flour and WEP-sugar carrier material was described in Chapter 2, Section 2.1.2. To achieve incurring various levels of WEP for cookies, pie crusts, and pasta, six concentrated carrier materials, ranging from 4000 to 4 ppm WEP, were prepared sequentially by mixing the desired amount of the higher concentration of the WEP-flour carrier materials with blank flour (**Table 4.1**). To prepare WEP-incurred ice cream, six concentrated carrier materials, ranging from 5769 to 6 ppm WEP, were prepared sequentially by mixing the desired amount of the WEP-sugar carrier materials with blank sugar (**Table 4.2**). WEP-flour mixtures were freshly prepared per food matrix and stored at 4°C until use. Between the preparation of different levels of WEP-flour carrier materials, all utensils and benchtops were properly cleaned to avoid carryover.

The highest (level A) and lowest (level G) of the WEP-flour carrier materials and WEP-sugar carrier materials were evaluated for their homogeneity before food matrix preparation. Eight 1 ± 0.1 g of the carrier materials were extracted (1:25 w/v) and analyzed in duplicate wells using Neogen® Veratox Egg ELISA kits (catalog No. 8450) following the manufacturer's instructions. The average coefficient of variation (%CV) of 16 data points below 25% demonstrated the acceptable homogeneity of the WEP-flour mixture.

Table 4.1 Preparation of six concentrated WEP-flour carrier materials to make various WEP-incurred levels in cookies, pie crusts, and pasta

To make incurred level at (ppm WEP)	Actual ppm of WEP-flour carrier material (ppm WEP)	Previously incurred mixture (g)	Egg-free flour* (g)	Total WEP-flour mixture (g)
10,000	40,000 (A)	30 of WEP	720	750
1000	4000 (B)	70 of A	630	700
100	400 (C)	75 of B	675	750
10	40 (D)	70 of C	630	700
5	20 (E)	35 of C	665	700
2.5	10 (F)	17.5 of C	682.5	700
1	4 (G)	70 of D	630	700

WEP, whole egg powder

* The wheat flour tested negative (below 2.5 ppm WEP) through Neogen® Veratox Egg ELISA kits.

Table 4.2 Formulation of concentrated WEP-sugar mixture to make various WEP-incurred levels in ice cream

To make incurred level at (ppm WEP)	Actual ppm of WEP-sugar carrier material (ppm WEP)	Previously incurred mixture (g)	Egg-free sugar* (g)	Total WEP-sugar mixture (g)
10,000	57,691 (A)	17.31 of WEP	282.69	300
1000	5769 (B)	30 of A	270	300
100	577 (C)	30 of B	270	300
10	58 (D)	30 of C	270	300
5	29 (E)	15 of C	285	300
2.5	14 (F)	7.5 of C	292.5	300
1	6 (G)	30 of D	270	300

WEP, whole egg powder

* The sugar tested negative (below 2.5 ppm WEP) through Neogen® Veratox Egg ELISA kits.

4.3.1.2 Preparation of whole egg powder-incurred cookie, pie crust, pasta, and ice cream

Sugar cookies,[30] pie crust,[31] pasta,[32] and ice cream were incurred with concentrations of 0, 1, 2.5, 5, 10, and 100 ppm WEP, following the existing AACC recipes or manufacturers. The detailed procedures for the preparation of cookies, pie crust, pasta, and ice cream are described in Chapter 2, Sections 2.1.3 to 2.1.6.

To make various WEP-incurred levels of cookies and pie crust, 500 g of the flour in the formulation was replaced by an equal amount of desired concentrated WEP-spiked flour (C to G, **Table 4.1**) to make 2000 g of WEP-incurred dough on a wet basis (ug/g of total mass). Cookie squares (2 x 2 cm squares with 0.6 cm thickness) were baked at 205°C for 10 minutes in the Reel oven. The pie crust squares (2 x 2 cm squares and 0.3

cm thickness) were baked at 190°C for 20 minutes in the Reel oven, with the tray rotated at 10 minutes. The baked cookies and pie crusts were weighed before and after baking to calculate water loss. Approximately ~100 g of the cookies and pie crusts were ground into a fine powder using a food processor and stored at -20°C until further use.

Five WEP-incurred levels of pasta (1 to 10000 ppm WEP) were prepared by mixing 300 g of the desired concentrated WEP-spiked flour (A to G, **Table 4.1**) with 900 g of blank flour on a dry basis (µg/g of total mass). The pasta dried in an environmental chamber at 70°C for 10 hours and at 40°C for another 12 hours with 65% humidity. The pasta was weighed before and after baking to calculate water loss. The dried pasta was ground into a fine powder using a coffee grinder and stored at -20°C until further use.

Ice cream was incurred with five WEP levels by mixing 150 g of the desired WEP-spiked sugar (C to G, **Table 4.2**) with the rest of the ingredients in the formulation to ice cream on a wet basis (µg/g of total mass). The mixture of all ingredients was cooled at 4°C for two hours, followed by the operations using the Cuisinart® ice cream maker (Model Ice-21) for 20 minutes. The final ice cream was dispensed into 50 mL Falcon tubes and stored at -20°C until further use.

4.3.1.3 Preparation of whole egg powder-incurred retorted pasta soup

The dry pasta incurred with 0, 10, 100, 1000, and 10,000 ppm WEP was used to prepare retorted pasta soup. The desired WEP-incurred dry pasta was first broken into approximately 5 cm long pieces to permit free movement in boiling vegetable stock. First, vegetable stock was preheated to reach 82°C in the glass jar. Then, 25 g of the small pieces of dry pasta were added to 250 mL of preheated vegetable stock and finger-tightened the lid. Subsequently, the sealed jars were placed on the rack in the pressure

cooker (The All American® Pressure Cooker, Model 921). The pasta soups were retorted at 15 pounds pressure and cooked at 118°C for 15 minutes. The pasta soups were homogenized using a blender to make final concentrations of 0, 0.9, 9, 91, and 909 ppm WEP. The homogenized pasta soup was dispensed into 50 mL Falcon tubes and stored at -20°C until further use.

4.3.2 Sample preparation for MS analysis

4.3.2.1 Food Sample Preparation

Sample preparation before MS analysis used MS-graded chemicals and reagents. Urea, dithiothreitol (DTT), optima water, methanal, and acetonitrile (ACN) were purchased from Thermo Fisher Scientific (Waltham, MA). Thiourea, Trizma Hydrochloride, Ammonium Bicarbonate (ABC), and Iodoacetamide (IAA) were purchased from Sigma-Aldrich (St.Louis, MO). Sequencing-grade modified trypsin (Promega) was used in peptide digestion.

Five levels of the WEP-incurred food samples (1 ± 0.01 g) were extracted (1:20 w/v) using chaotropic buffer (2 M Thiourea, 6 M Urea, 50 mM Tris-HCL, pH 8.9, and 20 mM DTT) with the addition of 0.1% (w/v) nonfat dry milk powder (NFDM), in triplicate. The protein extraction was incubated (10 min in a 60°C shaking water bath), resuspended (1 minute by vortex), and sonicated (10 minutes in a 60°C shaking water bath). Subsequently, 1000 µL of protein extract was centrifugated at 17,000 g for 10 minutes at room temperature. Then, 60 µL of supernatant of the protein extract was transferred into a LoBind® Eppendorf microcentrifuge tube. The extracted proteins were reduced with 22.5 µL of 100 mM DTT, 225 µL of 50 mM of ABC, and 97.5 µL of MS-grade water for 5 minutes in a 95°C hot block, followed by 20 minutes of alkylation with 45 µL of 50

mM IAA in the dark. The alkylated proteins were digested with 15 µL of trypsin solution (100 ng/µL) at 37°C for two hours and an additional 15 µL of trypsin solution at 30°C overnight to achieve 16 hours of digestion. The digested peptides were cleaned up with Pierce ™ peptide desalting spin columns (catalog No. 89852) following the manufacturer's instructions. The resultant peptides were eluted in 600 µL of elution buffer (55% ACN in water, v/v) and frozen at - 80°C for 60 minutes. Lastly, the frozen eluate was lyophilized in a SpeedVac vacuum evaporator (Model SPD120). The lyophilized peptides were stored at - 20°C until further analysis. The lyophilized peptides were resuspended with 50 µL of 5% (v/v) ACN and 0.1% (v/v) FA in water before MS analysis. A diluent that contains 80 ng NFDM peptide/µL in 5% (v/v) ACN and 0.1% (v/v) FA water was used to prepare the calibration curve and further dilute the resuspended samples if needed. The WEP-incurred food samples were analyzed using triplicate test portions, with each test portion subjected to duplicate injections for MS analysis (n = 6).

4.3.2.2 WEP calibration curve preparation

WEP and NFDM were used as calibrant and carrier peptides in the diluent, respectively. First, 0.04 g of WEP and 0.1 g of NFDM were extracted. Following the WEP protein extraction, the WEP extract was diluted with protein extraction buffer to make the concentration 0.5 µg of WEP/µL equivalent to the WEP concentration in the WEP-incurred food sample extract at 10,000 ppm WEP. Then, the diluted WEP and NFDM extracts were carried through the reduction, alkylation, desalting, and lyophilization procedures along with the food samples. To prepare a calibration curve, the lyophilized WEP and NFDM were resuspended with 50 µL and 1518.7 µL of 5% (v/v)

ACN and 0.1% (v/v) FA in water. The resuspended NFDM with a final concentration of 80 ng of NFDM peptides/µL was used as the diluent and as the 0 ppm of the calibration curve. The WEP calibration curves were serially diluted to make the concentrations ranging from 0.12 to 470 ppm total egg protein.

4.3.2.3 Internal Standard Preparation

Stable isotope-labeled target peptides (referred to as heavy peptides hereafter) were synthesized by Thermo Fisher Scientific with > 95% purity (AQUA™ Basic grade). First, each heavy peptide aliquot was resuspended in 300 µL of 50% ACN in water to make a final 10 pmol/mL concentration. Subsequently, 10 µL of each of the eleven heavy peptides (total of 100 pmoles) was pooled into a LoBind tube (Eppendorf) and mixed with 90 mL of 50% ACN to make a final concentration of 500 fmoles of each heavy peptide per µL. To avoid freeze-thaw cycles, 7 µL of the heavy peptide stock mixture was aliquoted into multiple 0.5 mL lo-bind microcentrifuge tubes and stored at -20°C. Before the MS analysis, 5 µL of the heavy peptide stock was mixed with 45 µL of 0.111% (v/v) formic acid and 450 µL of 5% (v/v) ACN and 0.1% (v/v) FA in water to make a final concentration of 5 fmoles of each peptide per µL heavy peptide working solution. Prior to the injection for MS analysis, 36 µL of each resuspended sample or calibrant was mixed with 3 µL of the heavy peptide working solution in the HPLC vial. The injection volume was 13 µL to achieve 5 fmoles of each heavy peptide on the column.

4.3.3 Targeted mass spectrometry analysis

Peptide separation was performed on a Thermo Fisher Dionex UltiMate 3000 RS ultrahigh performance liquid chromatography (UHPLC) system at 0.3 mL/min flow rate. The temperature-controlled autosampler was kept at 5°C, and the thermostat column

compartment was kept at 35°C. The column was a Hypersil GOLD C18 Selectivity LC Column (100 mm × 2 mm, 1.9 μm, 175 Å). A UHPLC filter cartridge (1 mm ID, 0.2 μm) was attached to the column. The mobile phase consisted of solvent A: 0.1% (v/v) FA in water and solvent B: 0.1% (v/v) FA in ACN. The gradient program was conducted as follows: 0 – 3 minutes: 2% – 12.5% of solvent B; 3 – 25 minutes: 12.5 – 27.5% of solvent B; 25 – 26 minutes: 27.5 – 98% of solvent B; 26 – 30 minutes: 98% of solvent B; 31 – 35 minutes: 98 – 2% of solvent B. From 0 – 26 minutes, the flow rate was 0.3 mL/min. From 26 – 35 minutes, the flow rate was 0.35 mL/min.

Following peptide separation, a targeted MS analysis was performed using Thermo Scientific™ Q Exactive™ Plus hybrid quadrupole-Orbitrap™ mass spectrometer with parallel reaction monitoring (PRM) in positive ionization mode. Source parameters were set as follows: sheath gas flow rate of 50 arbitrary units (au), aux gas flow rate of 10 au, positive ion spray voltage of 3500 V, heated electrospray ionization (HESI) capillary temperature of 320°C, S-lens RF of 60 au. For PRM-MS, parameters were set: resolution of 70,000, automatic gain control (AGC) of 1×10^6, and maximum injection time (IT) of 240 milliseconds. The mass isolation width for the precursor of interest was 0.8 m/z with no offset. The normalized collision energy was 27. Three selected product ions of the target peptides were monitored post-acquisition. The performance of the peptides was evaluated using Skyline Software.[33]

4.3.4 Detection criteria for positive detection

False-positive detection can occur due to interference from food matrix backgrounds. Objective criteria were established to evaluate the peptide's signal quality and performance and to determine the positive detection of the peptide. In the study, data

metrics generated in Skyline software were used to develop detection criteria for assessing the performance of the peptide signal and determining a positive detection of the peptide. The detection criteria involved four numerical metrics, including peak found ratio (PFR) = 1, library dot product (dotp) ≥ 0.8, reference dot product (rdotp) ≥ 0.8, and mass tolerate < |± 5 ppm|. The PFR =1 indicates that all three monitored product ions were detected; the dotp and rdotp score the correlation between the product ion ratios of the native peptide to the product ion ratio in the library spectrum (from previous DDA results) and its heavy peptide, respectively. Furthermore, these performance metrics can be easily applied to determine whether a peptide should be included or excluded from the calibration curve.

