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Can Mass Spectrometry Analysis of *In Vitro* Digestion Products Improve the Assessment of Allergenic Potential of a Newly Expressed Protein?

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Abstract

The rigorous safety assessment conducted on genetically modified crops includes an evaluation of allergenic potential for an associated newly expressed protein (NEP). Since no single method is recognized as a predictor for protein allergenicity, a weight of evidence approach (WOE) has been adopted. *In vitro* digestion is a part of the WOE approach and is used to evaluate the susceptibility of a NEP to digestion by gastrointestinal proteases. In 2017, the European Food Safety Authority outlined additional digestion conditions and suggested liquid chromatography tandem mass spectrometry (LC-MS/MS) as an analytical method to detect small post-digestion peptides. This technical review paper focuses on the question of whether LC-MS/MS can aid in assessing allergenic potential of *in vitro* digestion products generated under the newly proposed conditions. After an extensive review, it was determined that LC-MS/MS can detect very small digestion products. However, the method cannot provide relevant information to differentiate whether these products are allergenic or non-allergenic. Therefore, the use of LC-MS/MS for a standard *in vitro* digestibility assessment provides no improvement in allergenicity prediction.

Keywords: genetically modified crops, allergenicity assessment, *in vitro* digestion, mass spectrometry

1. Introduction

Genetically modified (GM) crops undergo an extensive safety assessment prior to commercialization. A key component of the food safety assessment for a GM crop is an assessment of allergenic potential of the newly expressed protein (NEP). Due to the lack of clear understanding of mechanisms of allergenic sensitization and elicitation, there is no single test or characteristic that can predict the allergenic potential of a protein or peptide [6, 27]. As a result, an overall weight-of-evidence (WOE) approach with a cumulative body of evidence was adopted to assess the allergenic potential of NEPs in GM crops [6, 9, 43]. The registration requirements to address the allergenicity potential of a GM crop include the source of the NEP, a review of the history of safe use for the NEP and its homologs, a bioinformatics analysis of amino acid sequences, the stability of the protein when heated, and the susceptibility of the protein to gastrointestinal enzyme digestion (e.g., pepsin). Resistance to pepsin digestion was initially linked to allergenicity by one research publication and an opinion paper [2, 25] under the premise that more stable proteins tended to be allergens via the potential for increased exposure to the gut immune system, one route for sensitization and elicitation of allergy. However, subsequent investigations have revealed that there is no direct correlation between pepsin resistance and allergenicity [3, 13, 17, 32]. Despite the lack of a clear correlation, the *in vitro* pepsin digestion test remains as one component in the WOE approach.

The current standardized *in vitro* digestion assay has been adopted and accepted globally for almost two decades; this assay consists of a pepsin resistance time course of the NEP followed by a qualitative assessment for the presence or absence of intact protein or degradation fragments (>3 kDa) on a stained sodium dodecyl sulfate-polyacrylamide gel and/or western blot [38]. In 2017, the European Food Safety Authority (EFSA) Panel on Genetically Modified Organisms outlined new *in vitro* digestion conditions and proposed an additional detection method, liquid chromatography tandem mass spectrometry

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(LC-MS/MS) [26]. The hypothesis is that the combination of newly outlined *in vitro* digestion conditions and detection of small peptides by LC-MS/MS could enhance the identification of the allergen potential of a NEP [11, 26]. The newly proposed *in vitro* digestion conditions consist of pepsin digestion to represent gastric digestion followed by trypsin and chymotrypsin digestion to represent intestinal digestion. For pepsin digestion, the EFSA GMO Panel recommended the use of classic (low pH and high enzyme-to-test protein ratio) and suboptimal (high pH and low enzyme-to-test protein ratio) conditions to consider populations with impaired or underdeveloped digestive systems [38]. Recent observations suggested that pepsin digestion tests under suboptimal conditions would not provide useful information because some proteins that are readily digested by pepsin could show resistance to degradation and lead to the inability to distinguish between pepsin labile and pepsin resistant proteins [1, 41].

