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Review

Immunoassays and Mass Spectrometry for Determination of Protein Concentrations in Genetically Modified Crops

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ABSTRACT: Quantifying protein levels in genetically modified (GM) crops is crucial in every phase of development, deregulation, and seed production. Immunoassays, particularly enzyme-linked immunosorbent assay, have been the primary protein quantitation techniques for decades within the industry due to their efficiency, adaptability, and credibility. Newer immunoassay technologies like Meso Scale Discovery and Luminex offer enhanced sensitivity and multiplexing capabilities. While mass spectrometry (MS) has been widely used for small molecules and protein detection in the pharmaceutical and agricultural industries (e.g., biomarkers, endogenous allergens), its use in quantifying protein levels in GM crops has been limited. However, as trait portfolios for GM crop have expanded, MS has been increasingly adopted due to its comparable sensitivity, increased specificity, and multiplexing capabilities. This review contrasts the benefits and limitations of immunoassays and MS technologies for protein measurement in GM crops, considering factors such as cost, convenience, and specific analytical needs. Ultimately, both techniques are suitable for assessing protein concentrations in GM crops, with MS offering complementary capabilities to immunoassays. This comparison aims to provide insights into selecting between these techniques based on the user's end point needs.

KEYWORDS: *quantitation, quantification, protein concentration, GM crops, immunoassay, mass spectrometry, LC−MS/MS*

INTRODUCTION

The global genetically modified (GM) crops market is expected to grow from US$19 billion in 2018 to reach US $26 billion by 2024 (Genetically Modified (GM) Food Market—Forecasts from 2019 to 2024 (researchandmarkets.com)), reflecting the demand for technologies to better manage pressures from weeds, plant pests, and environmental challenges. Most GM crop products are designed to express novel proteins that contribute to the desired trait technologies. Developing methods to detect and quantify these proteins in GM crops is essential to support GM crop product research, development, seed production, monitoring, and international trade. From a regulatory perspective, precise and accurate protein quantitation tools are crucial to ensure the safety of GM crops, whether to demonstrate the presence or absence of a transgenic trait or to quantify protein levels for regulatory compliance and safety evaluation.[1] This becomes more challenging when dealing with the expression of multiple trait proteins in a crop product[1,2] or when the proteins are difficult to isolate due to low expression levels[3] or other factors.[4] As a result, alternative protein analytical techniques may be required for some complex scenarios.

Immunoassays, which rely on specific interactions between antibodies and their target proteins, have historically been powerful techniques for protein detection and quantitation in GM crops.[5−11] Immunoassays can provide a relatively high throughput method with readily available reagents and instrumentation, resulting in widespread adoption across various industries.[12,13] Newer immunoassay technologies support the simultaneous analysis of multiple proteins, often termed multiplexing, and the potential for greater sensitivity.[14] However, the development of immunoassay methods can be time-consuming, due to the need for production of antibodies, and standard proteins.[15,16] Additionally, when the analyte proteins are expressed at very low levels or are membrane proteins, the challenge of developing suitable immunoassay methods is increased.[17,18] Multiplexing individual immunoassays for GM products with multiple trait proteins can lead to difficulties when there is homology between proteins or when the assay conditions are not compatible. For these reasons, the availability of alternative analytical techniques for quantifying GM proteins can be important.

In recent years, the use of liquid chromatography (LC) coupled to mass spectrometry (MS) has become an alternative approach for protein quantitation in a wide range of sectors, including biotechnology, environmental monitoring, food processing, pharmaceuticals, agrochemicals, and cosmetics.[19,20] The pairing of LC to tandem MS (LC−MS/MS) combines the physical separation capabilities of LC with the specific mass identification capabilities of MS. The LC−MS/MS technology

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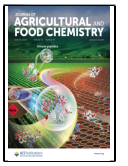

also supports multiplexed and high through put protein analysis.

In this article, we review and compare the advantages and disadvantages of immunoassay and LC−MS/MS techniques for protein quantitation in the agriculture biotech industry and discuss their suitability for detecting and quantifying GM proteins.

DESCRIPTION OF TECHNIQUES

Immunoassays. Immunoassays have been used for decades by the agricultural biotech industry to detect and quantify proteins in GM crops.[21,22] This widespread adoption of immunoassay methods is attributed to their inherent specificity, high sensitivity, and relatively high level of throughput. In addition, they are amenable to automation, which further enables throughput and minimizes human errors in sample handling, storage, and testing. Lastly, immunoassays can be readily transferred across global markets due to the low cost of instrumentation and simple analytical procedures.

Immunoassays are critical tools for protein analysis due to their ability to quantify biological molecules through specific antibody interactions. Analysis with immunoassays involves measuring the activity from certain signaling molecules, which provide colorimetric, radioactive, fluorescent, or electrochemiluminescent signaling.[23−25] A calibration curve or standard curve representing the output signal as a function of analyte concentration is generated by using known concentrations of the target protein, and a curve fit is used to interpolate protein concentrations within the samples.