4.3.5 Comparison with egg-specific ELISA kits

BioFront MonoTrace™ Egg (limit of quantification (LOQ) of 1 ppm whole egg powder, catalog NO. EOM-EK-96), Morinaga Egg (Ovalbumin) (LOQ of 0.31 ppm egg protein, catalog NO. M2111), r-biopharm RIDASCREEN® Egg (LOQ of 0.25 ppm whole egg powder, catalog NO. R6411), and Neogen® Veratox Egg (LOQ of 2.5 ppm whole egg powder) were used for the comparison with the performance of the MS methods. The unprocessed dough of cookie, pie crust, pasta, and processed foods of all five food matrices incurred at 0, 4.7, and 47 ppm total egg protein were extracted and conducted for ELISA analysis following each of the kits' instruction manuals. The food samples were analyzed with duplicate extractions on duplicate wells in ELISA.

4.3.6 Statistic analysis

GraphPad Prism was used to perform one-way ANOVA, followed by Tukey's comparison. Nested ANOVA and percent relative standard deviation (%RSD) were calculated in R.

4.4 Results and Discussion

4.4.1 Sensitivity assessment of the calibration curve

External factors, such as system suitability, environmental conditions, sample preparations, etc., can impact the sensitivity of an MS method. To evaluate the external impacts on the overall performances of the MS method, we conducted 18 WEP calibration curves from nine independent experiments across eight months (August 2023 – March 2024). Two WEP replicate extracts were prepared on the same day for each independent experiment with identical reagents, buffers, etc. The calibration curves were diluted from the resulting WEP digests to make ten total egg protein concentrations from 0.12 to 47 ppm and a 0 ppm total egg protein calibration point (i.e., the diluent). Ten egg-specific target peptides and their heavy peptides were analyzed using the targeted PRM-MS method.

4.4.1.1 Detectability of the calibration curve

The probability of detection (POD) model based on the Bernoulli statistical analysis was designed to validate qualitative methods for the detection of microorganisms or chemical contaminants.[34] In the present study, the POD model was used to assess the performance of the PRM-MS method with 10 target peptides in the inclusion list from WEP calibration curves. Because the POD model is limited to binary output, the detection criteria described in section 4.2.4 were used to determine the 1 (positive) or 0

(negative) result of the peptide across ten calibrant concentrations. At each concentration, a peptide that met all four detection criteria was counted as one positive event. The probability of positive events among 18 independent WEP calibration curves (i.e., 36 total MS measurements) across the calibrant concentrations was plotted to draw POD curves (**Figure 4.1**).

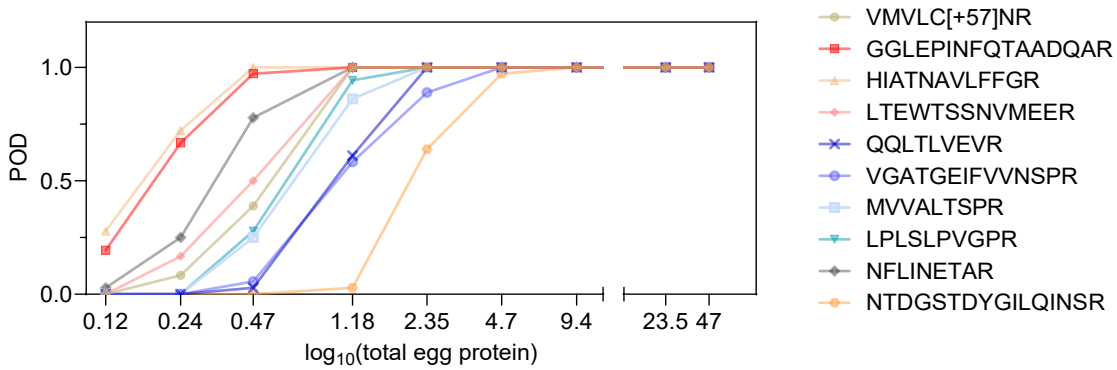

Figure 4.1 Probability of detection curve (POD) of ten target peptides across total egg protein concentrations. The POD curve of a longitude whole egg powder calibration curves ranging from 0.12 to 47 ppm total egg protein. The calibration curves were conducted on 18 independent samples across eight months with duplicate injections for MS analysis (n = 36). POD =1 indicates that the peptide is detectable in 36 MS measurements.

Because the POD curve was constructed using a log scale on the x-axis to distribute the ten calibrant concentrations better, the POD at the 0 ppm samples was not shown (**Figure 4.1**). Most target peptides were not detected in all 36 measurements (POD = 0) at 0.12 ppm total egg protein. At this concentration, the GGL, NFL, and HIA peptides obtained seven, one, and ten positive events, respectively, out of 36 measurements. Among these three peptides, for GGL and NFL, there were no positive detection events in the 0 ppm samples. As illustrated in example chromatograms at 0 and 0.12 ppm, the GGL and NFL delivered a clean background in the 0 ppm sample, while some 0.12 ppm samples delivered positive detection satisfying the detection criteria (**Figure 4.2 A and B)**. Even though some product ion signals can be observed within the schedule window, peptides that missed one or two product ions were reported as negative detection (**Figure 4.2**). These results also revealed that it is possible to obtain some positive detections at low concentrations of total egg protein mainly because of reaching the limit of the sensitivity of the method. Surprisingly, the HIA peptides delivered 2 out of 36 positive detections in the 0 ppm samples (**Figure 4.2**). These unexpected results were suspected to be peptide carryover from the previous injection, which was confirmed with further carryover experiments.

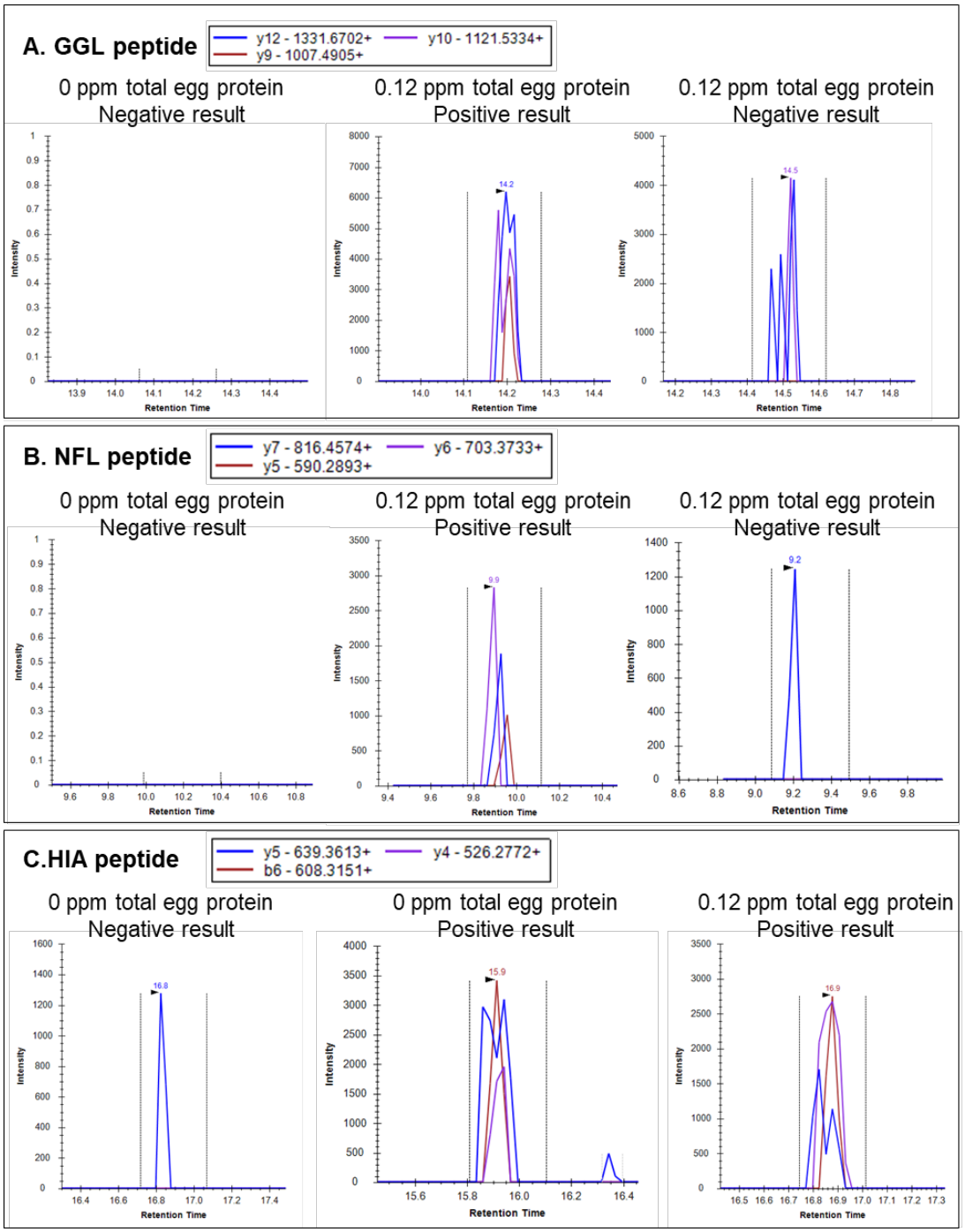

Figure 4.2 Example chromatogram for GGL (A), NFL (B), and HIA (C) peptides in 0 and 0.12 ppm total egg protein samples. The peptide signal that met the detection criteria was considered a positive result; otherwise, it was a negative result. The detection criteria include peak found ratio = 1, library dot product ≥ 0.8, reference dot product ≥ 0.8, and mass tolerate < |± 5 ppm|.

Therefore, using a statistical-based POD approach to estimate the limit of detection (LOD) allows for statistical confidence in the positive detection at LOD. A modified Wilson method (R package 'binom') was used to estimate the binomial 95% confidence interval (CI) based on the calculation of POD at each concentration. If the POD is 0.94, indicating 34 positive detections out of 36 total measurements, a lower bound of the 95% CI is greater than 80%. In this study, therefore, the calibrant concentration with POD ≥ 0.94 was estimated as LOD for the peptide. Our results showed that all peptides had LOD ≤ 4.7 ppm total egg protein (**Table 4.3**), and the lowest LOD was 0.47 ppm total egg protein for the GGL and HIA peptides.

Furthermore, an assessment was conducted to determine whether the LOD of the MS method complies with the risk-based ALs of five food matrices. To calculate the ALs, the reference doses of the FAO/WHO (ED05, 2 mg total egg protein) or the VITAL 3.0 (ED01, 0.2 mg total egg protein) were divided by estimated food consumption.[18] The quantity of food consumed for each of the food matrices (cookies, pasta, ice cream, and reported pasta soup) was based on the 75th percentile food consumption (P75) provided in the FAO/WHO meeting report (**Table 4.4**).[20] It is noted that the P75 was determined by food consumption distribution, which was created based on data from three European countries' food consumption surveys, which were published by Birot et al.[35] Because Birot et al.'s food consumption data did not include pie crust,[35] the consumption of pie crust used to calculate AL in this study was based on the servicing size of a toaster pastry (96 g). In **Table 4.4**, the ALs were reported as ppm total egg protein in each food matrix, referring to mg total egg protein from whole egg powder per kg food. The results found that all ten target peptides had LOD ≤ 4.7 ppm total egg protein, which is lower than the

FAO/WHO-suggested ALs of all five food matrices. Six of ten peptides are detectable at 1.18 ppm total egg protein, meeting the VITAL-suggested ALs of cookies, pie crusts, pasta, and ice cream. The GGL and HIA were detectable at 0.47 ppm total egg protein, meeting the VITAL 3.0-suggested ALs for pasta soup.

Table 4.3 Summary analytical features of the developed method according to 18 independent calibration curves (n = 36).

