

SPECIAL GUEST EDITOR SECTION

From Signal to Analytical Reporting for Allergen Detection by Mass Spectrometry

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MS offers a flexible and precise alternative to traditional methods for allergen detection and quantitation. However, this flexibility also engenders many ways of acquiring information and translating it to simple, clear data useful to end-users. Currently, methods for performing data analysis for allergen detection by MS are unstandardized, and it is therefore difficult to compare different analytical methods. We identify three key components of data analysis: detection of positive signals, calibration, and signal integration. For each of these components, there are multiple pathways available for method developers. We discuss these alternative methods, giving examples from literature. Assuming that the end result of an allergen analysis should be clear, unambiguous, and understandable to all relevant stakeholders, we pay particular attention to the consequences of each choice to the analysis in question and, where appropriate, suggest best practices. We also identify data analysis criteria that should be clearly delineated in the reporting of a method. Establishment of community-wide standards for unambiguous reporting of data analysis workflows will improve the evaluation, comparability, and transferability of methods.

The properties of MS detection make it a viable and potentially powerful tool for allergen detection in foods (1). The inherently high resolution of MS instruments combined with pairing of parent and fragment ion detection leads to the potential for high specificity. The ability to discriminate small mass differences in peptides raises the possibility for internal control and calibration of MS-based assays using heavy isotope-labeled peptides. Developments in ion transfer and detector technology result in methods that approach, and in some cases exceed, the sensitivity of ELISA. The high (three to four orders of magnitude) dynamic range of detection reduces the need to frequently dilute and reanalyze samples. Of particular interest has been the inherently multichannel nature of MS instruments, which lends itself to the detection of multiple analytes during a

single analysis. These analytes may be from the same allergenic food, for increased rigor and utility, or from multiple allergenic foods, creating multiallergen methods capable of screening without additional analyses (2). The need for such screening methods capable of detecting unforeseen allergen risks has been highlighted by regulators (3), increasing the drive toward multiplexing.

There are many challenges facing the analytical community in developing MS methods. The selection of peptide targets for detection and quantitation relies on the availability of sequence information, usually derived from mRNA or, preferably, genomic sequencing efforts. The ability to select peptides that are present in the targeted allergenic food is obviously important. While substantial amounts of proteomic and/or genomic data are publicly available for some allergenic foods (e.g., soybean and cow's milk), very limited data are available for others (e.g., some tree nuts). Equally important, however, is a broad range of sequence information from other organisms that may be taxonomically similar to the allergen or comprise components of commonly used food matrices. Sequence information from these sources help select targets that are likely to be specific to the food allergen in question and therefore avoid false-positive detection, such as that which occurs by cross-reaction in ELISA. Given the wide array of organisms used to produce food ingredients, the amount of currently available protein sequence data is extremely small.

Additional data add complexity to analysis and interpretation. The advantages of MS can lead to multiple pathways from raw data to a final value used for reporting. MS data are typically acquired as multiple data points from ion signals within a defined mass tolerance across a chromatographic peak, leading to choices in how method developers define a signal. There are also options in how calibration is applied as a result of available options that are not applicable to traditional methods such as ELISA. Finally, how multiple signals from fragment ions, peptides, and proteins are used to produce an understandable and clearly derived reporting value is currently unstandardized. In this manuscript, we describe the options available for positive detection criteria, calibration, and signal integration. We address the consequences of each option and suggest ways in which MS method developers can improve the use of raw data in the particular circumstance of food allergen analysis. We also suggest that more clarity is needed in describing how such operations are performed along with recommendations for reporting how data analysis is performed.

Positive Detection Criteria

One of the key pieces of information necessary to evaluate and replicate an MS method for food allergen detection is a

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clear description of the criteria used to verify the selectivity and specificity of the signal obtained from the analysis. As is considered best practice in targeted proteomics applications (4, 5), food allergen method developers do frequently indicate that multiple transitions were monitored from multiple peptides. In many cases, however, one peptide or one transition demonstrates better performance in terms of detection and/or quantitative accuracy, and the majority of the reported data come from this best performing transition, with other transitions used as qualifier ions. Authors frequently do not give thorough descriptions about how the data from the qualifier ion(s) are incorporated into the overall data analysis procedure. For example, it is often unclear whether the presence of the qualifier ion was determined by manual inspection or whether objective metrics, such as product ion ratios, were utilized.