For the proposed detection of smaller digestion peptide fragments, the EFSA GMO Panel recommended LC-MS/MS to identify and track abundance of small peptides ($\geq$ 9 amino acids in length) in conjunction with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for intact protein and large peptide observations. The premise is that an LC-MS/MS detection method would aid in identification of a hazard and its exposure by providing valuable information through distinguishing allergens from non-allergens and tracking their abundance. At a first glance, the recommendation to use LC-MS/MS as an alternative detection method seems reasonable considering it has been used for peptide identification since the 1990s and applications have advanced with continued improvements in modern separation, detection, and computation technologies [33]. For example, LC-MS/MS has been used to detect and identify peptide-based biomarkers involved in disease, like the beta-amyloid fragments associated with Alzheimer's disease [7, 18, 22]. Researchers have used this technology to understand protein degradation in a variety of matrices, such as the digestive fluid, milk, urine, and stool [4, 19, 28]. This technology has been used to detect and identify *in vitro* digestion products of several allergenic and non-allergenic proteins [14, 39]; and theoretically, LC-MS/MS methods can be optimized to detect peptides from *in vitro* digestion of NEPs expressed in GM crops. However, prior to implementing a new detection method to the evaluation of the *in vitro* digestion, the LC-MS/MS method should be evaluated as to whether it adds improved utility with respect to the assessment of allergenic potential. This paper examines the technical capability of LC-MS/MS analysis for detection, identification and tracking of *in vitro* digestion products under the proposed conditions, and whether LC-MS/MS provides improved utility to differentiate allergens from non-allergens [11, 26]. Although a discussion of the proposed *in vitro* digestion conditions is important, the focus of this communication is the proposal to use LC-MS/MS for identification and its ability to assess allergenic potential.

2. Applicability of LC-MS/MS to Evaluate *In Vitro* Digestion

Mass spectrometry (MS) is an instrumental technique for separation of electrically-charged molecules as ions in a gas phase. When paired with liquid chromatography (LC) separation capabilities followed by powerful software tools, mass spectrometry can give detailed information on both the identity and abundance of peptides. This technology has demonstrated great analytical potential for purified proteins and complex matrices; it is crucial to apply this technology properly to understand the unique aspects of mass spectrometry data generation and analysis. The LC-MS/MS method requires technical establishment for GM crop *in vitro* digestion analyses with respect to sample preparation (digestion and desalting), LC-MS/MS analysis (ionization and detection), and data analysis. Ultimately, the resulting data are critically linked to the efficiency of each step and the peptides' physiochemical properties, which have significant variations in size, structure, and abundance. Some peptide fragments from the digestion could be lost during sample preparation or become essentially undetectable to the LC-MS/MS [35]. For instance, there can be high variability in the ranges of peptide abundance, which could lead to masking of less abundant peptides [36]. Also, some high-abundance peptides may not ionize with commonly used positive-charged ions mode proteomics due to the presence of acidic amino acids or cysteine residues [10, 12]. For these reasons and others outlined below, some peptides may not be detected by LC-MS/MS and others may appear to have an artificially enhanced relative abundance. To evaluate LC-MS/MS in perspective for *in vitro* digestion analysis of NEPs, we provide a technical review of mass spectrometry methods based on sample preparation, MS analysis, and data analysis, as well as a discussion of its utility for *in vitro* digestibility assessment.

2.1. Sample Preparation

Many factors are considered when designing MS sample preparation strategies, including sample source, type, physical properties, abundance, and complexity. As a result, it is important to describe how the *in vitro* digestion samples are prepared. The *in vitro* digestion sample preparation steps that the EFSA GMO Panel recommended are outlined in Figure 1. Briefly, a NEP hydrolysis time course is performed with a gastric enzyme (i.e., pepsin) under classical or suboptimal conditions. Pepsin has relatively low specificity, with preferential hydrolysis of the peptide bonds for aromatic amino acids, and it can cleave other peptide bonds with hydrophobic amino acids. The lack of specificity could result in variability between repeated digestion assays. Next, the enzyme is deactivated, and the sample is further hydrolyzed by intestinal enzymes (i.e., trypsin and chymotrypsin) with bile salts. Trypsin and chymotrypsin have relatively high specificity to basic amino acids and aromatic amino acids, respectively. In addition, bile salts are considered an important component for intestinal digestion; therefore, they may be included during trypsin and chymotrypsin digestion [26]. After hydrolysis, trypsin and chymotrypsin are deactivated. The precise timing