Peptide[a]	Qualitative analysis[b]		Quantitative analysis[c]					r^2
	LOD	Lower 95% CI (no. of positive results)	LOQ	RSD (i)	Relative Weight[d] D	DR	M	
VMV	1.18	90.4% (36)	2.35	26.3%	79.8%	17.0%	3.2%	0.9477
GGL	0.47	85.8% (35)	1.18	22.6%	76.3%	14.8%	8.9%	0.9739
HIA	0.47	90.4% (36)	9.4	24.2%	80.1%	14.1%	5.8%	0.9545
LTE	1.18	90.4% (36)	1.18	26.0%	71.7%	14.8%	13.5%	0.9704
QQL	2.35	90.4% (36)	4.7	21.2%	48.5%	22.8%	28.7%	0.9697
VGA	4.7	90.4% (36)	4.7	22.0%	82.9%	11.6%	5.5%	0.9682
MVV	2.35	90.4% (36)	2.35	24.3%	84.9%	10.2%	4.9%	0.9782
LPL	1.18	81.9% (34)	1.18	28.6%	66.7%	10.9%	22.4%	0.9604
NFL	1.18	90.4% (36)	1.18	23.0%	63.3%	28.9%	7.9%	0.9667
NTD	4.7	85.8% (35)	4.7	27.5%	56.6%	24.4%	19.0%	0.9542

[a] The abbreviation of the peptide is three initial amino acid letters of the sequence.
[b] The qualitative analysis was conducted based on the probability of detection (POD) across 18 independent calibration curves (36 measurements). The POD ≥ 0.94 obtained a lower 95% confidence interval (CI) above 80%. The limit of detection (LOD) was determined at the calibrant concentrations with POD ≥ 0.94.
[c] At each calibrant concentration, the relative standard deviation [RSD(i)] was calculated across 36 measurements using nested ANOVA analysis (R package, fitVAC). The concentration that had RSD(i) ≤ 30% was determined as the limit of quantification (LOQ) of the peptide.
[d] Relative weight indicates the relative contribution of the day of analysis (D), test portion replicates (DR), and measurement (M).
r^2 was calculated using simple linear regression on GraphPad Prism.
The LOD and LOQ were reported in ppm total egg protein.

Table 4.4 The estimation of action levels (mg total egg protein from allergenic source per kg food consumption) according to the FAO/WHO and VITAL 3.0 references dose of total egg protein.

Food matrix	Food consumption (g)	FAO/WHO 2 mg total protein from an allergenic source	ED01 (VITAL 3.0) 0.2 mg whole egg protein
Cookie[a]	50	40	4
Pie crust[b]	100	20	2
Pasta	200	10	1
Ice cream	100	20	2
Pasta soup	400	5	0.5

VITAL, voluntary incidental trace allergen labeling
[a] The 75th percentile of food consumption of cookies is 42g. In this study, cookies consumption was rounded up to 50 g.
[b] There was no matching food group for pie crust in Birot et al.'s food consumption data.[35] Therefore, the pie crust's consumption was based on the servicing size of a toaster pastry (96 g).

4.4.1.2 Consistency of the calibration curve

To evaluate the consistency of the PRM-MS method, a combined WEP calibration curve derived from 18 independent WEP calibration curves was plotted by ppm total egg protein against the light-to-heavy ratio. Simple linear regression (GraphPad Prism 10) was conducted to generate r^2 of each peptide from the combined calibration curve. The r^2 was greater than 0.95 (**Table 4.3**) for all ten target peptides. An additional simple regression comparison showed no significant differences among the slopes across 18 independent WEP calibration curves (data not shown). These results showed that the 18 independent WEP calibration curves remained statistically stable and consistent over eight months. This suggests that the calibration method was highly reliable and could consistently produce accurate results.

In addition, to evaluate the accuracy of the calibration method and ensure the reliability of the protein quantification, the intermediate precision of the MS method was analyzed. The nested ANOVA analysis was performed R with the fitVAC function to calculate the intermediate precision. The nested experiment involved two test portion replicates of WEP that were nested within the day of analysis, which generated 18 independent calibration curves. Each test portion replicate of WEP included two MS measurements (i.e., duplicate injections). Relative standard deviation [RSD(i)] was calculated across the obtained light-to-heavy ratio from the 36 MS measurements. Essentially, the RSD(i) considers within-day and day-to-day variability across 18 independent calibration curves. A small RSD(i) in the longitudinal experiment can indicate that the method provides reliable precision at the given concentration. Referring to the AOAC guidance on food allergen immunoassay validation, the limit of quantification (LOQ) should be estimated with RSD(i) ≤ 30%.[36] In this study, similarly, the LOQ was estimated at the calibrant concentration that had RSD(i) ≤ 30% from the longitudinal experiment. The estimated LOQ of ppm total egg protein was 2.35 ppm for VMV, 1.18 ppm for GGL, and 4.7 ppm for QQL. For the other peptides, the LOQ was estimated to be the same as the LOD (**Table 4.3**). In addition, the nested ANOVA analysis revealed that the variables among days contributed the most relative weight (56.6% to 84.9%) to total variation, followed by the variables from the technical duplicate within a day and the replicate MS measurements (**Table 4.3**).

It was noted that the HIA peptide had 35.3 ± 8.4% RSD(i) in the 0.47 – 4.7 ppm total egg protein range. Even though the HIA showed high sensitivity, being detectable at 0.47 ppm total egg protein, this finding suggested that the HIA peptide may not be

suitable for egg protein quantification. Furthermore, upon further investigation, it was found that the HIA peptide was undesirable for quantitative analysis due to carryover issues. After two injections of the WEP calibrant at 235 ppm total egg protein, it was noticed that five blanks (0.1%(v/v) FA and 5% (v/v) ACN in water) were required to remove the HIA peptide and to achieve undetected peaks (dotp < 0.8) in the subsequent blank sample. A further carryover investigation revealed that after ten blank washes (~ 5 hours), the HIA was still detectable (dotp > 0.8) in the blank sample when the 4700 ppm total egg protein was analyzed. Therefore, the carryover HIA peptide was excluded from quantifying egg proteins in the incurred food samples.

It is important to assess if the LOQ of the MS method complies with the requirements of an allergen detection method. FAO/WHO suggests the LOQ of an allergen detection method should be three times lower than the AL of the food matrix.[20] Among the five food matrices tested, retorted pasta soup has the lowest AL, 5 ppm total egg protein based on 400 g of food consumption. Therefore, to accurately quantify egg protein in all five food matrices, the LOQ of the MS method should be less than 1.7 ppm total egg protein. Among monitored peptides, GGL, LTE, LPL, and NFL peptides complied with the LOQ requirement for the retorted pasta soup, while except for HIA and NTD, all other peptides met the LOQ requirement for cookies, pie crust, pasta, and ice cream.

4.4.2 Sensitivity assessment from WEP-incurred food matrices

4.4.2.1 Detectability of egg protein in incurred food matrices

The POD approach was used to assess the peptide's detectability in incurred food samples at various egg protein concentrations. Only the peptides that met the detection

criteria (described in section 4.2.4) were considered for one positive event. The POD was then measured and compared across the incurred levels. Most peptides have varied POD curves in different incurred food matrices, but all ten peptides were detectable in approximately 47 ppm total egg protein across five food matrices (**Figure 4.3**). Only the GGL peptide was detectable at 0.47 ppm total egg protein incurred level across five food matrices. Moreover, the linear curves (light-to-heavy ratio vs. the incurred total egg protein concentrations) in **Figure 4.4** show that the detectability of GGL is consistent across all food matrices. This indicated that GGL is the desired quantifier in the PRM-MS method.

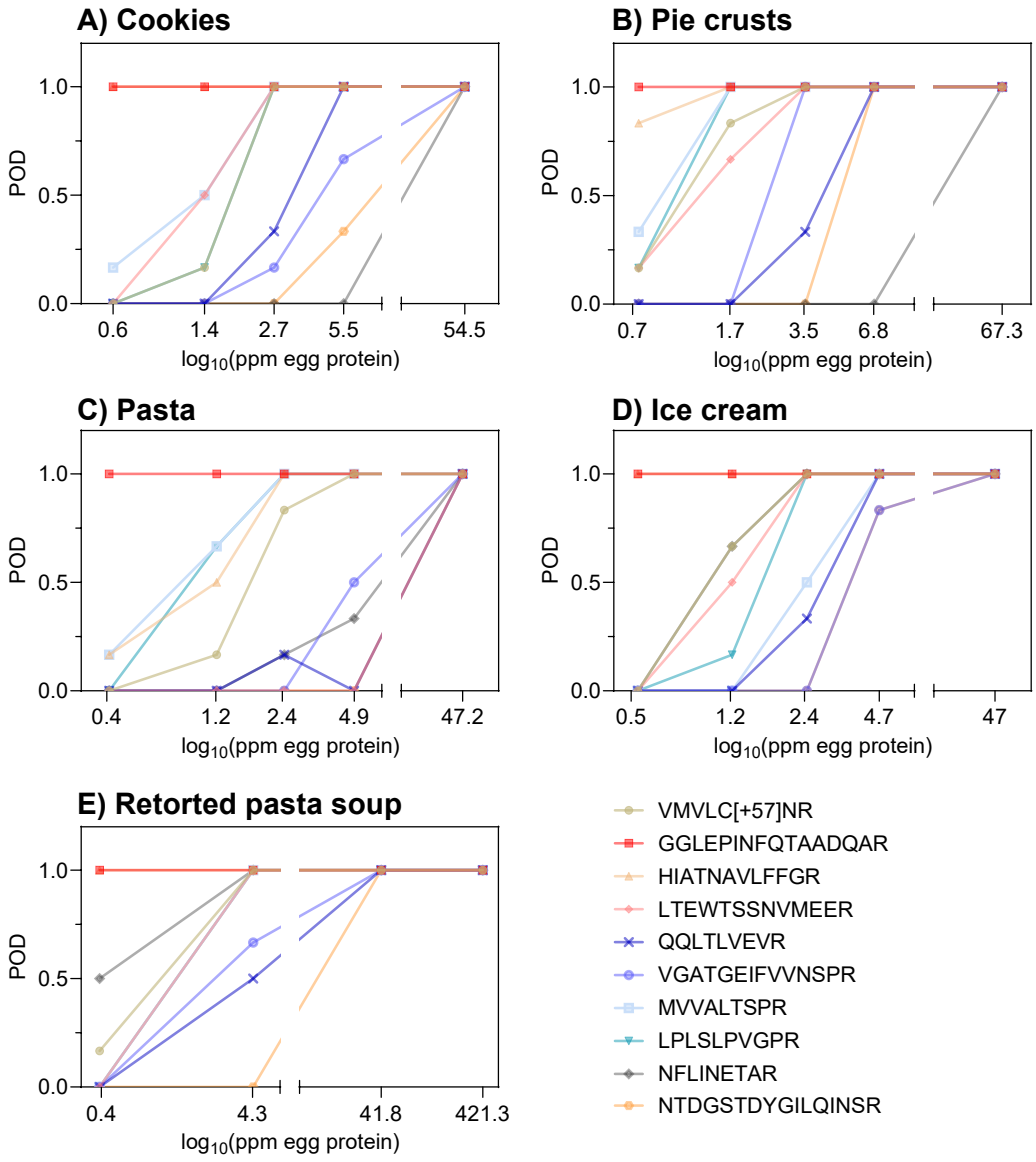

Figure 4.3 Probability of detection curve (POD) of ten target peptides from incurred food matrices: A) cookies, B) pie crust, C) pasta, D) ice cream, and E) retorted pasta soup. The POD = 1 indicates the peptide is detectable in 6 out of 6 MS measurements (triplicate test portions with duplicate injections; n = 6). The y-axis of the POD curve is the theoretical ppm total egg protein incurred levels, accounting for any water loss during processing.

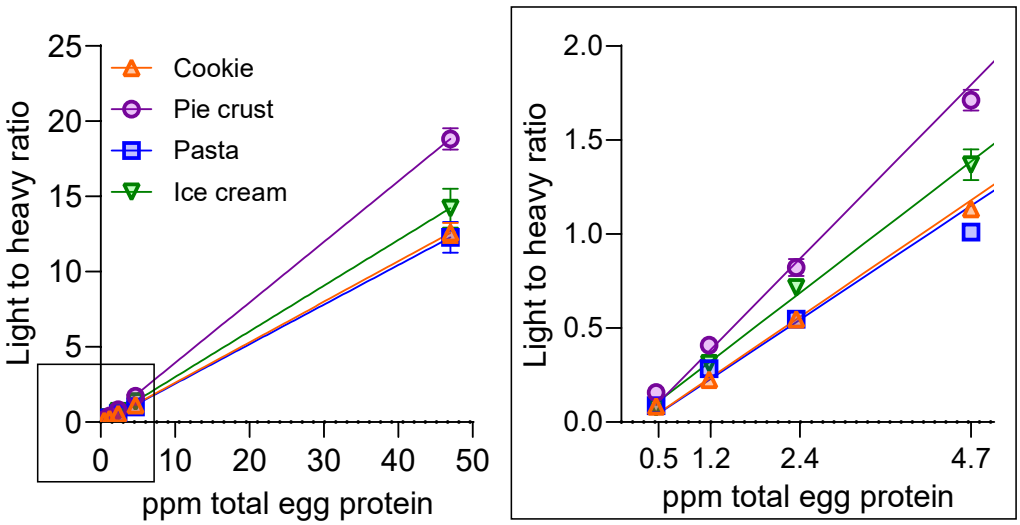

Figure 4.4 The light-to-heavy ratio of the GGL peptide against incurred egg protein concentration in the food matrix. Data reported as mean ± standard deviation (n = 6, three technical replicates duplicate injections on MS). Some error bars are not visible due to small standard deviations. The linear regressions were generated by GraphPad Prism 10.