Given the wide diversity and variations in food products and formulations (e.g., sugar cookies, chocolate chip cookies, ginger cookies, etc.) that may require analysis for food allergens, determining possible background ion interference from exactly matched blank matrices for every sample seems impractical, if not impossible. In the absence of such determinations, implementing stringent, predetermined, objective criteria for signal confirmation will likely be critical to maintain sufficient specificity across food product formulations. Monitoring multiple transitions for each target peptide is one of the primary means to improve method specificity. However, in the case of complex food matrices, the simple presence of multiple target product ions may not provide sufficient selectivity. More definitive confirmation can be obtained by using additional metrics to verify the quality of the transitions. As product ions from the same precursor should be generated in reproducible patterns and over concurrent retention times, comparison of the observed product ion ratios with those obtained from a known sample are among the most informative metrics to verify that the observed production ion signals were from the target peptide.

If internal standard (IS) peptides are incorporated into a method, they can be utilized to verify the product ion ratios as well as the retention time profile of transitions from a target peptide. For methods without IS peptides, reference spectra from external standard analyses can be utilized to establish expected product ion ratios. Targeted proteomics software platforms frequently include some variation of a product ion ratio measurement (6). Defining a tolerance for production ratio variation (e.g., $\pm 10\%$) in advance removes the need for manual assessment of product ion signals, which can lead to variability in interpretation of results. Additional tools, initially developed for clinical proteomics, are available to perform statistical comparisons between sample and IS signals to identify problematic transitions (7). To assess method quality and allow for replication, method developers should clearly indicate how many and which transitions were required for confirmation, how transition or product ion quality was assessed, and whether IS peptides or external standard spectra were used as references. The guidelines developed by the clinical quantitative proteomics community may serve as a useful reference for what information is necessary for evaluating and replicating targeted methods (8).

LOD and LOQ

Among the most widely discussed attributes of detection methods, including those for food allergens, are LOD and LOQ.

LOD, while a widely used term, is often used with varying definitions. LOD is defined by some as the lowest concentration or amount that can be reliably distinguished from a true negative sample (9). Often, the LOD is inferred by users to mean the amount or concentration of a particular analyte that can be detected by the method in question. These types of definitions, however, lack specificity with respect to the level of statistical certainty associated with the detection. The International Organization for Standardization and the International Union of Pure and Applied Chemistry have therefore developed nomenclature for detection limits (L_D) based on statistical methods for hypothesis testing (10). AOAC INTERNATIONAL has also endorsed this type of L_D determination for food allergen ELISA methods (11). Hypothesis testing methods can take into consideration two types of errors. Type I errors (with a probability designated by α) occur when a sample is determined to contain the analyte when in fact the analyte is not present, otherwise referred to as a false positive. Type II errors (with a probability of β) occur when a sample is determined to not contain the analyte when in fact it is present, also called a false negative. Both types of errors are issues for food allergen analysis, with Type II errors or false negatives possibly representing health risks.

In order to clearly illustrate these types of errors and their relationships to L_D , additional metrics are required. First, the distribution of observed results for a blank sample should be considered. If a normal distribution of observations is assumed, the results obtained from an analysis of a true blank sample would deliver a theoretical distribution of observed results as shown in Figure 1. The observed value from an analysis that would need to be exceeded to provide a specified level of confidence ($1-\alpha$) that the sample was different from the blank can be estimated from the distribution of results from analysis of blank samples. This level is established with an accepted false-positive error rate and is often referred to as the critical limit, the limit of the blank, or decision limit (L_C ; 10, 12, 13). As a default, $\alpha = 0.05$, indicating a 5% chance that the determined result will be a false positive. In contrast, the L_D is an a priori characteristic of the method that establishes the true concentration that would be detected as a positive result, with a specified confidence ($1-\beta$), given the use of the L_C (Figure 1A). The L_D can also be referred to as the LOD or detection capability (10, 12, 13). The false-negative error rate tolerance (β) is generally also set to 5% as a default.