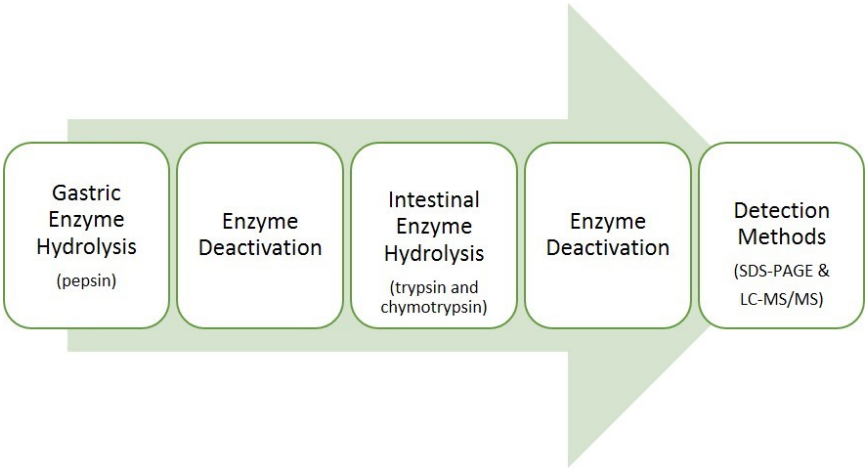

Figure 1: The proposed *in vitro* digestion steps include the following steps sequentially: gastric enzyme hydrolysis (e.g., pepsin) 60 min time course of NEP at low pH/high enzyme (classic) or high pH/low enzyme (suboptimal) with bile salts; deactivation of gastric enzyme by pH neutralization; intestinal enzyme (e.g., trypsin and chymotrypsin) hydrolysis 60 min time course with bile salts; deactivation of intestinal enzymes by acid or inhibitors; and application of SDS-PAGE and LC-MS/MS detection methods.

of enzyme activity deactivation is important for reproducible observations of stable peptides. The inhibition of pepsin can be achieved by neutralizing the pH, since pepsin is irreversibly deactivated at pH 7 [29], while inhibition of trypsin and chymotrypsin can be achieved by addition of acids or protease inhibitors such as 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), Tosyl phenylalanyl chloromethyl ketone (TPCK), and Tosyl-L-lysine chloromethyl ketone (TLCK). Finally, the digestion products are analyzed by SDS-PAGE and LC MS/MS.

Considering the steps outlined above, sample preparation and the removal of unwanted components are essential for MS analysis. For the proposed *in vitro* digestion of a NEP, purified protein (1-5 mg/ml) is generally used. The absence of other biological matrix components (lipids, starch, etc.) make the three-enzyme (pepsin, trypsin, and chymotrypsin) *in vitro* digestion system less complex compared to biological samples from tissues or fluids [26]. However, the purified protein sample may be at a pH required for purification or stable storage that is unsuitable for LC-MS/MS or contain incompatible buffer components, such as non-volatile salts, detergents and bile salts. The final quenched digestion mixture may include components that are incompatible with the electrospray ionization step, interfering with the MS detection process (see Mass Spectrometry Ionization Methods section). Surfactants, such as bile salts, could be detrimental and suppress ionization of peptides. Therefore, the removal of bile salts may require a desalting step. Optimization of desalting is necessary to prevent any significant loss of peptides.

Top-down proteomic methodology analyzes intact protein while bottom-up proteomic methodology analyzes enzyme hydrolyzed peptides. In traditional bottom-up proteomics, there is a common workflow to prepare proteins for enzyme digestion [15]. Concurrently or after treatment with a chaotropic reagent, disulfide bonds within the proteins and peptides are cleaved to allow further processing. Dithiothreitol is commonly used for this purpose and would modify any cysteine amino acids involved in forming disulfide bridges at these specific sites within the protein. Once the bonds are disrupted, an alkylating reagent is typically used to "cap" the residues capable of forming disulfide bonds and thus prevent any bond reformation. When assessing *in vitro* digestion of NEPs, typical pre-MS proteomic sample preparation, such as reduction and alkylation, would be eliminated to avoid breaking disulfide bonds and artificially impacting NEP stability. The absence of these steps allows detection of disulfide-bond linked protein/peptides but the resulting peptides with multiple disulfide bonds may be difficult to characterize due to incomplete fragmentation [20].

After digestion, sample enrichment and/or clean-up is a critical step. The enrichment can be accomplished in several ways, including, but not limited to, acid precipitation, solvent extraction, and/or molecular weight-based filtration (e.g., 10 kDa cut-off selects for peptides less than 10 kDa). Peptides may be lost with enrichment steps due to non-specific binding or inability to resolubilize. Samples could be cleaned-up by solid phase extractions (e.g., C18 cartridges or filter plates) to remove intact protein and bile salts for improved peptide detection; however, it is important to note that very hydrophobic peptides may never be recovered. These steps need to be carefully evaluated and the sample preparation process must be controlled to achieve reproducible results.