Immunoassays can be developed to be very specific and sensitive by selecting antibodies that exhibit high affinity for the analyte protein and low binding or cross-reactivity with other molecules in the sample matrix. Sample dilution and the use of blocking reagents may help overcome some nonspecific background signal, however, it may challenging to avoid cross-reactivity for related endogenous proteins or homologous proteins that are expressed together in a GM crop,[1] such as different members of the Cry protein family.[26] It may also be difficult to create antibodies that recognize post-translational modifications on the analyte protein.

Another challenge related to antibody selection is the need for a constant supply of antibodies and other method critical reagent(s) throughout the lifecycle of the method. Preparing and maintaining a large batch of critical reagents is one way to overcome this challenge but may be costly. Changes in any of the critical reagents may trigger the need for revalidation of the entire method. When necessary, bridging an old lot of critical reagents to a newer lot of reagents should be performed.

Enzyme-Linked Immunosorbent Assay (ELISA). ELISA is widely used to quantify newly expressed proteins in GM crops. ELISA requires one (direct) or two (indirect or sandwich) antibodies that bind specifically to the protein analyte. In the most common form of a sandwich ELISA (Figure 1), one antibody (capture antibody) is immobilized on solid surface and binds the analyte present in the sample, after which a second antibody (detection antibody) is added to form a complex with any captured protein analyte. The detection antibody is either conjugated directly to a signaling enzyme or recognized by an antispecies secondary antibody conjugated to the enzyme. The inclusion of a standard curve on the ELISA plate, with known amounts of analyte protein, turns the ELISA method into a quantitative assay. On the basis of a pair of antibodies that bind different epitopes of the protein, ELISA methods can be highly specific. Commercial ELISA kits can demonstrate sensitivity as low as 0.1 to 1 ng/mL (or ppb) of the target protein in a plant tissue extract and a quantitative range spanning two to 3 orders of magnitude.[27] ELISA requires a purified and characterized protein standard that is either isolated directly from a trait-specific crop or expressed and purified using a heterologous system (e.g., microbial expression or chemical synthesis).

Luminex. Luminex is another newer type of immunoassay technology and these assays have been developed for the clinical diagnostics, pharmaceutical, and life science research markets.[28−32]

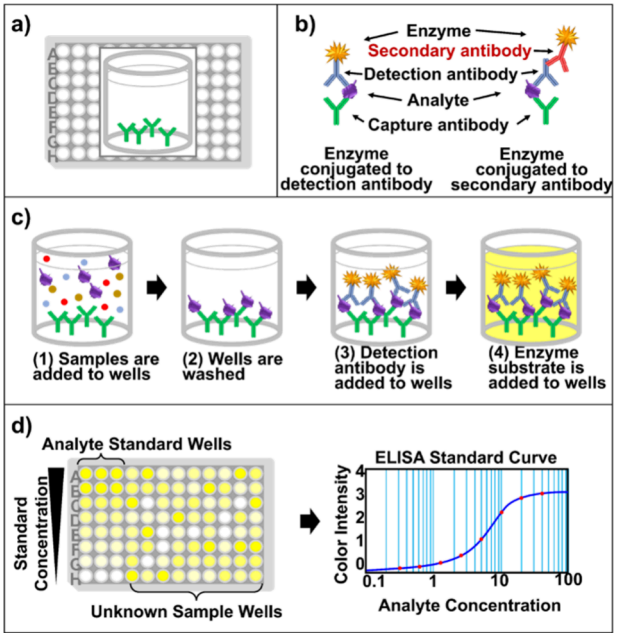

Figure 1. Overview of the sandwich ELISA immunoassay technique. (a) The wells of an ELISA plate are coated with analyte-specific capture antibodies. (b) When analyte is present, interactions with the capture antibody and a separate analyte-specific detection antibody allow formation of a "sandwich" complex, which provides for analyte detection and quantitation. The signaling enzyme (e.g., Horseradish Peroxidase) can be directly conjugated to the detection antibody or to a secondary antibody with specificity for the detection antibody. (c) In the ELISA workflow, (1) samples containing a complex mixture of molecules (e.g., plant tissue extracts) are added to the ELISA plate wells; (2) analyte molecules are specifically bound by the capture antibody and the wells are washed to remove nonspecific molecules; (3) a solution of detection antibody is added to the wells and the antibody binds specifically to the captured analyte; and (4) the enzyme substrate is added to the wells and allowed to develop a visible colored signal. (d) Known amounts of an analyte standard are added to wells on the plate to generate a standard curve. The intensity of the signal in the sample wells is directly proportional to those produced by the standard curve allowing for quantitation of the analyte.

The use of Luminex in the agricultural biotechnology field is increasing.[33] Currently, most commercially available Luminex assays for protein analysis are based on the use of a Luminex 100/200 instrument with xMAP technology (Luminex Corp., Austin, TX) (Figure 2). Luminex xMAP is a multiplexing capable technology that combines microfluidics, optics, and digital processing with antibody-linked magnetic microbeads. The microscopic size and low density of the beads used in Luminex assays allow reactions to exhibit virtually solution-phase kinetics, while the incorporated magnetic properties further simplify analysis. The separation of beads containing different antibodies that specifically bind different analyte proteins can be conducted based on color coding, bead size, or magnetic force. Fluorescence is a commonly used detection system for Luminex assays since it typically detects samples with a high signal-to-noise ratio. A highly multiplexed Luminex immunoassay can potentially measure hundreds of analytes simultaneously.