The estimation of L_C and L_D can be accomplished by a number of means. If the results of blank sample analysis can be assumed to follow a normal distribution with a standard deviation of σ_B , the L_C can be estimated as $1.645 \times \sigma_B$ (for $\alpha=0.05$) as shown in Figure 1A. If the SD of the blank is equal to the SD of a sample at the L_D (σ_D), the L_D can be estimated as $3.29 \times \sigma_B$ (10). Some guidance documents recommend using $3 \times \sigma_B$ to estimate LOD (14). This method, however, can have a number of complications. First, if the L_D is then also used as the L_C , which is often the case, β will be 50% (i.e., a 50% chance of a false negative), as shown in Figure 1B. In addition, often the assumption is that $\sigma_B = \sigma_D$ is incorrect and σ increases as the signal and/or concentration increases, resulting in a false-negative rate greater than 5% ($\beta > 0.05$, given L_C , Figure 1C). A wide array of statistical methods have been developed to account for different assumptions and characteristics of result distributions, and these methods are reviewed elsewhere (10, 14–17). Even in cases in which method developers attempt to estimate both σ_B and σ_D , they are often faced with the fact that many instrument platforms or

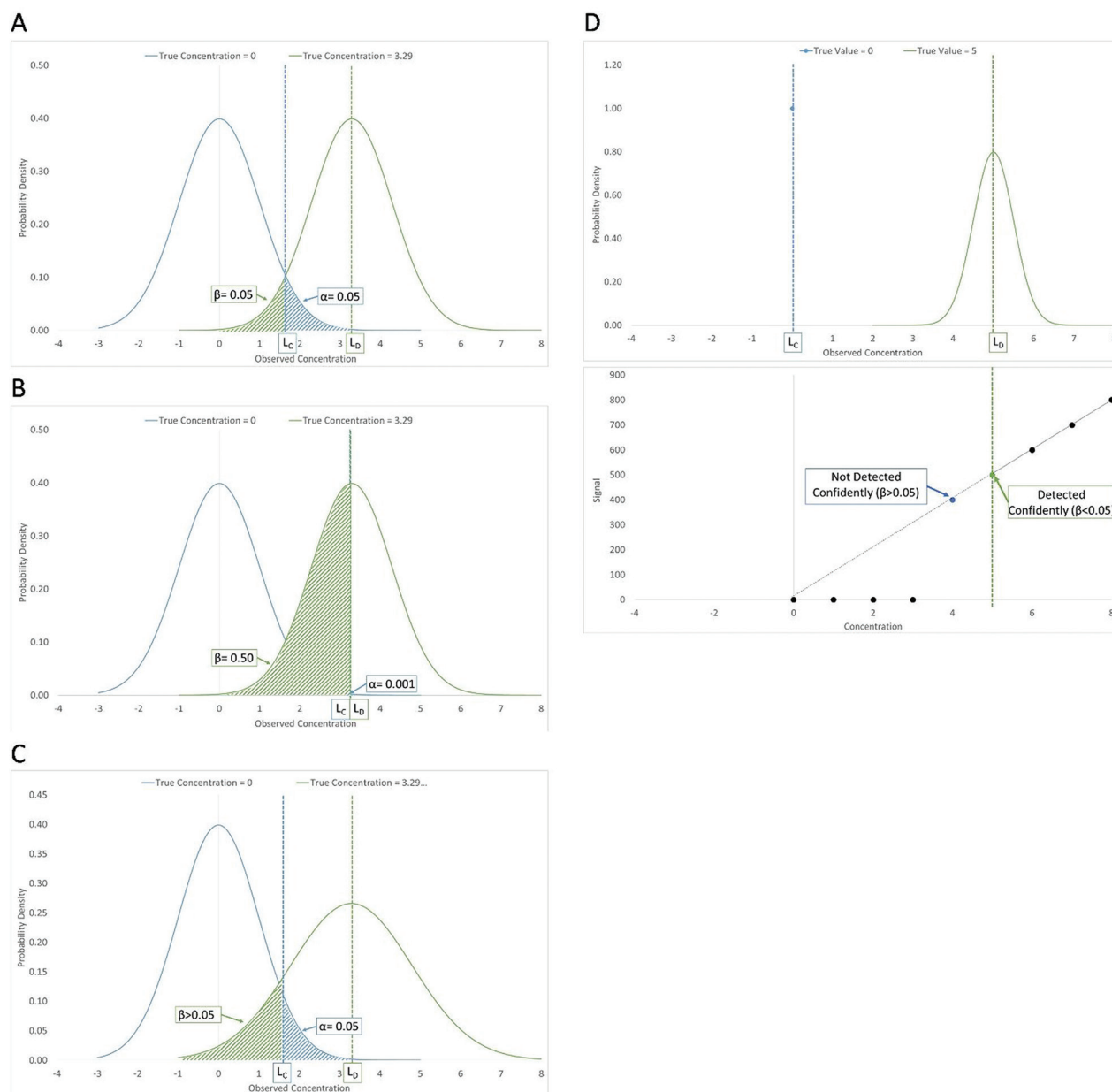


Figure 1. Decision limits (L_C) and detection limits (L_D). Hypothetical distribution curves are shown representing different scenarios, with shaded areas indicating Type I (α) and Type II (β) errors. Distribution curves represent normal distributions of observed results for a true negative sample (blue) and a positive sample, with a true concentration of 3.29 (green). (A) Ideal scenario, where $\alpha = \beta = 0.05$. (B) L_C set equivalent to L_D , resulting in $\beta = 0.50$ and $\alpha = 0.001$. (C) Variance of observed results increases with concentration, resulting in $\beta > 0.05$. (D) Very low or no observed signal in blank, resulting in $L_C = 0$.

analysis methods (including MS) do not provide negative results, resulting in a non-normal distribution of blank results. In these cases, nonparametric methods are required to estimate the L_C and L_D (15). Lastly, in relation to MS method for proteins, if the specificity criteria for a positive signal described above are utilized, it is possible for no (or very low) positive signals to be determined in the blank (making $\sigma_B = 0$) and therefore making $L_C = 0$ (Figure 1D). L_D is therefore dependent only on the true sample concentration at which a positive signal is reliably obtained (12).

Unlike the L_D and L_C , the LOQ of a method is not defined by hypothesis testing statistical measures. Instead, LOQ is generally