The lowest egg protein concentrations at which the peptides had POD = 1 in the incurred food matrix were summarized in **Table 4.5**. The results show that 7 of 10 target peptides were detectable in cookies with POD = 1, 9 in pie crust, 5 in pasta, 8 in ice cream, and 7 in retorted pasta soup when the incurred total egg protein concentration was 4.7 ppm (**Table 4.5**). This means that these peptides could be detected at FAO/WHO-suggested ALs across the five tested food matrices. However, VITAL 3.0 suggests a much lower reference dose for egg protein, resulting in the VITAL-suggested ALs being ten times lower than the FAO/WHO-suggested ALs. If the VITAL 3.0 egg reference dose is considered, fewer peptides were obtained POD = 1 at the ALs (6 peptides in cookies, 4 in pie crust, 1 in pasta, 2 in ice cream, and 2 in retorted pasta soup) (**Table 4.5**). The results demonstrated that the PRM-MS method can detect 0.47 ppm total egg protein in the incurred food matrix, which is below the ALs according to the FAO/WHO and VITAL 3.0 reference doses. It should be noted that this is the first study that tested both egg white and egg yolk peptides in these five food matrices that incurred egg protein concentrations closer to or below the ALs.

Table 4.5 The lowest egg protein concentration of the peptide with the probability of detection of 1 in the incurred food matrix.

	Cookie	Pie crust	Pasta	Ice cream	Pasta soup
FAO/WHO ALs	40	20	10	20	5
VITAL 3.0 ALs	4	2	1	2	0.5
K.VMVLC[+57]NR.A	2.74[a,b]	3.45[a]	4.9[a]	2.35[a]	4.26[a]
R.GGLEPINFQTAADQAR.E	0.55[a,b]	0.66[a,b]	0.39[a,b]	0.47[a,b]	0.4[a,b]
K.HIATNAVLFFGR.C	0.55[a,b]	1.69[a,b]	2.35[a]	0.47[a,b]	0.4[a,b]
K.LTEWTSSNVMEER.K	2.74[a,b]	3.45[a]	47.18	2.35[a]	4.2[a]
R.QQLTLVEVR.S	5.48[a]	6.77[a]	47.18	4.7[a]	41.78
R.VGATGEIFVVNSPR.T	54.52	3.45[a]	47.18	47	41.78
R.MVVALTSPR.T	2.74[a,b]	1.69[a,b]	2.35[a]	4.7[a]	4.2[a]
R.LPLSLPVGPR.I	2.74[a,b]	1.69[a,b]	2.35[a]	2.35[a]	4.26[a]
R.NFLINETAR.L	54.52	67.3	47.18	2.35[a]	4.26[a]
R.NTDGSTDYGILQINSR.W	54.52	6.77[a]	47.18	47	41.78

[a] The peptide met the FAO/WHO reference dose (2 mg total protein from an allergenic source) in the corresponding food matrix.[20]
[b] indicates that the peptide met the VITAL 3.0 reference dose (0.2 mg total protein) in the corresponding food matrix.[18]

4.4.2.2 Quantification of egg protein in incurred food matrices

The quantification of the total egg protein in the incurred food matrices was determined by an external WEP calibration curve coupled with internal standard peptides. Two WEP extracts were prepared as calibrants alongside the food samples for each of the five food matrices. The WEP calibration curve was prepared by progressively diluting the desalted WEP digest with a diluent of 80 ng milk peptides/μL in 0.1% (v/v) FA/5% (v/v) ACN in water to obtain known total egg protein concentrations from 0.12 to 235 ppm. The run sequence for each sample set began with the analysis of one WEP calibration curve from 0 to 235 ppm total egg protein, followed by analyzing food samples ranging from low to high incurred levels, and concluded with the analysis of the second WEP calibration curve. These two calibration curves were then combined and generated into one WEP calibration curve for quantifying total egg protein in incurred food samples. A constant amount of heavy peptide mix was added to the calibration curve at each calibrant concentration and to each food sample, resulting in constant peak areas of the heavy peptides obtained across the calibrant concentrations and the samples. Then, the ratio of peak areas between the light and heavy peptides (light-to-heavy ratio) in the food sample was compared against the corresponding ratio in the calibration curve to determine the total egg protein concentration in the food sample. Each of the nine peptides, except HIA, generated a measured concentration of total egg protein per MS measurement. The concentrations of egg protein for each independent sample were calculated from the average concentrations from the two MS measurements.

4.4.2.3 Comparison of average and maximum reporting strategy

In this study, two quantification approaches were evaluated to report the concentration of egg protein in food samples. The first approach calculated the average concentration of measured ppm total egg protein in all monitored egg peptides, while the second approach reported the maximum measured concentration among any of the monitored egg peptides. These two reporting approaches (average across peptides vs maximum single peptides) were evaluated in the lower incurred concentrations of egg protein (0.47 – 47 ppm total egg protein). In **Figures 4.5 – 4.9**, the horizontal lines display the average ppm concentration across monitored peptides, while the highlighted dots indicate the maximum value reported from a single peptide. Subsequently, the average and maximum reported values were used to calculate the protein recovery from the incurred food matrix. The protein recovery was determined by dividing the theoretically incurred egg protein concentration from the reported total egg protein concentration. For instance, in the case of the cookie (**Figure 4.5**), at incurred levels of 54.5, 5.5, and 2.7 ppm total egg protein, the average recovery rate was significantly lower than the maximum protein recovery ($p < 0.05$, unpaired t-test). The significant differences in protein recovery were caused by the nine monitored peptides reported in various quantities of measured ppm concentration, where the broad range of measured ppm concentration skewed the average protein recovery. The lowest measured egg protein was approximately 20-fold lower than the maximum measured egg protein at 54.5 ppm total egg protein incurred level, and 2-fold differences were observed at 2.7 and 5.5 ppm total egg protein incurred levels. In cases of lower incurred total egg protein concentration at 1.4 and 0.6 ppm (**Figure 4.5**), there was no significant difference between the average

protein recovery and maximum protein recovery. These findings can be explained by the detection criteria, which were only met by a limited number of peptides, making fewer peptides used for quantification. For example, the cookie incurred 0.6 ppm total egg protein, and only GGL remained quantifiable, resulting in both the average and maximum protein recovery being the same. Similar findings were observed in other food matrices (**Figure 4.6 – 4.9**), whereby the maximum measured egg protein reported higher protein recovery than using the average measurement across monitored peptides at the high incurred levels (>1.18 ppm total egg protein incurred level) and no significant differences at lower incurred levels.

In contrast to the interpretation, Parker et al.[21] found that there was no statistical difference between the protein recovery calculated by the average of the monitored peptides and a single maximum reported peptide. This can likely be explained by the fact that fewer peptides are being monitored in their method. Moreover, a conversion factor that considered the relative allergen protein content was used to adjust the measured egg protein concentrations. Such adjustment on the measured concentration results in similar reporting egg protein from the monitored peptides. In the present study, the measured egg protein concentration was directly interpreted based on the WEP calibration curve without further adjustment. This resulted in a broad range of measured protein concentrations in the food matrix at high incurred levels. In the case of the food matrix incurred with higher egg protein concentrations, the skewing effect can occur because the average reported values were influenced by all the monitored peptides. The skewing effect then caused significantly lower reported values than the maximum reported values.

Because fewer peptides were quantifiable in the food matrices at lower incurred levels, the average and maximum approaches make no difference in such cases.

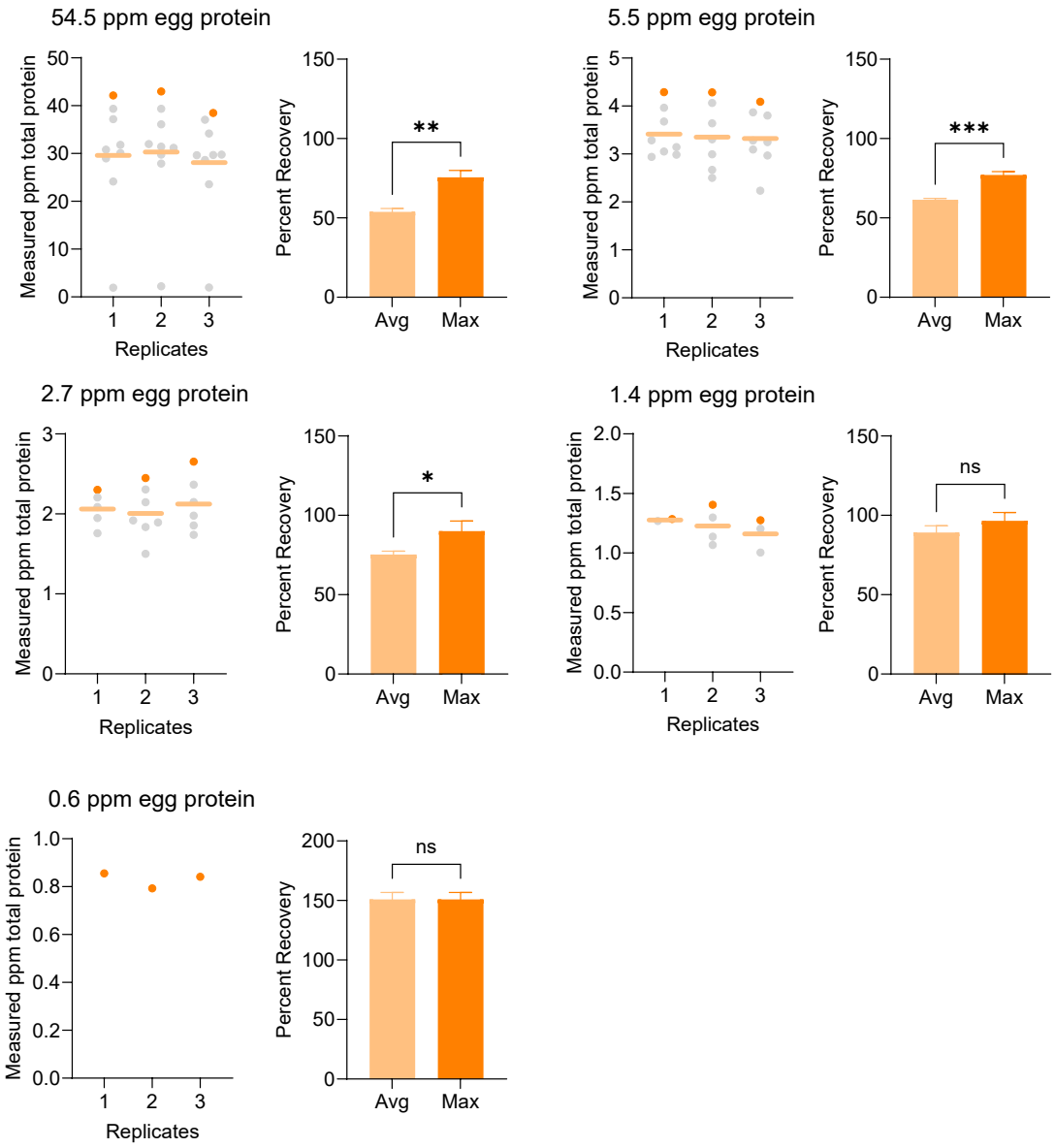

Figure 4.5 Comparison of the average and maximum reported concentrations of total egg protein from egg protein-incurred cookies. The scatter plot shows the measured egg protein concentration for the monitored peptides across various incurred levels. The

horizontal lines indicate the average egg protein concentration from all the monitored peptides; the highlighted dots indicate the maximum reported concentration among the monitored peptides. The bar chart shows the comparison between the use of average and maximum reported concentration. The percent recovery was reported as the average reported concentration with standard deviation as an error bar (n = 3). The unpaired t-test was conducted at each incurred level. Significant differences are denoted as *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and ns: no significant.