As with other immunoassays, antibody specificity is a critical parameter during method development and validation in the multiplexed Luminex environment. Typically, assays are developed and validated for accuracy either individually for single trait proteins or together for stacked traits. Protein buffer compatibility and the dilution factor required for each protein also need to be considered during method development. Luminex also requires extra effort for the proper storage and handling of the microbeads. Freezing will

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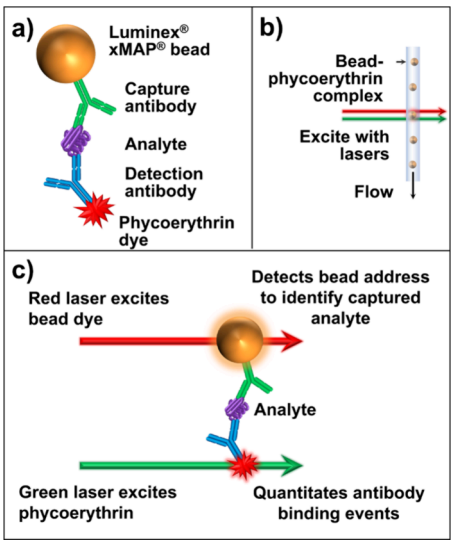

Figure 2. Overview of the Luminex xMAP immunoassay detection scheme. (a) The presence of the analyte protein targeted by the specific capture and detection antibodies allows the formation of a bead-phycoerythrin complex. (b) Individual bead-phycoerythrin complexes are separated in a flow cell and detected by fluorescence excitation. (c) The bead fluorescence is excited to allow indexing of the specific bead color address being detected. Bead addresses are correlated with different analyte-specific antibodies, allowing identification of the analyte in each complex. Excitation of the phycoerythrin fluorescence level from each complex provides the output signal that allows quantitation.

damage the antibody-conjugated beads, which may incur some practical inconvenience for transferring them between locations.

On top of the advantages of multiplexing capability, Luminex assays can have dynamic ranges of up to 5 orders of magnitude. Reading time for a Luminex plate is relatively long (e.g., 15−20 min for the FlexMAP 3D system) compared to the under 1 min read time for an ELISA plate. However, while the results from an ELISA plate must be captured within approximately 30 min, the Luminex fluorescence is quite stable and can be measured after a few days storage at 4 °C.[34]

Meso Scale Discovery. Meso Scale Diagnostics (MSD, LLC., Rockville, MD) is another immunoassay technology that incorporates an electrochemiluminescent SULFO-TAG detection technology and micro plates with carbon electrodes integrated into each well (Figure 3).

The wells of MSD plates are divided into several well-defined spots that can be coated with different protein-specific capture antibodies and the plates are available with up to 10 spots per well. Similar to ELISA, the samples are incubated in the wells with the capture antibodies and then the wells are washed to remove unbound molecules. A mixture of detection antibodies that have been conjugated to SULFO-TAG labels are then added to each well for binding to the protein analytes captured on each spot. Following electrical stimulation of the electrodes in each well by the MSD instrument, the light emitted by the SULFO-TAG labels is measured for each spot. The intensity of the light emitted at each spot is proportional to the amount of protein captured and the amount of protein in the samples is interpolated from a standard curve produced with known amounts of proteins. Like the single plex ELISA, it is important to use highly specific antibodies for MSD assays to minimize cross-reactivity.

Few publications address the use of MSD multiplex technology for protein expression in the agriculture biotechnology industry.[35] Nevertheless, there has been widespread use of this technology in the pharmaceutical industry and in clinical research.[36−40] MSD technology is very sensitive, with ultralow pico-gram level detection limits and a wide dynamic range of five or more orders of

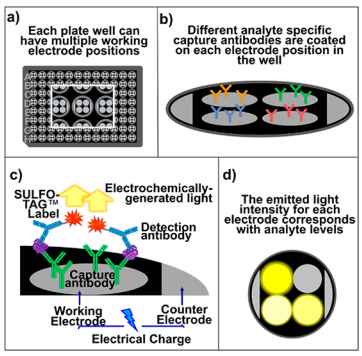

Figure 3. Overview of the MSD immunoassay detection scheme. (a) Each well of an MSD plate can have multiple electrode positions that can each be set up for detection of a specific analyte contained in the sample extract. (b) Analyte specific capture antibodies are coated onto the respective electrode positions within the wells. (c) Following incubation steps first with samples and second with detection antibodies labeled with a SULFO-TAG label, bound analyte levels are detected by adding an electrochemiluminescent substrate solution and applying an electrical charge across the well electrodes. (d) The amount of light emitted by each electrode position corresponds to the amount of bound analyte and SULFO-TAG, and the relative light levels for the electrodes are quantified using an MSD instrument.

magnitude.[14] This is particularly useful when analyzing samples with multiple traits that express trait proteins at widely varying levels. Despite the wide dynamic range, dilution factors still need to be optimized to minimize matrix effects. If considerations for multiple analyte proteins cannot be balanced in one multiplexed assay, then it may become necessary to split off some analytes into a separate assay.