defined as a true sample concentration at which a method will deliver a sufficiently reproducible quantitative result. Often, the requirements for reproducibility are established as 10–20% relative SD (RSD), depending on the application (10, 17). If the σ remains constant across sample concentrations, LOQ can be estimated as $(1/\text{target RSD}) \times \sigma_B$ (i.e., $\text{LOQ} = 10 \times \sigma_B$ when target RSD = 10%). As noted above, however, σ rarely remains constant as concentration increases, so other methods often are required to estimate LOQ (10).

Despite the existence of guidance to estimate and use L_D based on hypothesis testing statistical measures, these methods rarely have been used to express detection capabilities in food

allergen methods, including MS-based methods. In part, there are practical issues with having two separate concentrations used for a priori descriptions of method capabilities and for decision making with actual sample data. In addition to potentially leading to some confusion among users, having these two distinct limits may also require users to occasionally interpret results that fall between the L_C and L_D . These types of scenarios can be particularly problematic given the wide array of matrices to which food allergen analysis methods may be applied. While the ideal situation would be to have validation of L_C and L_D in each type of food matrix, this is impractical, if not impossible. As noted in the previous section, even obtaining an exactly matched blank to use for determining the L_C can be challenging. However, users are also unlikely to understand that if the true concentration of a sample is equal to the stated L_D and the L_D is used as the L_C , half of the results would be predicted to be negative and half to be positive.

Given all of these challenges and restraints, it has remained unclear how developers of MS-based food allergen methods should proceed with determining and expressing the detection capabilities of a method. The outcome has been methods with a wide variety of definitions and approaches used to determine LOD or LOQ, often times with very little information about how the authors arrived at those values. In many instances, the values expressed as LODs and/or LOQs would more precisely be called lower limits of applicability (LLAs), as they are not estimated using the statistical methods discussed above but rather represent the level below which the developer does not claim that the method can produce reliable results. Regardless of whether method developers wish to use true LODs, LOQs, or LLAs, the definition of those values and the methods used to arrive at or validate the values should be unambiguous. If the hypothesis-testing-based methods are used to estimate the values (including simplified methods such as $LoD = 3 \times \sigma_B$), developers should also demonstrate that key assumptions underlying these methods are met (e.g., normality, uniform variance, etc.). In the end, the level of confidence in determining true positives and true negatives at the stated LODs, LOQs, or LLAs should be clear to a reader or end user of the method.