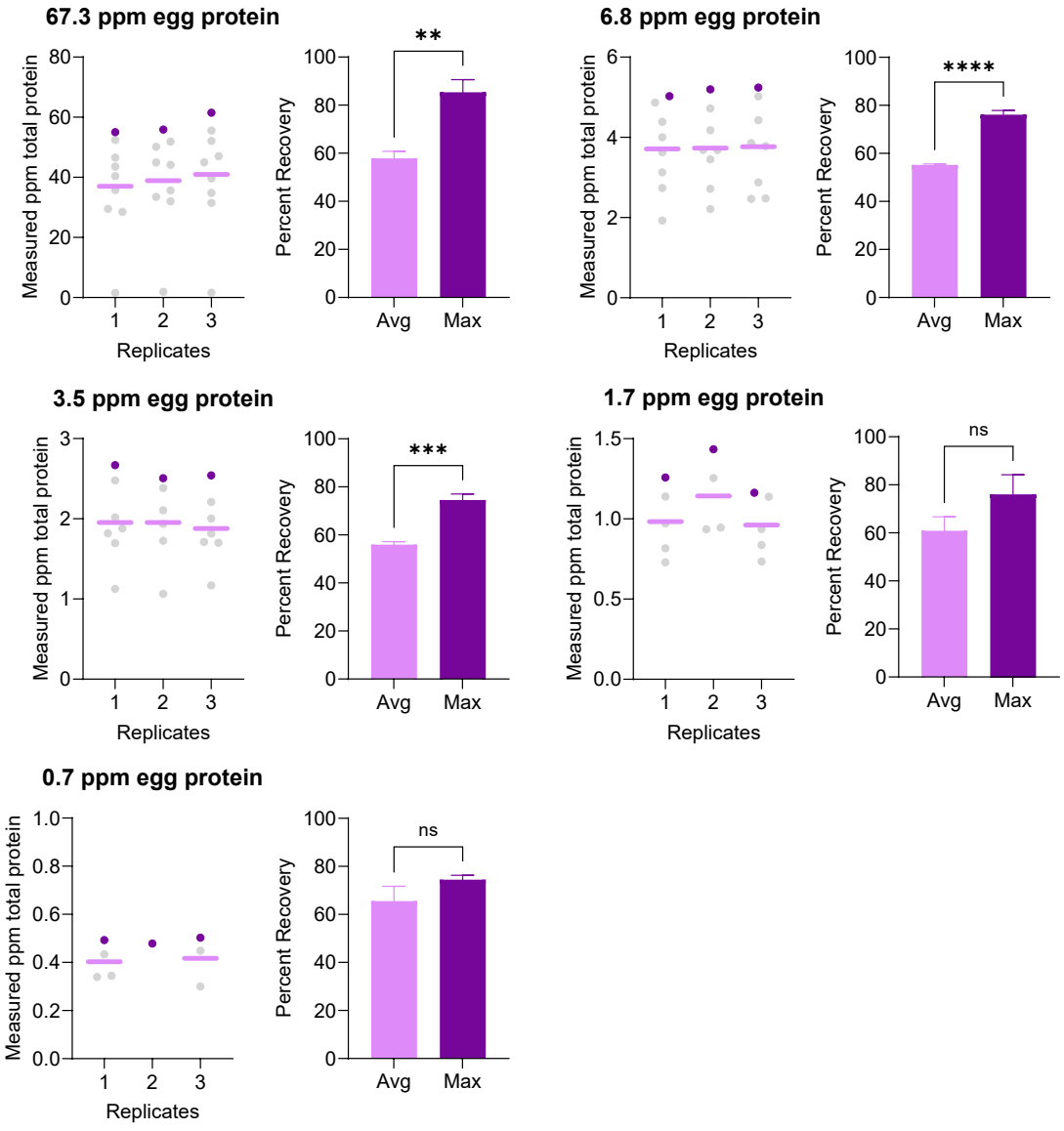

Figure 4.6 Comparison of the average and maximum reported concentrations of total egg protein from egg protein-incurred pie crusts. The scatter plot shows the measured egg protein concentration for the monitored peptides across various incurred levels. The horizontal lines indicate the average egg protein concentration from all the monitored peptides; the highlighted dots indicate the maximum reported concentration among the monitored peptides. The bar chart shows the comparison between the use of average and

maximum reported concentration. The percent recovery was reported as the average reported concentration with standard deviation as an error bar (n = 3). The unpaired t-test was conducted at each incurred level. Significant differences are denoted as *p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, and ns: no significant.

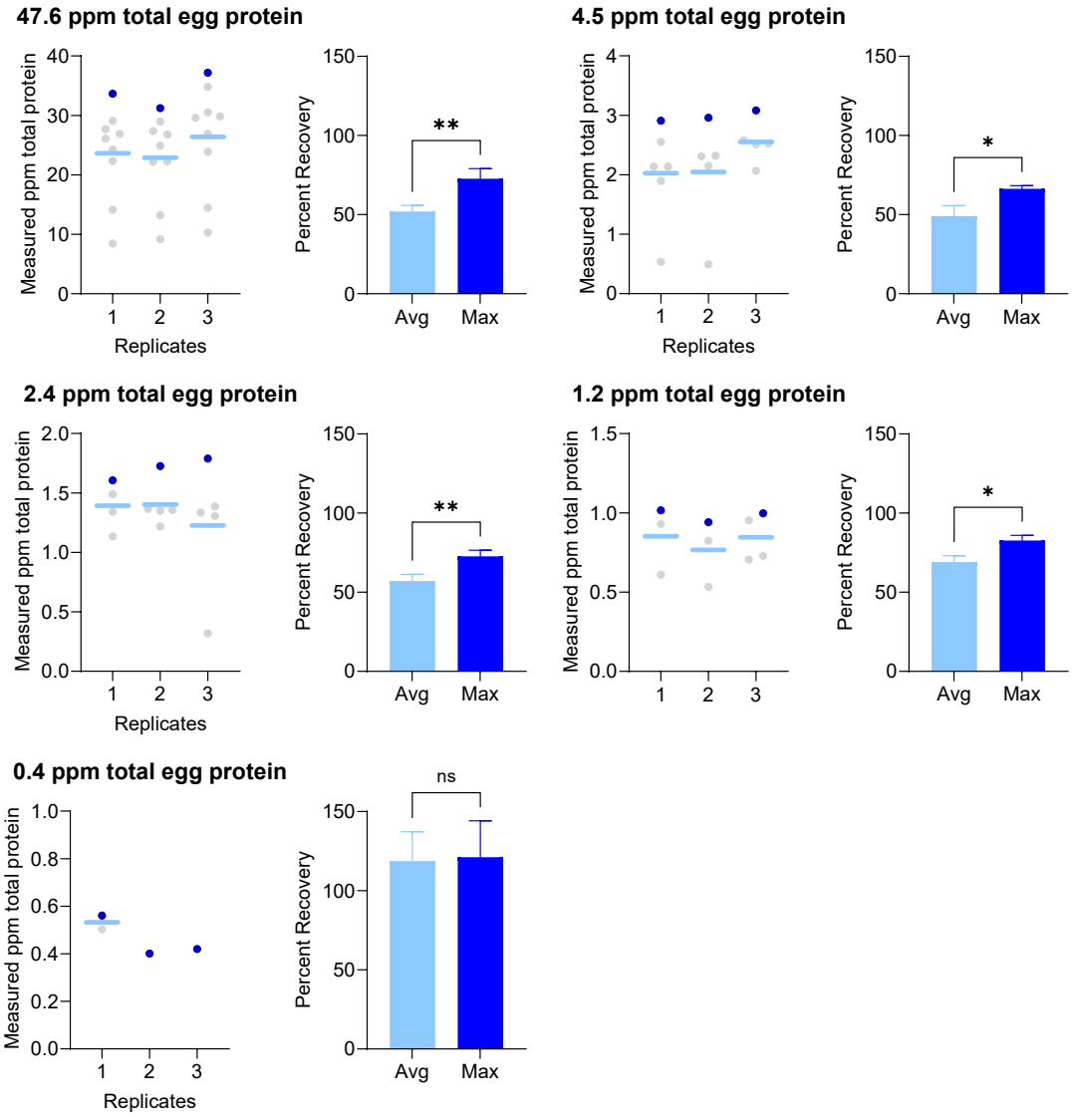

Figure 4.7 Comparison of the average and maximum reported concentrations of total egg protein from egg protein-incurred pasta. The scatter plot shows the measured egg protein

concentration for the monitored peptides across various incurred levels. The horizontal lines indicate the average egg protein concentration from all the monitored peptides; the highlighted dots indicate the maximum reported concentration among the monitored peptides. The bar chart shows the comparison between the use of average and maximum reported concentration. The percent recovery was reported as the average reported concentration with standard deviation as an error bar (n = 3). The unpaired t-test was conducted at each incurred level. Significant differences are denoted as *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and ns: no significant.

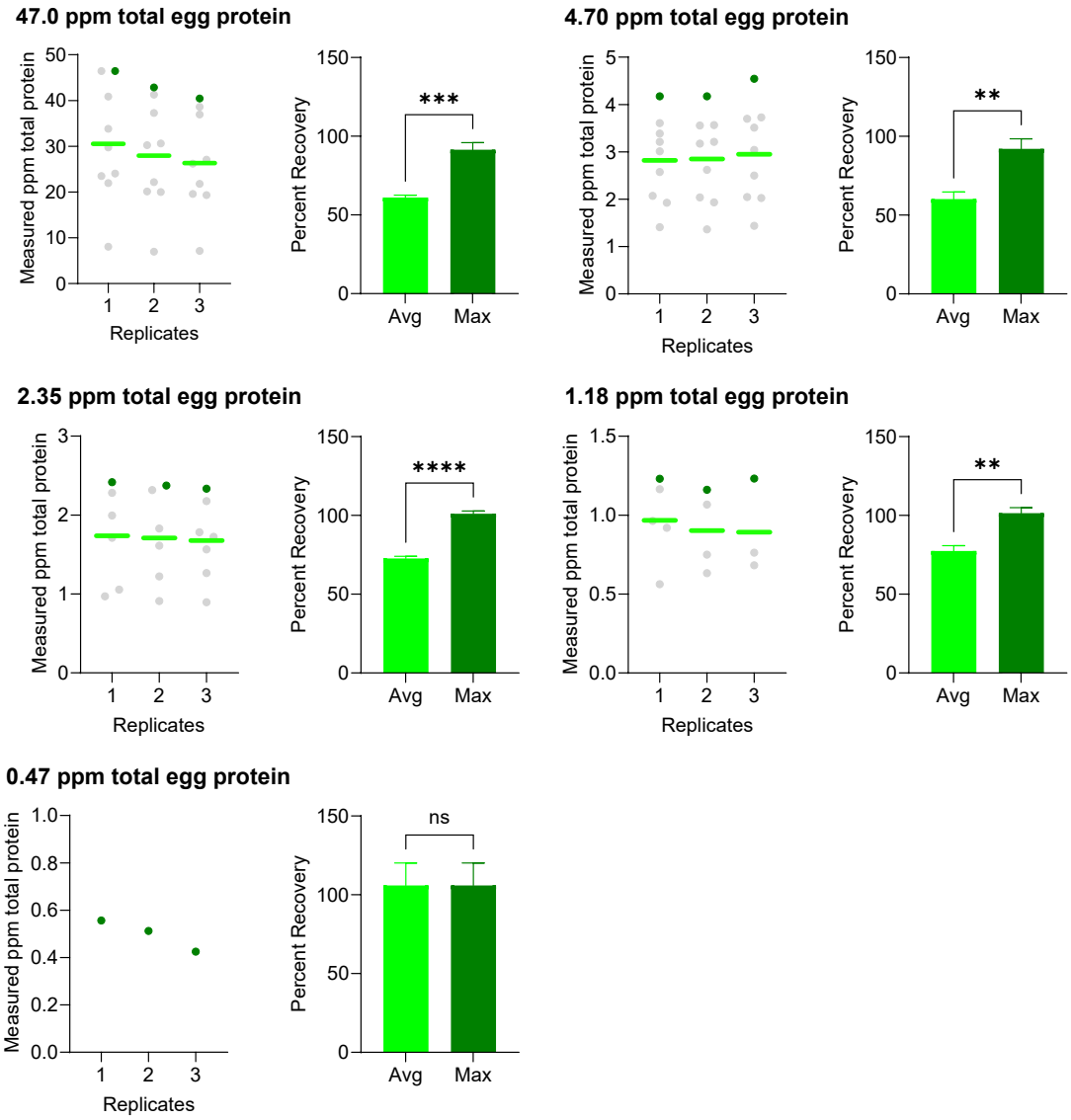

Figure 4.8 Comparison of the average and maximum reported concentrations of total egg protein from egg protein-incurred ice cream. The scatter plot shows the measured egg protein concentration for the monitored peptides across various incurred levels. The horizontal lines indicate the average egg protein concentration from all the monitored peptides; the highlighted dots indicate the maximum reported concentration among the monitored peptides. The bar chart shows the comparison between the use of average and maximum reported concentration. The percent recovery was reported as the average

reported concentration with standard deviation as an error bar (n = 3). The unpaired t-test was conducted at each incurred level. Significant differences are denoted as *p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, and ns: no significant.

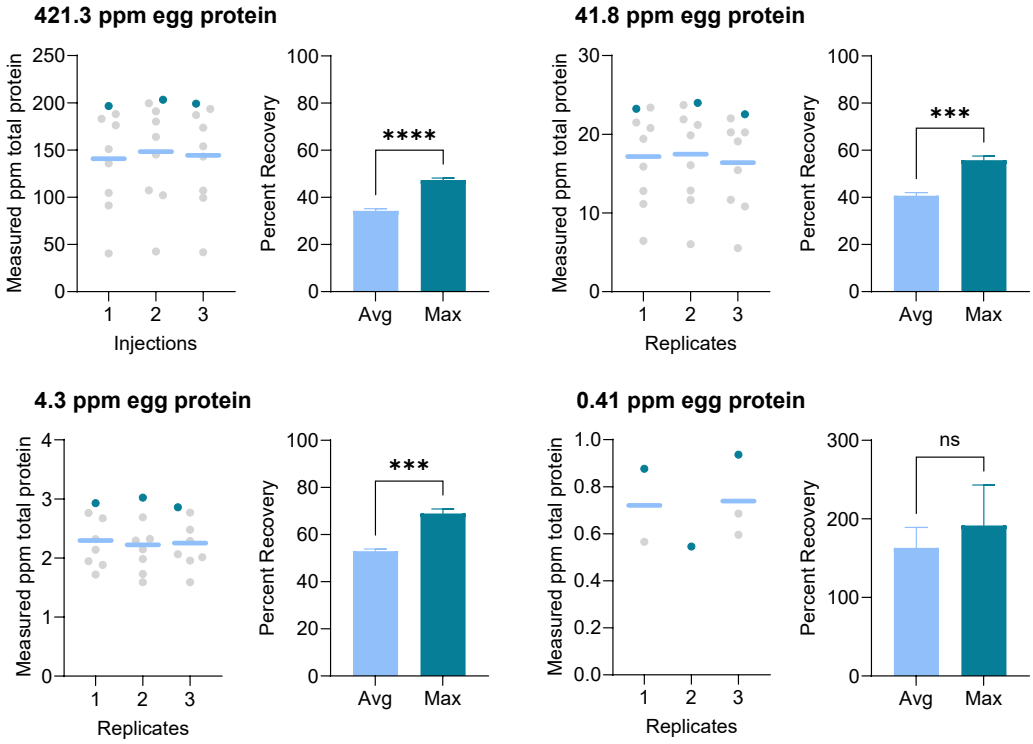

Figure 4.9 Comparison of the average and maximum reported concentrations of total egg protein from egg protein-incurred retorted pasta soups. The scatter plot shows the measured egg protein concentration for the monitored peptides across various incurred levels. The horizontal lines indicate the average egg protein concentration from all the monitored peptides; the highlighted dots indicate the maximum reported concentration among the monitored peptides. The bar chart shows the comparison between the use of average and maximum reported concentration. The percent recovery was reported as the average reported concentration with standard deviation as an error bar (n = 3). The

unpaired t-test was conducted at each incurred level. Significant differences are denoted as *p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, and ns: no significant.