Immunoassay Summary. Overall, immunoassays are powerful bioanalytical methods for quantifying traits and endogenous proteins in GM crops. However, the relatively costly and time-consuming method development process for immunoassays, the potential cross-reactivity with other proteins and multiplex assay reagents,[40] and the lower tolerance for the harsh extraction buffers required for some analyte proteins may limit their applicability for quantifying some proteins in agricultural biotechnology (Table 1). Therefore, MS technology is being implemented more often as an alternative approach for the quantification of proteins of GM crops.

Table 1. Key Advantages and Limitations for Immunoassays (ELISA, MSD, Luminex, etc.)

advantages	readily transferrable between laboratories
	amenable to automation and high throughput
	high sensitivity
limitations	potential for cross-reactivity or nonspecific detection
	antibody development and maintenance required
	maintenance of critical reagents needed

Mass Spectrometry. MS has been widely used in qualitative and quantitative protein analysis. MS enables the direct identification of molecules based on their mass-to-charge ratios with tandem MS (MS/MS), allowing exploration of unique fragmentation patterns, serving as a fingerprint for each molecule. MS quantitation of proteins can be categorized into nontargeted or targeted strategies.

Targeted bottom-up quantitation is the most commonly used method for protein MS quantitation and has been widely employed for the assessment of specific protein concentrations in a variety of industries.[41−43] Targeted quantification strategies allow total protein to be isolated from a sample and a subset of specific proteins to be selectively compared or quantified.[44−49] This approach offers high specificity due to its ability to selectively quantify compounds within complex mixtures. The quantitation typically uses selected-reaction

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monitoring/multiple-reaction monitoring (SRM/MRM) or parallel-reaction monitoring (PRM) with high resolution MS improvements.

The use of liquid chromatography in LC−MS/MS allows for sample molecule separation and potential automation, making it advantageous for handling multiple proteins in a high throughput manner. However, challenges include the need for sophisticated and expensive instruments, skilled analysts, and knowledge that is not easily transferred. Identifying a surrogate peptide that can specifically represent the protein of interest, has acceptable properties for MS analysis, and has no interference from the sample matrix can sometimes be difficult. Sample preparation could be limited to volatile buffers because the analyte must be in the gaseous phase and effectively ionized prior to analysis by MS. Therefore, all method parameters must be examined in detail during method development and validated extensively, similar to immunoassays. Some key advantages and limitations for LC−MS/MS are listed in Table 2.

Table 2. Key Advantages and Limitations of LC−MS/MS Protein Quantitation

advantages	wide dynamic range of 4 or 5 orders of magnitude
	no need for protein standard or antibody
	multiplexing
	amenable to automation, medium to high throughput
	high sensitivity, high specificity
limitations	need for highly skilled personnel
	limited to volatile buffer systems
	difficulty in identifying surrogate peptides
	efficient ionization of targets required

Plant Tissue Protein Quantitation Workflow. For bottom-up targeted protein quantitation, proteins undergo enzymatic digestion to produce a mixture of peptides. Predicting the resultant peptides from a given enzymatic digestion can be achieved in silico, and one or several of these peptides can serve as surrogate(s) for the intact target protein. Identifying appropriate surrogate peptides that specifically represent the protein of interest without interference from the matrix can be challenging. Synthetic peptides or pure protein standards can be used to generate a standard curve to quantitate the protein(s) of interest in samples.

A typical targeted bottom-up proteomics LC−MS/MS flow diagram for plant tissue protein quantitation is shown in Figure 4. Proteins are extracted using a specialized protocol involving cell lysis, fractionation, and removal of high-abundance proteins. The use of compatible buffers is essential, as incompatible buffers can complicate the process. After extraction, proteins undergo enzymatic digestion to create peptide fragments, followed by removal of salts and detergents.

Labeled Peptides. An important component for accurate quantitative MS procedures is an appropriate label or internal standard (IS). Heavy isotope labeled peptides are spiked into the digested peptides to normalize the subsequent LC−MS/MS ionization at any step in the process. Ideally, a high purity version of the analyte of interest with ^{13}C, ^{15}N, or ^{2}H heavy isotope labels replacing native atoms can be used. Since the compound of interest and internal standard will have different masses. The IS can be used to correct for differential ionization at the MS source to normalize the signal observed and account for any losses, depending on where it was added in the workflow. Synthetic peptides or heavy isotope-labeled peptides have become easier and faster to source from multiple vendor's relatively low cost. These heavy-isotope labeled peptides allow analysis of very complex mixtures of digested proteins.[47]

Multiple-Reaction Monitoring (MRM). MRM, also known as SRM, is an important LC−MS/MS technique for the quantitation of target proteins in complex samples. MRMs use the signal of selected MS/MS fragment ions for quantitation and are typically performed on triple quadrupole (QQQ) or quadrupole-ion trap (Q-Trap) type mass spectrometers. Both tandem instruments use the first mass analyzer (Q1) to selectively transmit ions of a particular mass-to-charge ratio (m/z) (Figure 5); these target ions are called precursor ions. The

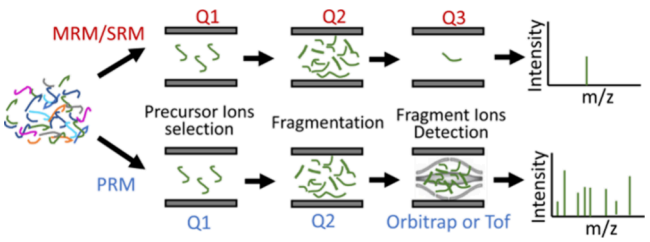

Figure 5. Protein quantitation by MRM/SRM and PRM. MRM/SRM monitors each precursor ion transition at a time, while PRM analyzes all fragment ions derived from a precursor ion.

precursor ions are then subjected to collision-induced dissociation (CID) in a collision cell to generate product ions, which give excellent specificity for a targeted peptide. The targeted product ions are detected selectively either by the third quadrupole in or by the ion trap.