For many other analytical applications, the estimation or selection of LOD is not terribly consequential, as the measurement range relevant for taking actions is typically higher than the LOD of the analytical method. In the current situation in which no regulatory limits or action levels have been established for undeclared allergens, however, this becomes very problematic, as the LOD of the analytical method becomes the de facto action limit. A report from a U.S. Food and Drug Administration Threshold Working Group tasked with identifying and evaluating approaches to establish thresholds for food allergens found that while an analytical-methods-based approach could be used to establish thresholds for major food allergens in the short term, this approach was fraught with sufficient issues, so if it were to be used, the thresholds should be replaced with those established under another approach as soon as possible (18).

Calibration

Calibration is the process of converting an observed signal to a quantity of analyte. Most quantitative detection methods for chemicals (including peptides and proteins) depend on the comparison of a signal obtained from an analyte to that obtained

from a known concentration of the same analyte. There are many options when selecting a calibration strategy for an MS method for allergen quantitation, which we summarize here. The diversity of calibration options is due in part to the lack of AOAC, or other standardization body, guidelines for the overall calibration strategy or composition of calibrants to be used in an MS method for quantifying allergens. However, guidance for such methods may be extrapolated from either MS methods for other analytes or from that provided for the use of other methodology (typically ELISA-based) to detect allergens. Regardless of the type of calibration chosen, the fine detail of calibrant preparation has the capacity to greatly effect resulting quantitation. Minor differences in the source of the calibrant material, any processing applied, and extraction methodology will likely affect the result and therefore hinder reproducibility and comparability. Precise detail (particularly cultivar or genotype of calibrant material, time/temperature of processing, any defatting applied) is often lacking from descriptions of methodology. Until a time when reference materials are generally available to the community, such detail will remain crucial in allowing method comparability and evaluation.

External Calibration

A calibration in which analysis of the calibrant is performed within a separate analysis to that of the sample is referred to as external. Application to an MS allergen detection method would typically involve the preparation of a standard curve of allergen-containing food, with subsequent preparation following the same procedure as that used for unknown samples. As this type of calibration is used by existing commercial allergen detection methods, best practice may be drawn from guidelines for calibration of, for example, ELISA methods such as in Abbott et al. (11), who suggest the information that should be provided regarding calibrators but largely leave the selection of materials to method developers. A simple option would be to digest a known quantity of allergen-containing food and dilute to achieve concentrations of calibrant, the analysis of which yields a standard curve. However, this approach ignores digestion and sample preparation efficiency at different peptide target concentrations. A more rigorous approach would therefore involve the preparation of powdered calibrants at different concentrations within an inert and easily extractable matrix (such as sorbitol). However, the use of inert matrices ignores the effects that complex real-world food matrices may have on sample digestion and processing. It is well established that extraction and digestion of allergens in incurred foodstuffs severely impacts recovery and that some peptides may behave differently to others after matrix and/or thermal processing is applied (19), in a similar fashion to which ELISA detection is affected by matrix and processing (20). However, the use of standard curves generated using similar matrices and processing to that expected in samples would require prior knowledge regarding the state of unknown samples and that different calibration curves be generated for different types of sample.

Internal Calibration

MS offers the possibility of using mass-modified but chemically identical analytes as calibrants. For protein quantitation, heavy labeled peptides, usually labeled at the C-terminus