4.4.2.4 Maximum reported peptide

Using the maximum reported values from any of the monitored peptides raises intriguing questions about which peptides were consistently detected in different food matrices. To answer these questions, we analyzed the frequency of the peptide that obtained the maximum measured egg protein concentration in each food sample across various incurred concentrations. Out of the nine monitored target peptides, six peptides were selected as the maximum reported value across five food matrices (**Figure 4.10**). It is worth noting that it is likely to choose more than one peptide among the triplicate samples from each matrix. Despite GGL and LPL being considerable quantifiers in the cookie and pie crust matrix, the LPL was preferred in the pie crust over the cookie matrix. As for the pasta, GGL was the most desired quantifier, whereas there was one instance in which the MVV peptide was reported as the maximum egg protein concentration. As for the ice cream, GGL, VMV, and LTE were considered as the maximum reported concentrations. The GGL peptide was the most frequently reported maximum peptide across all five food matrices, even at the lowest incurred concentrations.

The most striking finding was the substantial difference in the frequency profile in the retorted pasta soup matrix. The NFL was the most frequent maximum report peptide. Meanwhile, the detectability of the NFL was greater in pasta soup compared with other food matrices, detectable in retorted pasta soup at 0.4 ppm total egg protein (POD = 0.5).

Controversially, the NFL peptide can only be detected in the 47 ppm total egg protein incurred in cookies, pie crust, pasta, and ice cream, despite the LOD and LOQ of the NFL peptide being estimated at 1.18 ppm total egg protein according to the WEP calibration curve. The NFL peptide has also been discovered as a target peptide for egg yolk in chocolate and mayonnaise but is yet to be used for a quantitative MS method.[27]

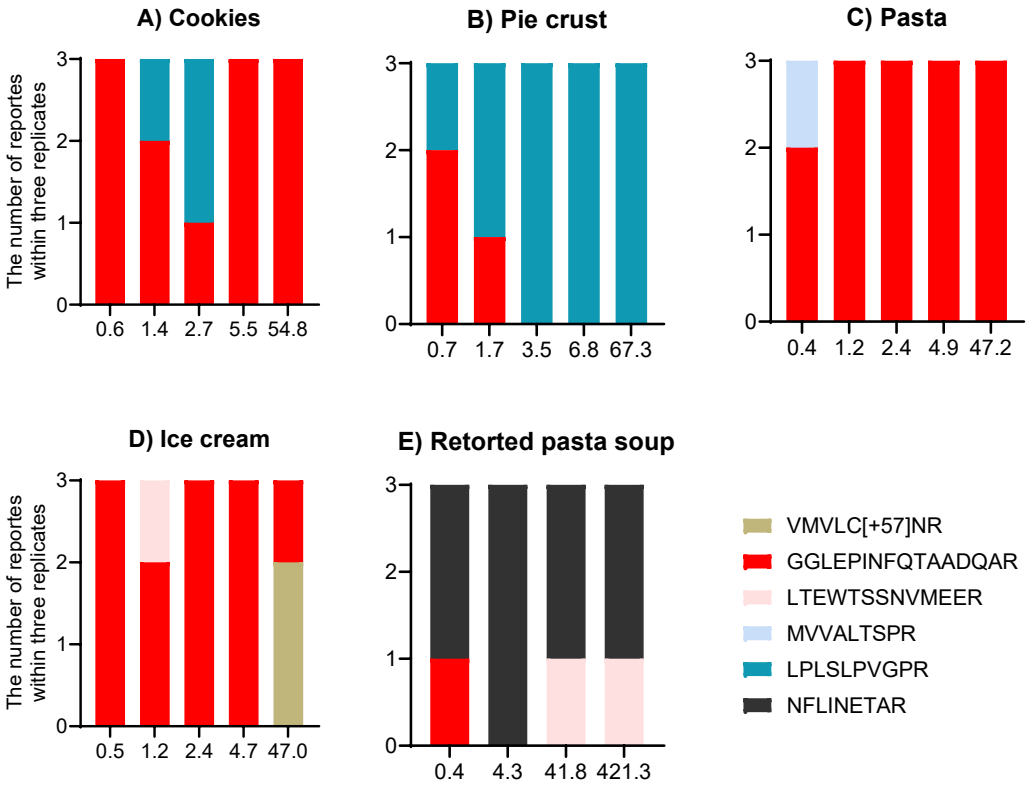

Figure 4.10 The frequency at which an egg target peptide was selected as the maximum reported egg protein concentration in triplicate samples across five food matrices at various incurred egg protein concentrations [(A) cookies, (B) pie crust, (C) pasta, (D) ice cream, and (E) retorted pasta soup]

4.4.3 Sensitivity assessment from egg powder-spiked food samples

Egg is a ubiquitous ingredient and can be used not only as whole eggs but also as egg whites, egg yolks, or purified protein in food products because of their unique functional properties. For example, egg white powder is used as a gelling agent in gluten-free bread;[37] egg yolk is used as an emulsifier in mayonnaise;[38] lysozyme is used as an antimicrobial agent in winemaking.[39] Therefore, it is important to understand how fractionated egg-derived ingredients would impact the protein determination based solely on the WEP calibration curve. To address it, we conducted an experiment in which three types of egg powders [(whole egg powder (WEP), egg white powder (EW), and egg yolk powder (EY)] were spiked in three different matrix backgrounds (protein extraction buffer, blank cookies, and blank ice cream). Each matrix background was spiked with 100 ppm egg powder, equivalent to 47 ppm total egg protein in the WEP, 84 ppm total egg protein in EW, and 35 ppm total egg protein in EY. Nine combinations of egg powder and matrices were conducted in duplicate with duplicate injections by the MS analysis.

4.4.3.1 Peptide profile of three types of egg powder

The detection profile among the nine monitored peptides was evaluated by the proportion of specific peptides among the sum of nine targe peptides (**Figure 4.11**). This was calculated by dividing the obtained light-to-heavy ratio of a specific peptide by the sum of the light-to-heavy ratio of nine target peptides and multiplying by 100. The results showed that the EW and EY have different peptide profiles than the peptide profiles in the WEP. Four egg yolk peptides (QQL, VGA, MVV, and LPL), originating from the vitellogenin-2, were absent in the EW (**Figure 4.11B**). While all nine monitored peptides were present in the EY, the proportions of the four egg yolk peptides were increased

(**Figure 4.11C**) compared to the egg yolk peptides in the WEP. What is interesting about the peptide profile is that NFL, originating from apovitellenin-1, was not only undetectable in the incurred cookie but also undetectable in the spiked cookie matrix background. It is difficult to explain the observation, but this finding may suggest that the cookie matrix can impede the detection of NFL. Overall, from the detection profile among these three types of egg powders, it can, therefore, be assumed that the proportion of the egg white and egg protein peptides can be used to infer the composition of egg yolk and egg white proteins in the food products.

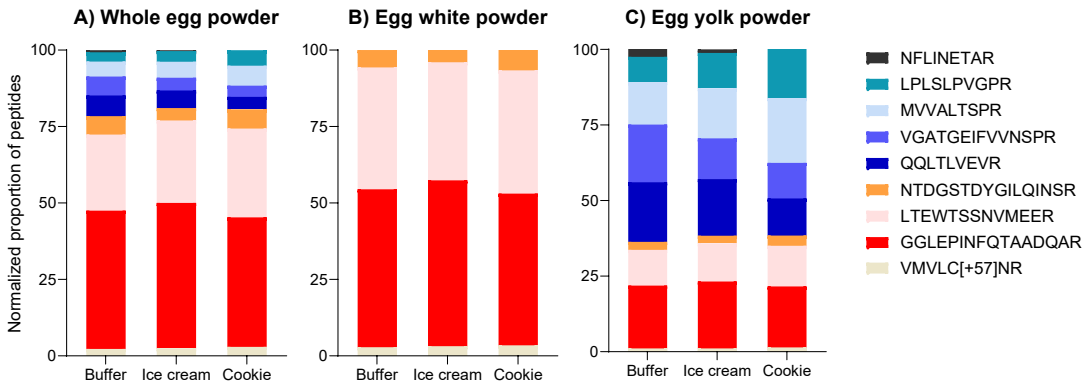

Figure 4.11 Peptide profile of whole egg powder (A), egg white powder (B), and egg yolk powder (A) in three different matrix backgrounds (protein extraction buffer, blank ice cream, and blank cookie).

Among the nine monitored peptides, LTE obtained the maximum measured total egg protein concentration for EW in all three matrix backgrounds. In the case of the EY, despite egg yolk peptides obtaining higher measured total egg protein concentration compared to other egg white peptides, the maximum reporting peptides varied in each of the three matrix backgrounds. The results found that the VGA was selected in the buffer background, the QQL in the ice cream, and LPL in the cookie. Another important finding is that the maximum reported peptides in the spiked samples were different from the maximum reported peptides in the WEP-incurred samples. LTE was the maximum reported peptide in the buffer background, and VMV was the maximum reported peptide in the cookie background. However, GGL tended to obtain the maximum measured egg protein concentration in the incurred food samples. The differences in the maximum reporting peptide between the spiked sample and the incurred samples can be explained by the physical and chemical interactions of the egg proteins with the food matrix during food processing, which may lead to various impacts on the detectability of the peptides.

4.4.3.2 Protein recovery from three types of egg powder

The egg protein concentrations in WEP, EW, and EY spiked samples were calculated using one WEP calibration curve and reported in ppm total egg protein. Subsequently, the average and maximum reporting approaches, described in section 4.3.2.3, were used to calculate the protein recovery. Then, protein recoveries (i.e., average percent protein recovery from duplicate samples) were compared across each type of egg powder-spiked sample. A two-way ANOVA analysis was conducted to compare the differences between the average protein recovery and the differences of the protein recovery among three matrix backgrounds using the same reporting approach.

Using the WEP calibration curve caused an overly reporting of the total protein in the EW and EY-spiked samples (**Figure 4.12**). Specifically, the protein recovery results for the EW-spiked samples were 185 – 212% of the average protein recovery and 258 – 278% of the maximum protein recovery. Next to the EY-spiked samples, the results were 54 – 70% of the average protein recovery and 111 – 160% of maximum protein recovery. For WEP-spiked samples, the results were 56 – 69% of average protein recovery and 77 – 106% of maximum protein recovery. The maximum protein recoveries from EW and EY were 3-fold and 1.5-fold greater than the maximum protein recoveries from the WEP, regardless of the matrix backgrounds. The overly-reporting protein recovery can be explained by the fractions of egg proteins enriched in egg white and egg yolk powders. Based on the estimation of the total protein in egg powders, the total protein in EW was expected to be 3-fold higher, and in EY, 2-fold higher, compared to the total protein in WEP. The increase in total protein in egg powder could result in the increase of peptide signal in the sample.