The MRM technique offers sensitive and repeatable measurements since the second mass analyzer targets selected product ions; only the specified transitions are recorded, and other product ions are not detected. Selective ion transmission affords MRM analysis with low limits of quantitation (LOQ), ranging in the nanomole to attomole levels of analyte.[50] and a wide dynamic range, which can cover a 4- to 5-orders of magnitude. Also, it provides high specificity to unique peptides and can theoretically distinguish proteins that differ in

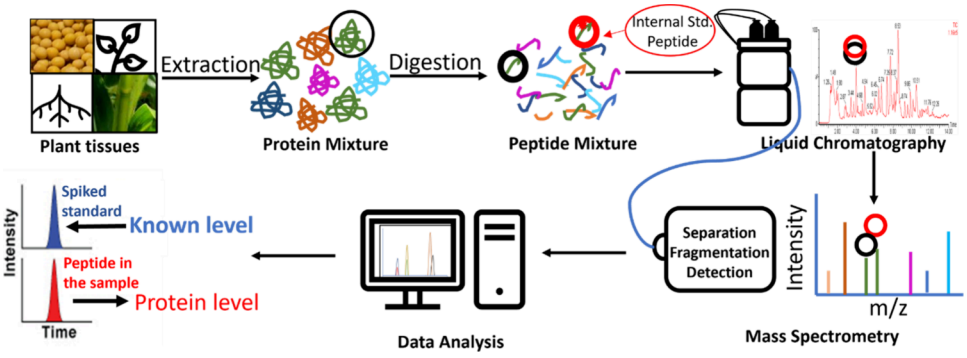

Figure 4. Protein quantitation workflow by LC−MS/MS. Proteins are prepared and extracted from plant tissue samples. After extraction, protein samples undergo enzymatic digestion to generate known or predicted peptide fragments. Heavy isotope labeled peptides are spiked in as internal standard. The peptide mixtures are separated by liquid chromatography and mass spectrometry. The ions are fragmented and detected. The data is plotted and used for quantifying samples.

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Table 3. Key Points/Differences for Immunoassays and Mass Spectrometry

information	immunoassay (ELISAs, Luminex, MSD)	mass spectrometry
specificity	measures whole/intact protein Quantitation might be affected by conformational changes. dependent upon immunogenicity	Measured surrogate peptides must be correlated to intact protein value. dependent upon physiochemical properties of protein/peptide
sample extraction and preparation	no need for cleanup required but harsh reagent use is limited	amenable to harsh reagents but need later treatment. Internal standard is used to normalize, can be advantageous when the analyte is in low abundance.
cost considerations	after kit development, inexpensive and rapid assay with, minimal equipment needed for ELISA; widely available with reasonable maintenance cost; cost added up with multiplexing instruments and standard protein, antibody production and method development	relative expensive instrumentation and software at start; increased cost associated with more sophisticated instruments and maintenance. The cost could be saved due to shorter development time and easy to add new analytes.
ease of implementation and acceptance	easier to implement and validate after kit development and hence easily adaptable to less skilled laboratories.	high level of technical expertise needs for data interpretation and troubleshooting aberrant data; recent development of performance and interface make the technique easier to use
method development time	development of a new assay is a lengthy process, months for polyclonal antibodies and years for monoclonal antibodies	relative quick process with target and matrix protein sequence information available, few weeks to months
multiplexing	well-developed multiplexing technique; sometimes difficult to develop capture antibody due to cross-reactivity or reduced sensitivity and specificity for certain proteins	well-developed multiplexing technique; attractive of specificity/sensitivity due to surrogate peptide detection
sensitivity	Good protein/antibody sensitivity ELISA kits can achieve LOQs as low as 0.1 to 1 ng/mL (or ppb) and multiplexing assays can reach similar or even lowers, down to 0.05 ng/mL.	newer instruments can have enhanced sensitivity and LOQ, with current LOQs ranging from low nanomole to attomole levels peptide dependent
accuracy and precision	The effect of the sample matrix is considered to impact specificity. high specificity due to the use of antibodies, provides accurate quantification of target proteins; limitations in accurately measuring multiple proteins simultaneously.	also faces challenges with extraction recovery and the labeled internal standards may obviate the need for matrix assessment precise quantitation with careful calibration and validation to ensure accuracy need consistence of sample preparation, instrument performance, and data analysis
intractable protein	challenge due to lack of protein production and antibody	advantage due to the use of peptide surrogates
validation	Most method validation parameters are common. Multiplexing immunoassays can be complex. technique specific parameters need to be evaluated such as antibody specificity and cross-reactivity testing for immunoassays, etc.	Most method validation parameters are common. Validation can be complex compared to ELISA, technique specific parameters need to be evaluated such as carry-over, digestion time course, interference, etc.
regulatory acceptance	Immunoassay analysis has been accepted by many global regulatory agencies.	currently accepted by FDA and EFSA, also accepted by many global reg agencies

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sequence by a single nonisobaric amino acid between homologous proteins.