(typically lysine and arginine for tryptic digests), with ^{13}C , ^{15}N are spiked into digesta before MS analysis (21). Although AOAC guidelines for detection of dietary supplements and botanicals (22) describe the use of internal or external calibrants for MS, there are currently no such guidelines for allergen analysis by MS. A considerable advantage of using internal mass-modified calibration is the accounting for instrument stability in measurements, variation in which was found to be second only to extraction variability in a study of brain tissue peptides (23). An example of the application of an internal calibration applied to allergen analysis (in this case, peanut, milk, and egg) is given by Parker et al. (2). The amount of light peptide (in moles) is first calculated using the observed light:heavy peptide ratio and the known amount of heavy peptide on column. The amount of peanut present (in grams) is then derived using a series of calculations to account for target protein mass, proportion of total peanut protein that is the target protein, moisture content, and protein content of the allergenic food material. In many cases, some of these variables (in particular the proportion of the protein present that is the target protein) may not be known or are poorly estimated. An additional disadvantage of IS calibration methods is that the number of targets that must be measured, and thus demand on instrument cycle time, is effectively doubled. As the calibration is performed at the peptide level, losses of peptide during sample extraction, digestion, or cleanup are not reflected in calculation of the amount of allergenic food present unless specifically included. Although such synthetic heavy peptides can typically be made to relatively high purity (>90% w/w) and concentrations estimated accurately, studies have suggested that quality assurance of these materials require vigilance lest avoidable errors impact analysis (24, 25). Recommendations for the production, quality assurance, and storage of such labeled peptides from the Clinical Proteomic Tumor Analysis Consortium of the National Cancer Institute (26) should be applicable to development of best practice for allergen detection.

External Calibration with ISS

Both external and internal calibration methods have advantages related to the detection of allergens in foods. The use of internal calibrants alone is particularly prevalent in published allergen-detection methodology. However, while internal calibration is particularly suited to the quantitation of individual peptide and proteins in a mixture, the assumptions required to calculate total allergenic food protein and total allergenic food commodity introduce potential error and, if different assumptions are made, variability between methods. External calibration involves multiple measurements at different analyte concentrations and allows for the possibility of using spiked, incurred, and matrix-matched calibrants.

In a study by Newsome and Scholl (27), the authors used an external calibration curve in which each dilution of calibrant was spiked with a set amount of heavy label. A calibration curve of calibrant concentration versus light-to-heavy ratio was thus obtained. Unknown samples were prepared with a spike of heavy peptides, as is traditionally performed when using an internal calibration approach, and the observed light-to-heavy ratio was used to determine the analyte concentration based on the external calibration curve. This calibration workflow elegantly combines many of the advantages of both internal and external calibration (Figure 2).

Signal Integration

Transition Information

The ratio of transitions of a given peptide under set fragmentation conditions should be consistent regardless of other experimental conditions (28). Therefore, if the selected fragment ions are specific to the peptide analyte (i.e., not generated from the matrix), any single transition or the summed total of such selected transitions should be suitable for quantitation. However,