2.2. Liquid Chromatography Separation

High-performance liquid chromatography (HPLC) has often been included as a physical separation technique prior to MS analysis to improve detection. This process involves incorporating the analytes (peptides and proteins) into a mobile phase that is pumped across a stationary phase contained in a column, and allows separation of the analytes by their relative affinity for either phase. Based on their properties, peptides

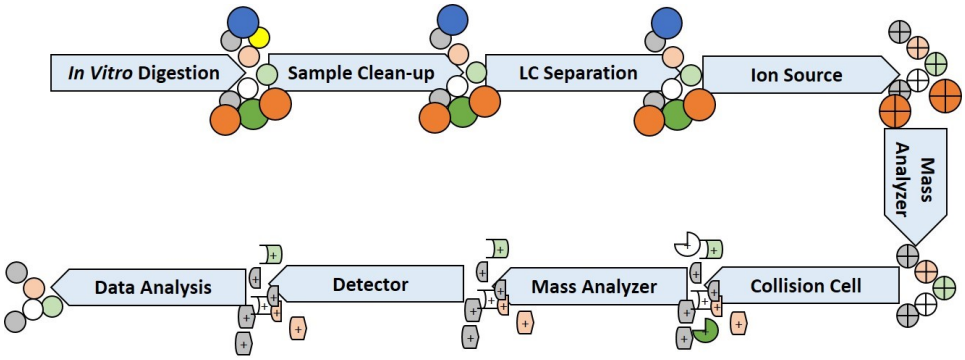

Figure 2: Peptides (circles) are generated during in vitro digestion. Sample clean-up removes incompatible components but may cause peptide loss (yellow circle is lost). Peptides are then separated during liquid chromatography prior to ionizations in the ion source. Ionization efficiency may lead to loss of peptide detected (blue circle is lost). Ions are separated in the mass analyzer and fragmented (large white and orange circle are lost). The resulting ions are separated and detected (partial circles are lost). Finally, peptides are identified during data analysis. As illustrated, peptide (ion) loss may occur at multiple steps.

present within a digestion mixture can be separated; this separation allows more peptides to be efficiently ionized and generate mass information, as there is less competition for the ionization potential at a given time. A variety of LC flow rates can be employed from high flow rates (>0.2 mL/min) to microflow (1-200 μL/min) or nanoflow (<1 μL/min), and there are compatible columns and systems used specifically for these conditions. The low flow rate in microflow and nanoflow allows ionization of a sample to last for minutes and enables orders of magnitude more observations compared with the observations from a regular flow of electrospray ionization (ESI) [24]. Nanoflow LC has unique advantages in supporting detection of more peptides than other techniques due to increased ionization efficiency and reduced ion suppression that leads to higher MS sensitivity. Likewise, microflow would also yield a nearly ideal platform for high sensitivity analysis by LC-MS/MS [42]. As a result, the technique exhibits high sensitivity and helps identify very minor populations of peptides at as low as femtomole concentrations.

2.3. Mass Spectrometry Ionization Methods

After sample preparation and LC separation, peptides would be analyzed on a mass spectrometer, which is an analytical instrument having multiple components, including an ion source, mass analyzer, collision cell and detector (Figure 2). The peptides in the liquid phase from the LC instrument go into the ion source and are converted to gas phase ions (ionization) that can be separated by the mass analyzer. There are many ionization techniques used in mass spectrometry. ESI is the most popular ionization technique due to its advantage of compatibility with LC. ESI creates an aerosol by applying a high voltage to a flow of liquid at atmospheric pressure. This aerosol is dried using gases and heat so that the protein or peptide of interest in the droplet retains a charge. ESI is very useful for biomolecules such as proteins and peptides because it is a "soft ionization" technique, which causes minimal fragmentation [5, 24].