Parallel-Reaction Monitoring (PRM). PRM, with the evolution of HRMS technology, has become an alternative platform for MS quantitative analysis.[51−53] Unlike MRM, all fragment ions are measured after fragmentation of a selected precursor in PRM, as diagrammed in Figure 5. PRM is typically performed on Orbitrap or Time-of-Flight (ToF) analyzers. Both full scan and product ion scan can be used for quantitative analysis. In full scan mode, all ions present in the sample of interest can be simultaneously achieved with the quantitative data. This qualitative information can be reanalyzed or reextracted at a later stage. This full scan mode reduces MS quantitation method development time due to its comprehensive data collection and could enhance sensitivity when fragmentation efficiency is poor. Quantitative processing of PRM data is performed based on narrow mass extraction windows rather than on nominal mass product ion chromatograms, as for MRM. Parameters also need to be examined in detail and kept consistent for data processing and reporting results. PRM and MRM exhibit have comparable linearity, dynamic range, precision, and repeatability for protein quantitation.[54]

Literature Highlights: Plant Protein Quantification. Well-developed and validated methods using either immunoassay or MS techniques should be considered acceptable for protein quantitation. There are, however, some advantages and limitations with both techniques that may guide selection of one technique or the other (Table 3). A literature search was performed to highlight these techniques in relevance to plant protein quantification. Two case studies from the literature search that are suitable for plant protein quantitation by either technique are presented here.

Literature Case Study 1: Endogenous Allergen Quantitation by ELISA and LC−MS/MS. One aspect of the safety assessment of GM crops is to evaluate whether the transformation process impacts the levels of endogenous allergens relative to conventional non-GM varieties.[55] To comply with the mandatory assessment of individual soybean allergens implemented by the European Commission,[56] Geng et al. (2017)[8] developed and validated ELISA methods for each of the allergen proteins Gly m 4, Gly m 5, Gly m 6, Gly m Bd 28k, and Gly m Bd 30k. Due to the wide range of expression levels (Figure 6) and allergen complexity (three or five

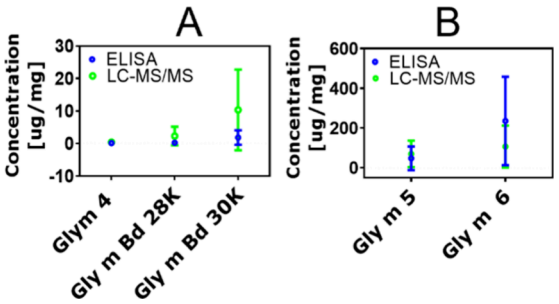

Figure 6. Summary of value ranges of five endogenous soybean allergens measured by using ELISA or LC−MS/MS. (A) Value ranges of endogenous soybean allergens Gly m 4, Gly m Bd 28K, and Gly m Bd 30K. (B) Value ranges of endogenous soybean allergens Gly m 5 and Gly m 6.

subunits in Gly m 5 or Gly m 6, respectively), recombinant and natively purified allergens were used as standards to generate the calibration curves. Also, Geng et al. (2017)[8] produced and purified mouse monoclonal antibodies, goat polyclonal antibodies, goat peptide polyclonal antibodies, and rabbit polyclonal antibodies to develop five specific and sensitive soy allergen ELISA methods.

Due to the increasing numbers of endogenous soybean allergens being requested for the GM crop safety assessment,[57] multiplexing protein detection methods such as LC−MS/MS have been investigated. For example, Hill et al. (2017)[58] showed that mass spectrometric analysis is ideal for endogenous protein assays due its high specificity. While there are differences in the specific methodology between the two protocols, and the growing seasons and soybean varieties are different for these two studies, endogenous allergen levels detected by Hill et al., using the described LC−MS/MS protocols are in the range of data from the ELISA methodologies[8,43] published by Geng et al. This agreement between methodologies demonstrates the acceptability of both techniques for collecting quantitative endogenous allergen data to address EFSA requirements.

Literature Case Study 2: GM Trait Proteins. In Hu and Owens,[45] a LC−MS/MS method was developed and validated to quantify three GM trait proteins expressed in GM maize leaves; the three proteins were a gene-shuffled glyphosate acetyltransferase variant (GAT4621), a highly resistant allele mutant of maize acetolactate synthase (zmHRA), and phosphinothricin acetyltransferase (PAT). These proteins are expressed in maize to achieve multiple herbicide resistance against a single herbicide or a combination of herbicides. Hu and Owens (2011)[45] quantified the protein levels in 10 transgenic GM leaf samples and compared using both ELISA and LC−MS/MS methods. The proteins were detected in a range of roughly 8−80 ppm/dry weight for both methods. Of the three proteins, GAT4261 had the highest detected values followed by PAT and zmHRA. The detected values suggested a lower day-to-day variation with LC−MS/MS compared to ELISA. The ranges of expression levels determined using LC−MS/MS and ELISA were similar for the three proteins but not identical. This was to be expected due to the different techniques and different experimental conditions. On the basis of statistical analyses, GAT4621 and zmHRA LC−MS/MS values were within ±3 standard deviations (SD), while ELISA values were within ± SD limits. LC−MS/MS results for PAT were consistently higher in comparison with ELISA values, and most of the LC−MS/MS values (78%) were well within the ±2 SD with ELISA value. Overall, Hu and Owens[45] found that all results demonstrated that the two independent analytical techniques, LC−MS/MS and ELISA, generated comparable results, indicating that both techniques were suitable for quantifying the GM trait proteins.