Moving to the comparison between the average reporting strategy and maximum reporting strategy for each type of egg powder. In both EW and EY-spiked samples, significant differences were found between the average and maximum reporting approaches ($p < 0.05$), but no significant differences were observed in WEP-spiked samples. In addition, it is somewhat unexpected that no significant difference was observed between the protein recovery obtained in the WEP-spiked cookie/ice cream matrix and the protein recovery obtained in the WEP-incurred cookie/ice cream matrix. These findings indicated that the monitored target peptides were less influenced by the food matrix effects during food processing. The findings from the egg powder-spiked

experiment emphasized the vital need to include egg white and egg yolk peptides in an MS-based egg detection method. Furthermore, by monitoring nine target peptides in five different food matrices, the results demonstrated that the types of food matrices had various impacts on the detectability of the peptides. Similarly, the types of food matrix cause different impacts on the protein-based immunochemical method.[40]

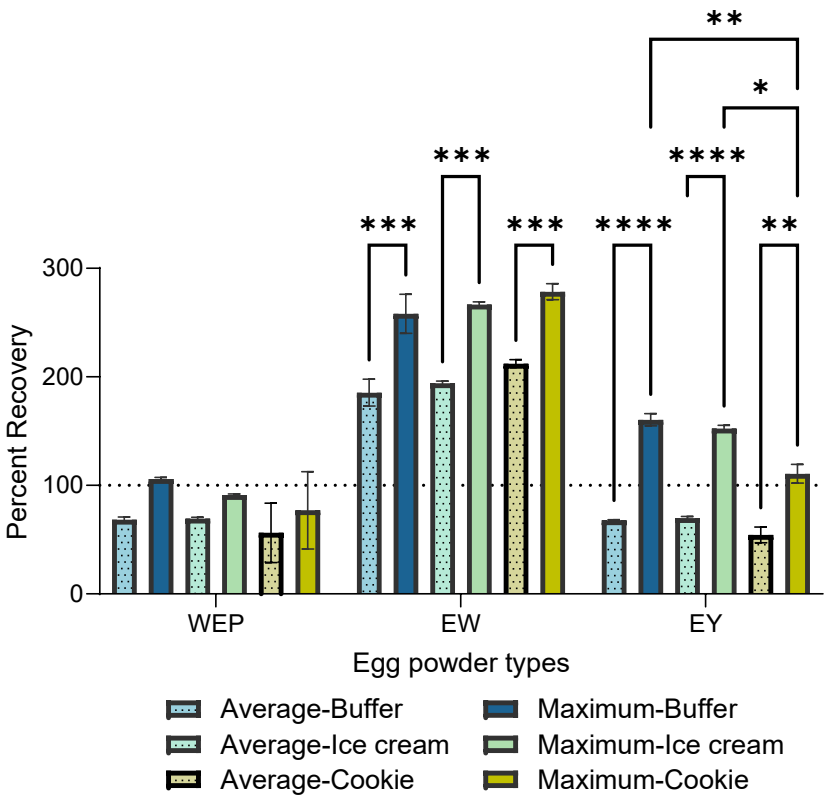

Figure 4.12 Average percent recovery from whole egg powder (WEP), egg white powder (EW), and egg yolk powder (EY) spiked in buffer, blank cookie, and blank ice cream. The average percent recovery was calculated by averaging the reported protein recovery from duplicate samples using either the average or the maximum reported approach. The two-way ANOVA was conducted for each egg powder type. Significant differences are denoted as *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

4.4.4 Comparison with commercial egg-specific ELISA kits

The developed PRM-MS method aims to serve as an alternative or confirmatory method to current egg ELISA kits. Therefore, the protein recovery was compared between the PRM-MS method and the commercial ELISA methods. Four egg ELISA kits (A, B, C, and D) were used to determine the egg protein concentrations in the unprocessed dough of cookies, pie crust, and pasta, as well as processed forms of cookies, pie crust, pasta, and ice cream incurred with 0, 4.7, and 47 ppm total egg protein. In addition to the mentioned food matrices, retorted pasta soup incurred with 0, 4.3, 43, and 431 ppm total egg protein was also analyzed by each of the ELISA kits. Depending on the manufacturer, the four egg ELISA kits vary from quantifying whole egg protein to egg white protein (ovalbumin and/or ovomucoid), which results in different reporting units. To harmonize the comparison across ELISA and the MS methods, the reporting units were converted into total egg protein by multiplying with conversion factors provided by each ELISA kit's instructional manual. In the case of blank samples (0 ppm total egg protein), the results were below LOD by all four ELISA kits. As for the five incurred food samples, the results of the average measured total egg protein using four egg ELISA kits were reported in **Table 4.6**. The comparison between the recovery from the dough and processed food samples revealed that after heating processing, the average recovery for cookies and pie crust was substantially reduced using kits A, C, and D. Specifically, the results of kits A and D were unable to detect protein in the processed cookies and pie crust incurred with 47 ppm total egg protein. Similarly, for pasta, which was dried at 70°C and 40°C, kits A, C, and D obtained substantial reductions in the

average recovery in pasta compared to the average recovery in pasta dough. In contrast, the egg ELISA kits work well in the spiked food matrix. A previous study demonstrated that seven egg ELISA kits, including the four ELISA kits tested in the present study, can quantify 3 ppm total egg protein in the spiked cookies, with an average recovery of 84 – 123%.[41] This demonstrates that the physical and chemical interactions of egg proteins during food processing impact the ELISA method's detection. As for kit B, the results obtained a comparable average recovery in pasta dough and dried pasta, with less difference in the average recovery between cookie/pie crust dough and the processed cookie/pie crust compared to the other three egg ELISA kits. These findings could indicate that the antibodies used in kit B can recognize heat-induced changed protein after heating.

Another important result was that all four ELISA kits encountered challenges detecting egg protein in the retorted pasta soup. Kit A could not quantify egg protein at the total egg protein concentration of 421 ppm in the pasta soup. Kit D could only quantify egg protein in the pasta soup, which incurred 421 ppm total egg protein but with less than 0.4% average recovery. Kits B and C could quantify egg protein in the pasta soup at three total egg protein concentrations. The average recovery across the three incurred concentrations was 14.6 ± 2.4% using kit B and 6.1 ± 1.8% using kit C.

Table 4.6 Average measurement of ppm total egg protein in incurred processed food matrices using four commercial egg ELISA kits.

Food matrices	Incurred levels[a]	Kit A		Kit B		Kit C		Kit D	
		ppm[b]	%CV	ppm[b]	%CV	ppm[b]	%CV	ppm[b]	%CV
Cookie dough	4.70	3.28	12.74	2.97	2.59	3.43	3.53	6.47	3.48
Cookie	5.48	BLQ	N/A	2.11	0.84	0.66	13.05	BLQ	N/A
Cookie dough	47.00	27.85	0.73	31.78	0.55	31.38	4.75	71.09	3.99
Cookie	54.52	BLQ	N/A	21.86	1.02	9.51	1.35	BLQ	N/A
Pie crust dough	4.70	2.51	6.37	2.34	1.03	3.39	6.14	5.04	10.79
Pie crust	6.77	BLQ	N/A	2.21	4.32	1.15	5.73	BLQ	N/A
Pie crust dough	47.00	25.43	7.96	25.04	4.41	33.27	10.21	58.55	2.55
Pie crust	67.30	BLQ	N/A	23.62	4.88	15.22	25.59	BLQ	N/A
Pasta dough	3.14	1.23	10.50	1.48	7.43	2.51	2.35	2.74	4.93
Pasta	4.49	0.73	12.52	2.14	8.05	2.57	5.94	2.70	15.47
Pasta dough	31.36	12.70	1.82	13.81	5.15	23.93	2.90	24.03	1.30
Pasta	46.82	10.81	1.13	22.66	7.27	23.18	13.90	30.92	2.34
Ice Cream	4.70	1.52	1.12	2.61	3.45	3.79	3.65	4.21	7.14
Ice Cream	47.00	26.12	8.70	27.40	2.15	38.37	8.34	52.94	12.65
Pasta soup	4.26	BLQ	N/A	0.67	9.45	0.26	5.78	BLQ	N/A
Pasta soup	41.78	BLQ	N/A	6.78	1.56	2.75	2.77	BLQ	N/A
Pasta soup	421.25	BLQ	N/A	49.55	1.84	23.01	1.72	1.85	2.39

Conversion factors provided by manufacturers were used to consolidate the reporting unit in ppm total egg protein for Kit A (0.481), C (0.49), and D (0.4801). After unit conversions, the limit of quantification for Kit A, B, C, and D is 0.48, 0.78, 0.12, and 1.20 ppm total egg protein, respectively.

BLQ: below the limit of quantification according to the specific ELISA kit.

Data reported as the average measurement of ppm total

[a] Food matrix was incurred with various ppm total egg protein. The actual concentration of total egg protein in food matrices accounted for any water loss during processing.

[b] Data was reported as the average ppm total egg protein in duplicate samples with a percentage of the coefficient of variation (%CV) (n = 4, duplicate extractions were analyzed in duplicate wells using ELISA kits).

The average protein recovery in the processed foods incurred at 4.7 and 47 ppm total egg protein was compared between the four egg commercial ELISA kits (A, B, C, and D) to the targeted MS method (**Figures 4.13 and 4.14**). When using the targeted MS method, the maximum reporting approach was used to quantify the concentration of total egg protein in the food matrices incurred at the same incurred levels. Then, the maximum reported concentrations were used to calculate the protein recovery.

Quantification of total egg protein using the targeted MS method was significantly improved in the cookies, pie crust, and pasta soup ($p < 0.05$). Given the example of food matrices incurred at 4.7 ppm total egg protein, the average recovery ranged from 69.0% to 77.0% (**Figure 4.13A, B, and E**). In contrast, the results showed that using ELISA kits was challenging in recovering protein from the highly processed food matrices. Among four egg ELISA kits, kit B resulted in the highest average recovery of 38.5% in cookies, 32.7% in pie crust, and 15.7% in pasta soup (**Figure 4.13A, B, and E**). For the pasta, the average recovery obtained from kits C and D was comparable with the average protein recovery obtained by the MS method ($p > 0.05$) (**Figure 4.13C**). Next to ice cream, only the average recovery obtained from kit D was comparable with the average protein recovery obtained by the MS method (**Figure 4.13D**). Similar results were seen in the food matrices incurred at approximately 47 ppm of total egg protein, with the average recovery ranging from 55.8% to 92.0% across the five food matrices investigated (**Figure 4.14**).

Moreover, the targeted MS method using the maximum reporting approach was used to quantify the total egg protein across five processed food matrices at various incurred concentrations (0.4 – 67 ppm total egg protein). The average protein recovery

across tested incurred concentrations above 1.18 ppm total egg protein (the lowest estimated LOQ of the method) was 84.8 ± 10.2% in cookies, 78.0 ± 5.0% in pie crust, 73.6 ± 6.8% in pasta, 96.5 ± 5.5% in ice cream, and 57.5 ± 10.8% in pasta soup. The %CVs at the quantifiable incurred concentration ranged from 1.2 – 10.8%. The low %CV demonstrated the reliable quantification of total egg protein in tested food matrices incurred with 1.18 and above ppm total egg protein. Overall, the PRM-MS method outperformed the four egg ELISA kits, demonstrating that the PRM-MS method can detect and quantify egg proteins in various processed foods.

Several MS-based methods have been developed to quantify total egg proteins in various food matrices, including muffins, cereal bars, chocolate, bread, salad dressing, red wine, ice cream, and cookies.[21,23–26,42] These methods have demonstrated LOQ ranging from 1.1 to 50 ppm of total egg protein depending on the target peptides and MS methods.[21,23–26,42] Planque et al.[42] reliably quantified total egg protein in cookies and ice cream incurred with 60 ppm total egg protein. New et al.[24] achieved a percent protein recovery of 111.2% in cookies containing 10 ppm of whole egg (approximately 4.7 ppm of total egg protein). In this study, the percent protein recovery in the cookies incurred at the same concentration was comparable to the results of New et al.[24] In addition to the incurred cookie matrix, the present study performed the sensitivity assessment of pie crust, pasta, ice cream, and pasta soup at various incurred concentrations. This study showed that the targeted MS method was able to quantify total egg protein in various processed food models, including baked, dried, frozen, and retorted food.

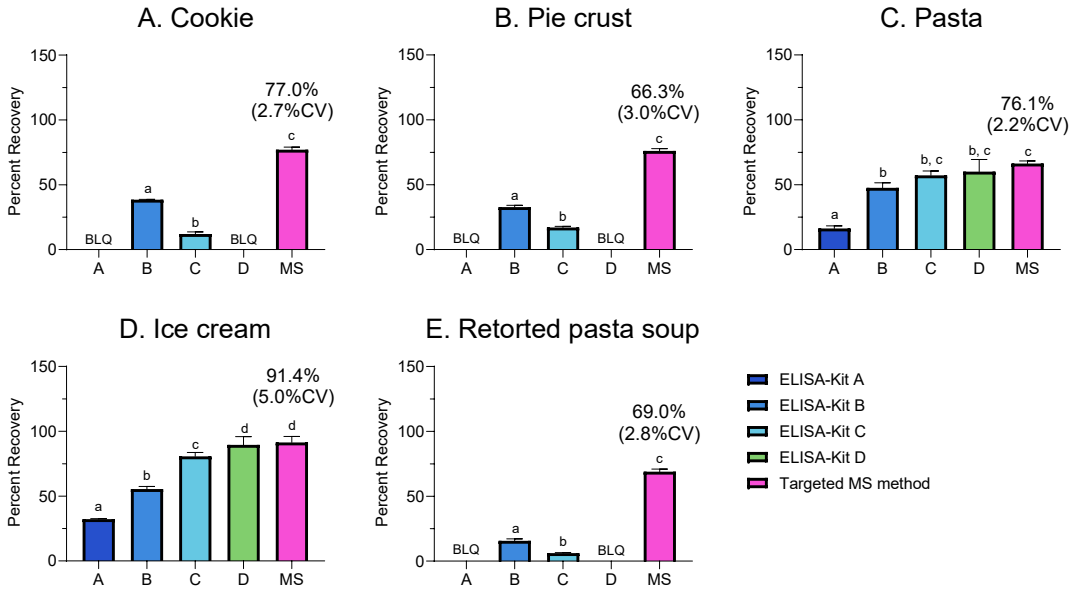

Figure 4.13 Comparison of the average recovery of total egg protein in food matrices incurred at 4.7 ppm total egg protein using the four egg ELISA kits and the targeted MS method. After accounting for the water loss during food processing, the actual incurred total egg protein concentration was 5.5 ppm in cookie (A), 6.8 ppm in pie crust (B), 4.5 ppm in pasta (C), 4.7 ppm in ice cream (D), and 4.3 ppm in pasta soup (E). The targeted MS method used the maximum reporting approach to quantify total egg protein in food matrices and then calculated the average recovery. The average percent recovery (%CV) was reported for the MS method. Data reported as average ± standard deviation (n = 4 for ELISA analysis, n = 6 for MS analysis). One-way ANOVA, followed by Tukey's comparisons, was conducted. The average recovery with the different letters indicated significant differences ($p < 0.05$).