Efficient ionization of peptides within the digested samples is critical for accurate LC-MS/MS detection and analysis. Each peptide from the *in vitro* digestion has unique physiochemical properties and capabilities for ionization. Ionization can theoretically occur in either the solution phase or the gas phase and is strongly affected by all components of the sample, including other peptides. Differential suppression or enhancement of specific ions can occur due to the differences in amino acid composition, mass and/or charges of individual peptides. In some cases, one peptide could be a source of ion suppression or enhancement for another. For example, acidic peptides do not readily accept a positive charge, which makes them difficult to ionize using positive mode methods, and therefore, less observable [10]. Ion suppression often occurs due to changes in the spray droplet solution properties depending on the presence of less volatile or non-volatile solutes such as salts, ion-pairing agents, or endogenous compounds like bile salts. These non-volatile materials can change the efficiency of droplet formation or evaporation and alter the amount of charged ions in the gas phase that ultimately reach the detector. When analyzing *in vitro* digestion samples, bile salts, phosphatidyl choline, and other lipids, if present in the assay, will reduce ionization efficiency; therefore, desalting, enrichment and/or clean-up post sample preparation and LC separation are needed to significantly reduce or remove incompatible compounds.

2.4. Mass Spectrometry Analyzers and Fragmentation

Once ionized, the mass analyzer (or filters) measures the mass-to-charge ratios (*m/z*) of the ions in a sample, and *m/z* detected from a sample provides an atomic signature. Currently, there are several types of mass analyzers suitable for a variety of applications. For example, low-resolution mass spectrometers have analyzers (e.g., triple quadrupole MS) that measure nominal mass and are often used for targeted quantification of peptides. On the other hand, high-resolution mass spectrometers have analyzers (e.g., Orbitrap) that measure exact mass and are used for identification and non-targeted analyses. The high-resolution Orbitrap MS analyzers can achieve *m/z* ratio up to 280,000 for mass range at 50-8,000 Da. As a result, MS instruments with different mass analyzers will likely yield results

that are very similar but not identical.

One key step to identifying peptides is to use tandem mass spectrometry (MS/MS). In MS/MS ions are separated and fragmented, and the resulting fragments are further separated and detected to yield spectra with characteristic peptide fragmentation patterns. Fragmentation of the peptide backbone generally produces characteristic ions, termed *a*, *b*, *c* and *x*, *y*, *z* ions, and allows reliable interpretation of spectra to predict amino acid sequences [31]. Collision-induced dissociation (CID) is the most widely applied fragmentation method for peptide identification with MS/MS. CID provided the largest contribution to the identified peptides from human blood plasma compared with high-energy collision dissociation (HCD) and electron transfer dissociation (ETD) [34], while ETD outperformed CID and HCD in the analysis of ubiquitylated proteome [30]. CID is suitable for identification of small peptides in digestion products, since it is most effective for small and low-charged peptides. Understanding fragmentation methods can help improve identification rates from digestion products. Like fragmentation, for reproducible peptide identification from *in vitro* digestion of NEPs, assay parameters, such as spray voltage, collision energy, dynamic range, limit of detection, and other parameters such as total injection amount, need to be harmonized among technology developers, contract research organizations (CROs), and research institutes.

2.5. Data Analysis

In traditional bottom-up proteomics the resulting spectra are searched against a known *in silico* spectra database generated from known protein sequences to compare observed and predicted masses of peptides. A query software with search algorithms, such as Mascot (Matrix Science, Boston, MA), XTandem, or Sequest, is needed to analyze peptides from *in vitro* digestion studies by predicting theoretical peptides based on potential pepsin, trypsin, and chymotrypsin cleavage sites [21]. The interpretation of amino acid sequences and their assignment to the spectra relies heavily on accurately predicted masses generated from the protein amino acid sequence of the protein, including potential modifications. This is especially true for a database generated with less specific enzymes such as pepsin. However, allowing for one or two amino acid mismatches during spectra analysis would minimize the impact. Accordingly, for the proposed *in vitro* digestion of a single NEP, protein analysis and the reliance on a database are not a factor, especially since the NEP sequence is known. The major limitation to data analysis is linked to the data input; any missing peptides or ions cannot be identified during data analysis.

Besides identification, the EFSA GMO Panel proposed using LC-MS/MS to evaluate the stability or persistence of a peptide by its temporal occurrence or presence throughout a digestion time course. Although there are several quantitative or semi-quantitative MS paradigms to choose, label-free methods would be most amenable to the *in vitro* digestion outlined above. Label-free methods do not require modification of the protein or peptides. In the absolute quantitative methods, protein abundance is calculated based on a linear correlation with MS collected data for each peptide [8]. Relative quantitative techniques compare peak areas intensities or spectral counts. Such relative techniques are simple and easy to perform but do not provide absolute concentrations, which can be used to compare multiple peptides. Relative parameters may be reported, such as the total number of unique peptides or the number of unique peptides derived from a specific segment of the protein during a time course. It is important to be aware that total peptide number does not directly correspond to protein stability.