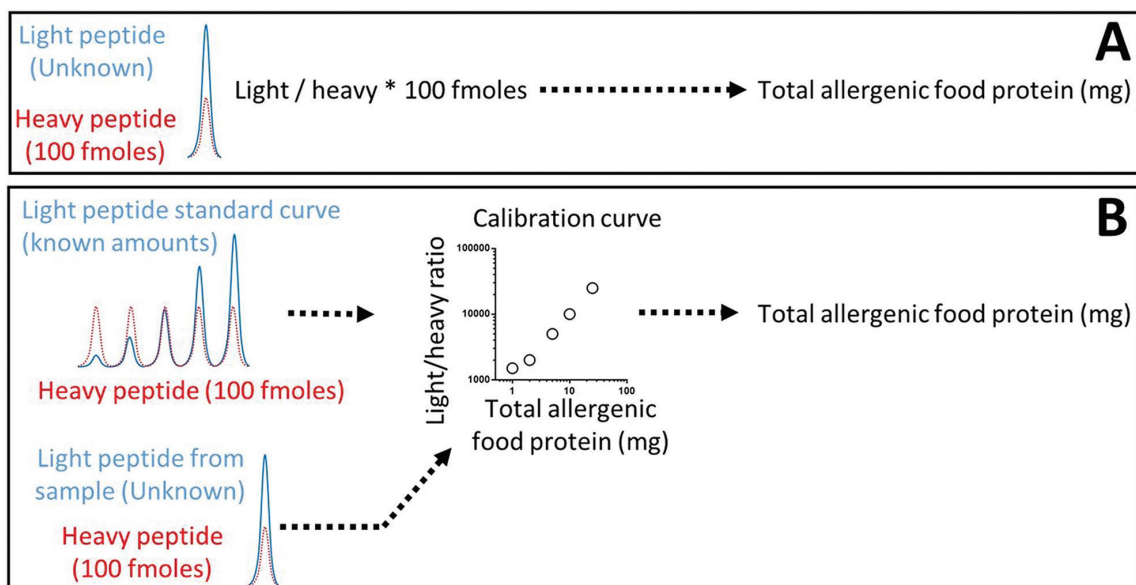


Figure 2. Consequences of integrating MS peptide signals with differing peptide target recoveries. Two integration methods are illustrated: taking a mean average of three peptide abundances and using one quantifier peptide (in red). Two theoretical sample analyses are used as examples: (A) consistent recovery of all three peptides and (B) good recovery of selected quantifier but poor recovery of two quantifiers.

selection of fragment ions, and how they are combined for quantitation, will affect analytical variability and LODs and LOQs. In multiple-reaction-modeling-based assays, it is common practice to select quantifier and qualifier secondary ions, with only the quantifier being subject to calibration and therefore used in the quantitative determination of target presence. The qualifier is intended to serve as a confirmation of analyte (e.g., peptide) presence, usually by calculation of the ratio of qualifier and quantifier ion signals present. One example of this approach (2) used a tolerance limit of $\pm 20\%$ of relative ion abundances. The sum of all measured transitions may also be used as the quantifier signal (19). However, in this case, care must be taken that acceptability limits are established for the quality of signal for each of the contributing ions. If the sum of, for example, three transitions is used to quantify, background food matrix ions may nonspecifically contribute to a transition signal, resulting in failed acceptability criteria and thus invalidating the measurement; however, the target allergen may still be present. Additionally, given the unequal intensity of product ions, the use of summed transitions for calibration would result in nonlinear standard curves at low peptide concentrations, at which some transitions are undetectable.

Integration of Multiple Peptide Signals

Most MS assays for allergenic food and proteins employ multiple peptide targets. Often, these peptides are derived from different proteins. Because of this, it is important that methods

have a valid strategy for combining the data that results from multiple peptide measurements. This strategy should be reproducibly applied and also provide the most favorable analytical characteristics in terms of method variation and quantitative limits. In a situation in which target protein and target peptide quantities are constant in a given allergenic food and can be expected to recover consistently from extraction and subsequent sample preparation, the ratio of peptide targets to one another should be consistent. However, in the case of allergen analysis from diverse foodstuffs, this is frequently not the case. Choosing which data is used and how it is processed therefore becomes a critical step that can greatly affect final results. A well-described example of differing target protein and peptide solubility is the loss of the peanut allergen Ara h 1 upon roasting of peanuts, described using both antibody-based (29) and MS (30) techniques. This loss is particularly noticeable when an MS-based assay detects peptide targets from both Ara h 1 and peptides from more soluble protein targets (19). The analysis of component parts (casein or whey fractions) is another example of a scenario in which the presence of different target peptides is expected. When differing target protein solubility or presence occurs, using a sum of all peptide targets to quantify allergenic food would tend to underestimate allergen content. Figure 3 describes this effect using a theoretical example of the detection of three peptide targets for an allergenic food, in three different foodstuffs, each different regarding protein recovery profile. In this example, two peptide signals are reduced in one of the foodstuffs (sample B), resulting in an underestimate of allergen content when the sum of all three peptide signals