DISCUSSION

In addition to the soybean and maize discussed in the case studies, immunoassay and LC−MS/MS techniques are suitable for quantitating proteins in various crops and agricultural matrices. The availability of protein and genetic sequence information enables the rapid development of multiple approaches to protein quantitation. Both techniques offer comparable sensitivity, providing multiplexed quantitative analytical data. When selecting a method for protein quantitation, user requirements and other criteria and various circumstances, such as breeding stacks, intractable proteins, convenience, cost, time, and personnel, play a significant role in selecting the analytical technique (immunoassay versus MS).

Specificity. One of the major differences between immunoassay and LC−MS/MS technologies is the manner in which the analyte protein of interest is targeted and quantified. Immunoassay methods target specific epitopes on the protein(s) of interest and detect these indirectly though specific antibody interactions which make the immunoassay specific. However, protein immunogenicity is a critical consideration for the assay and conformational changes may impact immunoreactivity and the quantitative results for some proteins. On the other hand, LC−MS/MS quantitation assays target direct detection of surrogate peptides for the full-length analyte protein. In this approach, peptides are quantified by monitoring several MRM/PRM transitions for each peptide (that span the intact protein) and typically one is selected for final quantification while another could be used for quality control. The surrogate peptide results are then correlated back

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to the amount of the full-length protein present in the sample. Correlating the peptide results to full-length protein results requires detailed method development to ensure proteins are extracted and digested to their representative fragments in a consistent manner, due to the large number of steps and modifications involved. Selecting unmodified unique peptides for proteins with multiple modifications can be challenging. LC−MS/MS methods quantify target proteins based on their primary sequence, allowing quantification regardless of conformation changes and provides high specificity to unique peptides and can theoretically distinguish proteins that differ in sequence by a single nonisobaric amino acid between homologous proteins.

Sample Extraction and Preparation. Immunoassays typically do not require a cleanup step for sample extracts, but the use of harsh reagent is limited due to potential disruption of antigen−antibody interaction. In a typical study, Geng et al. (2017)[8] applied ELISA analysis after extraction with a simple buffer of phosphate-buffered saline with 0.05% (v/v) Tween-20, pH 7.4 (PBST) for Soybean allergens Gly m 4, Gly m 5, and Gly m 6. In contrast, MS is amenable to harsh extraction buffers and solvents but requires additional steps. Hill et al. (2017)[58] demonstrated the use of buffer containing 5 M urea, 2 M thiourea, 50 mM Tris-HCl, and 65 mM dithiothreitol to extract proteins. Diluted extracts were denatured and reduced, followed by overnight incubation with enzyme. Typically, harsh extraction buffers are exchanged or diluted to buffers suitable for MS. Any losses throughout MS sample preparation can be assessed and normalized using IS, making the technique suitable for a wider range of proteins. The flexibility in sample handling with MS methods can be advantageous, especially when the analyte is in low abundance.

Cost Considerations. The relatively low cost of equipment and software used for immunoassay render it an attractive technology. The basic equipment for an immunoassay is the microplate reader, which is widely available and can cost less than ten thousand US dollars. Some laboratories may choose to purchase additional more expensive equipment such as plate washer or liquid handlers. The equipment and software that support multiplexing capabilities, such as Luminex or MSD, would also require increased capital expenditure. Although an increased cost is associated with these options, instrument and software costs could still be low. However, in addition to equipment costs, immunoassays require initial development and ongoing production of antigens, antibodies, and standard proteins, which increase the overall cost for assay development and cost per test. The quantitative analysis of proteins via LC−MS/MS normally involves the use of QQQ, Q-Trap, or HRMS instrumentation, coupled with LC systems. The overall cost typically starts in the hundreds of thousands of US dollars for the most basic equipment and quickly rises for more sensitive or sophisticated models. The software typically required for LC−MS/MS quantification can be both expensive and challenging to use. Due to these instrument and software needs alone, the up-front and operational costs for LC−MS/MS quantitation are typically much higher than most other analytical techniques. The added equipment maintenance, calibration, and automation for high throughput could easily lead to costs over a million US dollars. However, the technique normally needs shorter development time without antibody or even standard protein, it is also relatively easy to add new analyte proteins to a multiplexed method, which helps balance some of the increased cost of MS analysis.