The presence or number of peptides is directly related to digestion conditions, protein properties, LC MS/MS instrumentation, etc. Therefore, the same peptide from a protein may have a different occurrence profile under classic versus the sub-optimal pepsin digestion conditions. At pH 1.2, the optimal pH for pepsin, pepsin is fully active, yet the test protein may or may not be fully digested if it is not completely acid denatured and peptide bonds are not exposed for cleavage. For example, alpha lactalbumin is fully denatured at pH 1.2 while beta-lactoglobulin is still in its native structure at this pH [37]. As a result, there might be decreased observations of unique peptides from alpha-lactalbumin due to near completion digestion by pepsin at the end of time course. In contrast, the subsequent digestion by trypsin/chymotrypsin could result in increased observations of peptides from beta-lactoglobulin due to more effective degradation by the two enzymes. Such data can be easily generated by LC-MS/MS using the outline described above. Evidence suggests that peptides can be observed from digestion products of allergens or non-allergens at different digestion conditions [39, 42]. However, the connection between the presence of peptides and their allergenicity potential is unclear, indicating that the additional data from the LC-MS/MS analysis of the *in vitro* digestion assay does not add any information to inform the allergenic potential of the NEP.

3. Discussion

Recently, the EFSA GMO Panel outlined a suggested *in vitro* digestion protocol as a part of WOE of the allergenicity assessment for the GM crop regulatory submissions with a hope the new methods will have improved predictability of allergenicity potential of NEPs [26]. It described sequential digestions with additional sub-optimal digestion conditions and proposed the use of LC-MS/MS as a method to detect the digestion products. *In vivo* physiological digestion is highly complex, from oral to gastrointestinal digestions, and therefore it is impossible to mimic the physiological digestion *in vitro*. Currently, the *in vitro* digestion designed using purified protein at high concentration (1 to 5 mg/mL) along with the proposed three-enzyme system allows better forecasting for smaller numbers of potential peptides relative to the large numbers of potential peptides from more complex biological samples or food substances. Resistance to pepsin digestion was initially hypothesized to be linked to allergenicity by limited historical reports, but subsequent investigations have revealed that there is weak correlation between pepsin resistance and allergenicity [3, 32, 17, 13].

A few key points need to be addressed before answering the question posed in this manuscript, whether LC-MS/MS can

improve the risk assessment of NEPs. As part of WOE for allergenic assessment of an NEP, knowledge of the protein concentration, heat stability, and digestive stability of the protein are exposure criteria, while structural similarity with known allergens and history of safe use are hazard criteria [16]. Regardless of SDS-PAGE results that show the presence of an intact protein, LC-MS/MS methods are so sensitive that lead to observation of peptides from the protein; therefore, exposure assessment and allergenicity determination of that protein from LC-MS/MS analysis remain uncertain. It was reported that non-allergen soybean lipoxygenase has more resistant peptides than allergen beta-casein, from optimal or suboptimal digestion condition [39]. Similar observation was reported between non-allergen phosphofructokinase and allergen beta-lactoglobulin [42]. Evidence suggests that unique and stable peptides are present in digestion products from both allergen and non-allergens Also, no clear patterns in the persistence or abundance of peptides exist between allergen and non-allergen digestion products; therefore, the presence of peptides gives no information on their allergenic potential [39, 42]. Fundamentally, a simple correlation between protein digestibility and allergenicity has not been established, and therefore the presence of the protein or specific peptides may only be used in relation to the exposure assessment. It should be noted that most risk assessment bodies would consider that information on exposure is only informative for the risk assessment if a potential hazard has been identified. Therefore, it can be questioned if the mere presence of a peptide that is otherwise considered safe provides useful information for a risk assessment.