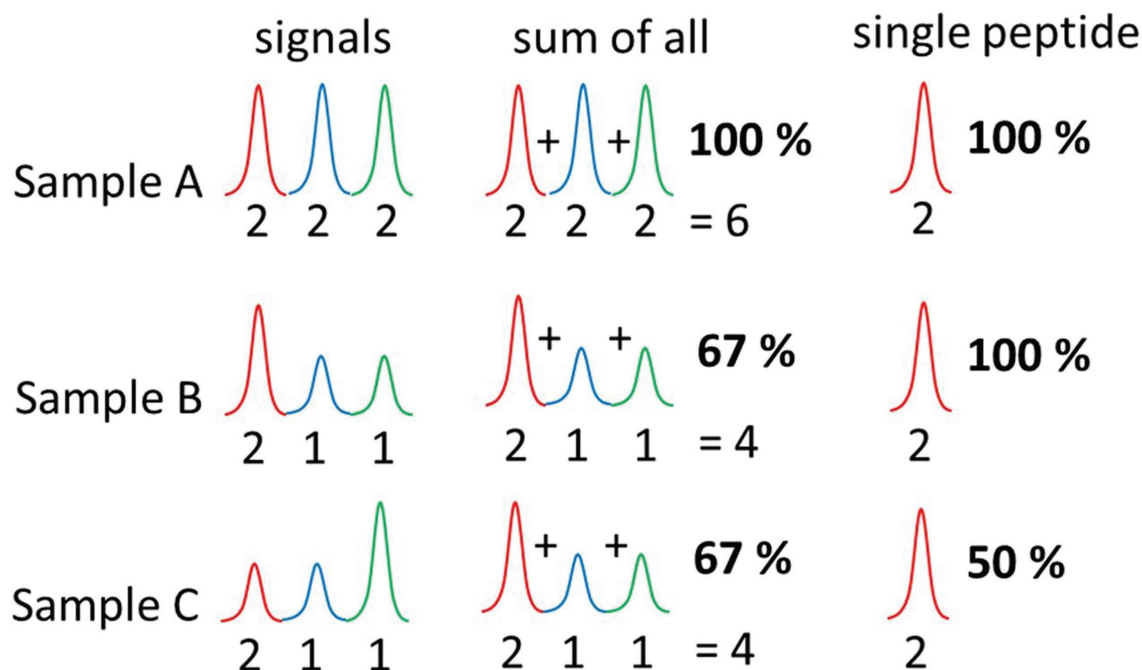


Figure 3. Illustration of two calibration workflows for MS quantification of allergens. (A) The use of IS peptides to quantify light peptide equivalents (molar amount). The amount of food allergen (commodity weight) present in an unknown sample is then calculated using assumptions of molar equivalence of peptide to protein, the known molecular weight of the protein, the proportion of the total protein complement of the allergenic food that is the protein target used, and the protein content of the allergenic food. Other factors (such as moisture content of the sample) may also be included in the calculation. (B) The use of an external calibration curve (from analysis of either the allergenic food commodity or a food matrix incurred with the allergenic food commodity) with SI peptides included in both the sample and calibrants. The calibration curve is generated using the light-to-heavy ratio for each calibrant sample. Allergenic food present in an unknown sample is determined by comparison the external calibration curve (typically using linear regression).

are used in quantitation. Where a single quantifier peptide is chosen, the allergen determination is consistent between the two food matrices. Sample C illustrates a scenario in which one peptide recovers well, but this is not the selected qualifier. In this case, the use of a flexible quantifier ion (selected after analysis is performed) would be beneficial. However, use of flexible quantifier ions would lead to a method with varying characteristics, including L_D , based on which was chosen and therefore dependent on the composition of the sample.

Recommendations for the Processing of Data for Allergen Analysis

Regarding signal integration, multiple measures of target peptides by MS offers new challenges to a data analysis workflow. The issues presented by calibration, and particularly those of extraction, are similar to those that analysts face with ELISA. Below, we provide suggestions for transparent and reproducible analysis of data for MS measurement of allergens. Some of these are applicable to allergen quantitation using ELISA.

Criteria for signal selection should be predetermined and clearly stated for the method. If at all possible, the necessity for manual inspection and assessment of signals should be removed, and predefined objective metrics should be used to assess signal quality.

Calibration materials should be fully described. The description should include source of material, processing of material, extraction, storage, and any characterization performed.

Calibration materials should allow for their use by other laboratories as much as is practicable. For this, and for other reasons, available reference materials, certified reference materials, and QC materials are preferred. Currently, few such materials exist.

The mathematical operations for a calibration should be described to a such a degree that someone experienced in the field could correctly replicate the calculations. Example calculations are often appropriate. A spreadsheet file allowing for the entry of MS signal data with automatic calculation of final reporting values, with fully described formulae for these operations, is preferable.

The strategy used to select signals for use in quantitation, and those to be used qualitatively, should be fully described. When qualitative data is used to make decisions regarding final reporting, the criteria used to inform these decisions should be described mathematically.

As developers are determining the data analysis workflows for their methods, consideration should be given to scenarios that could reasonably be encountered when analyzing diverse food products, paying particular attention to situations that could lead to false-negative results.

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