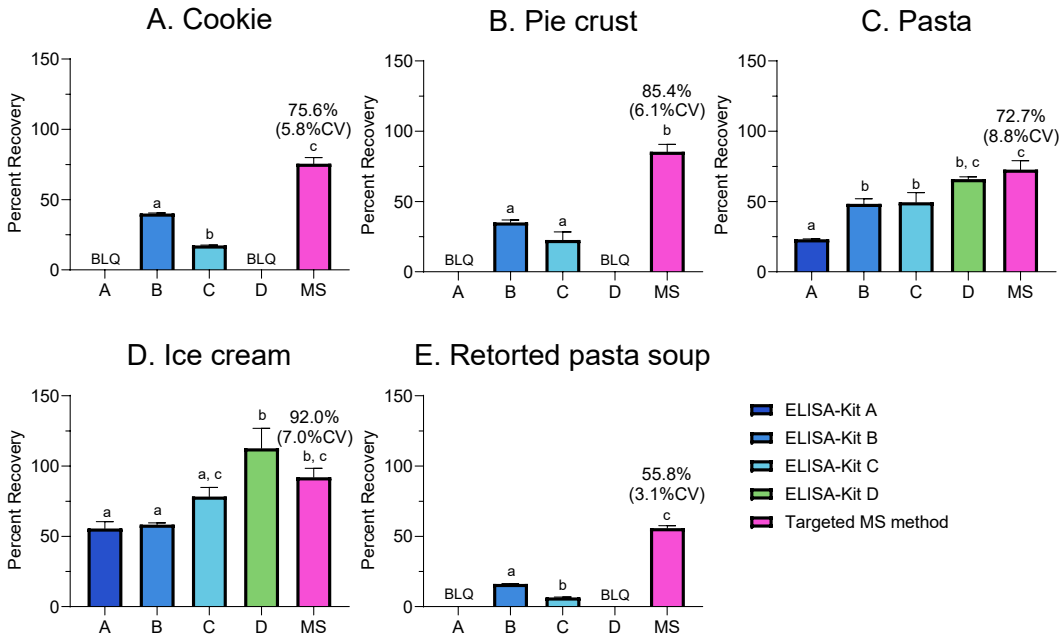

Figure 4.14 Comparison of the average recovery of total egg protein in food matrices incurred at 47 ppm total egg protein using the four egg ELISA kits and the targeted MS method. After accounting for the water loss during food processing, the actual incurred total egg protein concentration was 54.5 ppm in cookie (A), 67.3 ppm in pie crust (B), 46.8 ppm in pasta (C), 47.0 ppm in ice cream (D), and 41.8 ppm in pasta soup (E). The targeted MS method used the maximum reporting approach to quantify total egg protein in food matrices and then calculated the average recovery. The average percent recovery (%CV) was reported for the MS method. Data reported as average ± standard deviation (n = 4 for ELISA analysis, n = 6 for MS analysis). One-way ANOVA, followed by Tukey's comparisons, was conducted. The average recovery with the different letters indicated significant differences ($p < 0.05$).

4.5 Conclusion

This study set out to conduct a comprehensive assessment of the overall performance of the developed PRM-MS method in different aspects. To the best of our knowledge, this is the first study to estimate the LOD and LOQ of the egg peptides using the statistical-based POD approach from the pooled WEP calibration curve. The results showed all ten target peptides achieved LOD ≤ 4.7 ppm total egg protein, while nine of them had LOQ ≤ 4.7 ppm total egg protein. It was found that GGL was the most sensitive peptide, with an LOD of 0.47 ppm total egg protein and an LOQ of 1.18 ppm total egg protein. In addition, the PRM-MS method could detect the lowest incurred egg protein level (~0.4 ppm) and quantify at 1.18 ppm total egg protein across five food matrices. In general, 48 – 151% maximum protein recovery was obtained across five incurred food matrices. Moreover, compared to the quantification of egg protein using ELISA kits, the MS method showed improved protein recovery in processed foods, particularly in cookies, pie crust, and retorted pasta soup.

Due to practical constraints, this study only evaluated the food samples spiked with whole egg powder, egg white powder, or egg yolk powder. The egg powder-spiked experiment didn't mimic the real food processing effect or consider the different ratios of egg white and egg yolk powder. Notwithstanding, the results showed that using the WEP calibration curve can overestimate the egg protein concentration of the egg white and egg yolk spiked samples. Different types of egg powder can result in distinct proportions of the egg white and yolk peptides among the monitored peptides. In addition, the performance of the MS method may need to be evaluated in other complex food matrices. For example, egg white or egg-derived ingredients are used in sausage as a gelling agent

and in wine as an antimicrobial agent. The complexity of the meat (high protein background) and wine (high fat and polyphenols) matrices may pose challenges in quantifying egg proteins.

This study demonstrated that the PRM-MS method is a reliable and sensitive allergen analytical method for quantifying low levels of eggs in processed food matrices. Moreover, the PRM-MS method outperformed the existing commercial egg ELISA kits, particularly in its enhanced protein recovery in bakery products. The LOQ of the PRM-MS complied with risk-based action levels for cookie, pie crust, pasta, ice cream, and retorted pasta soup matrices according to a reference dose for the egg of 0.2 mg egg protein. The LOD of the PRM-MS method is 0.47 ppm total egg protein in the incurred food matrices, which translates to a consumption of 426 g of food product to reach 0.2 mg egg protein. Overall, the study highlighted the ability of the PRM-MS method to be an alternative and confirmatory analytical method for egg risk assessment and to improve allergen management in the food industries.

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CHAPTER 5

CONCLUSIONS

The main goal of the study was to develop a targeted mass spectrometry (MS) method for the accurate and reliable detection and quantification of total egg protein in processed foods. A final 9 egg-specific target peptides were identified and used to develop this quantitative MS method using parallel reaction monitoring. The development process included three critical steps: 1) selection of target peptides in food matrices using a discovery-based strategy, 2) optimization of the sample preparation workflow to enhance peptide detection, and 3) validation of the targeted MS method in whole egg powder (WEP) incurred food matrices.

First, four processed food matrices (cookies, pie crust, pasta, and ice cream) incurred with WEP were used to identify target peptides. These incurred matrices mimic the conditions of egg protein present in real food products. The use of incurred food matrices considers both physical and chemical interactions of egg proteins and matrix components during food processing, which can aid in selecting target peptides that are less prone to matrix effects. A discovery-based approach using data-dependent acquisition identified 88 candidate peptides, which were egg-specific, detectable in incurred food matrices, and most abundant. They were refined to 11 robust peptides using the PRM-MS method with inclusion lists. A set of criteria using Skyline parameters was used to select the best-performance peptides. Using clear and numerical metrics, this approach allows for the unbiased selection of target peptides without prior knowledge. These criteria can be adapted and applied to identify target peptides for other food allergens as well.

Second, the optimization phase focused on enhancing the sample preparation workflow. Key variables such as trypsin type, carrier protein inclusion, and types of desalting columns were optimized. The final sample preparation method added 0.1% non-fat dry milk powder (NFDM) (w/v) to the protein extraction buffer. A 5-fold increased protein extract (60 µL) was digested with Promega trypsin solution overnight (approximately 16 hours), then desalted using the C18 high-capacity desalting columns. The desalted resultant peptides were then lyophilized and resuspended in 0.1% (v/v) FA and 5% (v/v) ACN water. For further sample or calibration dilution, the resuspended sample was diluted with a diluent containing 80 ng NFDM peptide/µL. The results showed that 50 – 100 ng NFDM peptides/µL in diluent was optimal for obtaining the most peak area of the egg-specific heavy peptides. Using NFDM in both protein extraction buffer and diluent improved the precision of peptide detection. Additionally, an HPLC column with a larger diameter (2 mm) was used to separate peptides, and a shorter chromatography gradient (35 minutes) at a higher flow rate (300 µL/min) improved the peptide separations. These optimizations allowed for improved detection of the target peptides, which was essential for reliable quantification.

Finally, the quantification strategy used a matrix-independent external calibration curve (i.e., WEP, as calibrant) coupled with internal standards (i.e., stable-isotope labeled heavy peptides) to interpret the light-to-heavy peptide ratio into ppm total egg protein. Longitudinal calibration curves across eight months indicated that nine egg-specific target peptides could quantify total egg protein in processed food matrices, achieving limits of quantification (LOQ) between 1.18 and 4.7 ppm. Regarding conservative allergen risk assessment, a maximum reporting approach was used, reporting the

maximum measured concentration from any of the nine target peptides to determine the total egg protein in the food samples. The investigation of the WEP-incurred food matrices (cookie, pie crust, pasta, ice cream, and retorted pasta soup) and egg powder-spiked food samples found that the selected maximum reporting peptides varied across food matrices, total egg protein concentrations, and the type of egg powders (i.e., whole, white, and yolk powders). This demonstrated the benefit of monitoring a set of target peptides in accurate quantification for a diversity of food matrices. Among the nine monitored target peptides, GGL was the most frequently reported maximum peptide across all five food matrices. Moreover, the results demonstrated that the targeted MS method can quantify 1.18 ppm total egg protein across five incurred food matrices. This concentration is below the action levels relevant for the tested food matrices according to a reference dose of 2 mg total egg protein. The MS method showed superior protein recovery, especially in cookies, pie crust, and retorted pasta soup, compared to four commercial egg ELISA kits. Using the MS method, the average protein recovery across quantifiable incurred concentrations (above 1.18 ppm total egg protein) was approximately 84.8% in cookies, 78.0% in pie crust, 73.6% in pasta, 96.5% in ice cream, and 57.5% in pasta soup. These food matrices incurred with 4.7 and 47 ppm total egg protein were analyzed by four egg ELISA kits. The average protein recovery from two incurred concentrations ranged from below the limit of quantification (BLQ) to 39.3% in cookies, BLQ to 33.9% in pie crust, 19.7% to 63.1% in pasta, and 44.0% to 101.1% in ice cream. In addition, pasta soup incurred 4.3, 41.8, and 421.3 ppm total egg protein was analyzed by the ELISA kits, with an average protein recovery of BLQ to 14.6%. This comprehensive assessment revealed that the targeted MS method can quantify a relevant

reference dose for egg (2 mg of total egg protein) in a food consumption amount up to 1.69 kg.

The current targeted MS method was applicable for the reference dose of 2 mg total egg, which is expected to be safe for 95% of the allergenic population (ED_{05}), as recommended by an FAO/WHO expert consultation.[1] In some countries, a lower reference dose, 0.2 mg total egg protein recommended by VITAL 3.0 based on the ED_{01}, is used.[2] The current LOQ of the MS method can accurately quantify total egg protein concentrations applicable to VITAL 3.0 reference dose in cookies, pie crust, and ice cream but not pasta (1 ppm total egg protein, based on a consumption amount of 200 g) and retorted pasta soup (0.5 ppm total egg protein, based on a consumption amount of 400 g). To achieve accurate quantification at 0.5 ppm total egg protein, further optimization of the strategies may be needed to enhance the sensitivity of the MS method. Despite this challenge, the current LOD of the targeted MS method can detect egg protein in pasta and retorted pasta soup, which incurred 0.47 ppm total egg protein.

Future work can focus on improving the sensitivity of the MS method to meet the requirements of the VITAL 3.0 reference dose. Additionally, other complex matrices, such as salad dressing and mayonnaise, which are known to be challenging when using the ELISA method, could be evaluated to assess the performance of the MS method. Furthermore, the reproducibility of the MS method in terms of the transferability between instruments, laboratories, and MS methods could be validated (MRM vs PRM). Further evaluation of the applicability of the targeted MS method for quantifying total egg protein in commercial food products could be performed. The results from these food products can be used as input for allergen risk assessment for eggs.

In conclusion, this study demonstrated that the egg-specific targeted MS method is a robust, sensitive, and reliable tool for quantifying low levels of egg proteins in processed food matrices. The method's performance exceeds commercial ELISA kits, particularly in enhancing protein recovery in baked and retorted food matrices investigated. With LOD and LOQ levels complying with risk-based action levels, this targeted MS method is a promising alternative analytical method for egg allergen risk assessment and management in the food industry to improve food safety.

5.1 References

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