Although the proposed *in vitro* digestion conditions contain extra steps and enzymes compared with the classic pepsin resistance assay, they can be made amenable for LC-MS/MS analysis of the digested samples. LC-MS/MS technology can detect and identify unique peptides from *in vitro* digestion with some important technical challenges that could significantly impact the interpretation of the results and their utility in the WOE for allergenicity assessments. As discussed in previous sections, an inability to detect a peptide does not mean the peptide is absent from the digestion samples. The occurrence of a peptide over a time course may be directly related to properties of the parent protein and the digestion conditions, but may also result from the sensitivity of the instrument or other experimental conditions. A specific peptide may be present, but detection may not be guaranteed due to several factors, including sample preparation, low ionization, efficiency, and sensitivity of the mass analyzer. Despite this limitation, LC-MS/MS is still very sensitive and capable of detecting peptides missed in other technologies (e.g., SDS-PAGE). A peptide could be lost during the enrichment and desalting step due to its hydrophobicity, low abundance, or size; it could also be too small to be ionized or identifed because of repetitive amino acids within the sequence. Therefore, the ability to detect all small peptides in an in vitro digestion can be challenging (if not impossible).

The EFSA GMO Panel has not recommended a standard protocol for peptide identification or quantification using LC-MS/MS analysis. Criteria to achieve reproducible and consistent peptide identification from *in vitro* digestions are challenging to harmonize among different labs. There are many more parameters and settings, such as spray voltage, collision energy, and survey scan, required for a mass spectrometry instrument that are not relevant to SDS-PAGE. Different labs may have different models of mass spectrometers and parameters from one model do not perform the same way on another model. As a result, the lack of standardization seems likely considering the variability described previously. Nevertheless, modern LC-MS/MS exhibits sensitivity down to fmol concentrations, so it is technically possible for a peptide to be observed throughout the entire digestion. The LC-MS/MS methods for peptide identification from *in vitro* digestion cannot quantify the mass or concentration of the peptides. Moreover, observation of peptides is not necessarily linked directly to exposure.

Can mass spectrometry analysis of *in vitro* digestion products improve the assessment of allergenic potential of newly expressed proteins? Based on the technical review above, the answer is "no" or "not at this time". Like SDS-PAGE, LC-MS/MS can identify and track the abundance of digestion products albeit with technical challenges that may skew results. Results from an SDS-PAGE assay do not provide helpful information to assess whether a digestion product is an allergen or not. Unfortunately, results on digestion products using LC-MS/MS do not improve this shortcoming. Neither method, whether used in a tiered process or used alone, provides the capability of distinguishing between allergen and non-allergen.

A determining factor that could aid in the identification of an allergen from a non-allergen is sequence homology to known allergens. NEPs have been successfully screened and excluded from allergenicity concern using sequence homology and epitope searching that are done *in silico* with bioinformatics analysis on the intact protein, without conducting *in vitro* digestion and LC-MS/MS analysis. Use of LC-MS/MS can be informative if peptide presence from *in vitro* digestion is an indication of allergenicity of a NEP. The approach to monitor the peptide presence and abundance with a demonstration of the lasting presence of a peptide and counting the number of unique peptides throughout a digestion has a drawback because the number of peptides from a digestion of the protein is protein dependent rather than allergenicity dependent. In addition, the LC-MS/MS identification method is not for quantification, and therefore cannot provide concentration information. The value of LC-MS/MS analysis of digestions for allergenicity prediction needs to be demonstrated prior to adoption for allergenicity assessment of NEPs.

4. Conclusion

State-of-the-art, LC-MS/MS has become a familiar technology for the characterization of food proteins and peptides [23]. Detection of some stable peptides from *in vitro* digestion can be achieved through this technique. Nanoflow or microflow LC along with ESI are highly sensitive and can detect and identify a large number of peptides, including trace amounts of unique peptides. The relatively simple *in vitro* digestion design (purified test protein and three-enzyme system) and high concentration of test protein may be compatible with LC-MS/MS pep-

tide identification. However, LC-MS/MS methods need to be carefully developed and evaluated taking several factors into consideration, such as buffer compatibility, enrichment, signal suppression, etc., prior to data interpretation. Resistance to digestion has no direct correlation with allergenicity potential. The peptides observed from *in vitro* digestion may not reflect what occurs *in vivo*, and therefore, detection of peptides is unlikely to provide a good prediction of potential for allergenicity. More work needs be done for a better understanding of allergenic sensitization and elicitation before adding complexity to the *in vitro* digestion assay that does not translate into improved assessment of allergenic potential for NEPs from GM crop products, and which may only add to more confusion in evaluating assay results [40].

5. Declaration of Conflicting Interest

The authors are employed by the agricultural biotechnology industry. Employment affiliation of the authors is given on the first page of this paper.

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7. Article Information

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