Ease of Implementation and Acceptance. Immunoassay remains the technology of choice by laboratories in many geographic locations due to historical use and familiarity. ELISA methods can be streamlined and commercialized, making them readily adaptable to less experienced laboratories. In addition, there are well-established software packages available for data analysis. A tremendous amount of published data generated from immunoassay technologies exists from a broad variety of applications in laboratories across the globe, supporting broad acceptance of immunoassay methods. The complexity of the LC−MS/MS assay procedures, as well as the interpretation and troubleshooting of acquired data, demands well-trained and experienced staff to develop and validate these methods. In recent years, the performance and the software for the LC−MS/MS instruments has improved dramatically, making MS more approachable and user-friendly.

Method Development Time. When selecting one technology over the other, method development time is often an important decision factor. The development of a new immunoassay is a lengthy process. requiring purified protein, antibody development and testing for the protein(s) of interest. The design of custom antibodies also varies with the platform choice, involving immunogen design and preparation, antibody development, purification, and modification. This process normally takes two or more months for polyclonal antibodies and even longer for monoclonal antibodies, which might end up requiring years. In contrast, LC−MS/MS method development primarily uses the genetic and protein sequence information, as well as physiochemical properties of the target protein/peptide to develop the assay. This allows comparatively rapid development of an LC−MS/MS method. The target and matrix protein sequence information are evaluated to determine surrogate peptides unique to the protein of interest. LC−MS/MS methods can thus be developed and validated in weeks using preliminary protein information, making this an attractive choice when timelines are critical. If using HRMS bottom-up approaches and a variety of labels, then rich information on the observed proteome can be obtained in days.

Multiplexing. With the expansion of technological development in GM crops, more stacked event products are emerging, making multiplexing a critical consideration factor. Selectively detecting and quantitating several proteins in a single run is an extremely attractive feature for both immunoassay and LC−MS/MS based technologies.[2,59,60] Although assays for single traits in stacked events have already been developed, these assays do not directly translate to a multiplex assay. In some cases, immune-based techniques may not be the method of choice, especially when simultaneous monitoring of similar proteins is of importance. Immunoassays require full antibody generation for each protein involved and may be impacted by difficulty of developing capture antibodies due to cross-reactivity or reduced sensitivity and specificity for certain proteins when multiplexed together. Closely related proteins make the lack of selectivity in the assay extremely challenging due to increased matrix effects. The difference between proteins can be minimal or buried within regions of the protein structure that are not readily available to the antibodies. Given these circumstances, LC−MS/MS technology, with its multiplexing and proteolytic capabilities, may be more attractive. LC−MS/MS assays can quantify a larger number of proteins within the same assay, so long as targets share extraction and digestion conditions. The technique can

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differentiate and quantitate closely related proteins, such as proteins that exist in different isoforms or newly expressed proteins and their closely related endogenous counterparts.

Sensitivity. With the progress of technical advancements and improved instruments, most proteins can be detected with sufficient sensitivity. For immunoassays, protein/antibody sensitivity is typically very high,[61] ELISA kits can demonstrate limits of quantitation (LOQ) as low as 0.1 to 1 ng/mL (or ppb),[27] which is conserved excellent for many applications. Multiplexing techniques, offer comparable or even lower LOQ levels, and can reach approximately 0.05 ng/mL depending on the specific analyte and assay conditions.[14,61] For example, in the study by Yeaman et al. (2016), a multiplexed set of sandwich assays for quantifying multiple proteins in a multitrait GM cotton product was developed and validated using the Luminex system, and the results were compared with the ELISA technique.[62] In the study, they found that the LOQs of ELISA are in the 0.3−1.6 ng/mL range, while Luminex assays ranged from 0.1 to 1.7 ng/mL. Protein expression levels determined with this multiplexed Luminex assay were comparable to those determined by ELISA. Similarly, Bastarache et al. (2014) investigated the performance of a set of multiplexed MSD assays and compared the results to those from validated individual ELISA methods. The precision and accuracy of the MSD and ELISA methods were comparable, and protein measurements determined with both technologies were well correlated, with LOQs reported to be slightly more sensitive.[63] In the past, LC−MS/MS faced challenges in detection and quantitative sensitivity, but recent technical advancements have significantly enhanced instrument detection sensitivity. Currently, LC−MS/MS techniques can achieve low limits of quantitation, typically ranging from the low nanomole to attomole levels of analyte,[50] which are comparable to those of immunoassay techniques. This improvement in sensitivity has made LC−MS/MS a powerful tool for protein quantification in complex biological matrices.

Accuracy and Precision. When comparing the quantification of proteins, both immunoassay and MS offer similar ranges of accuracy and precision. According to the survey of Settlage et al.,[64] In ELISA and LC−MS/MS assays, the recovery accuracy ideally is at 70−120%, but currently accepted at 60−130%. The ELISA assay accuracy is impacted by sample matrix issues that reduce specificity. LC−MS/MS also faces challenges with extraction recovery, while the labeled internal standards may obviate the need for matrix assessment. The ELISA assay precision is ideally within 20% of the individual values but currently accepts 25%. Mass spec assay precision has a similar accept rate. While immunoassays provide accurate quantification, especially due to their high specificity, they may have limitations in measuring multiple proteins simultaneously. Conversely, mass spectrometry offers multiplexing capabilities and precise quantitation, but requires careful calibration and validation for accuracy. Factors such as sample preparation, instrument performance, and data analysis are critical in achieving precise protein quantification using mass spectrometry.

Intractable Protein. When dealing with challenging proteins, both technologies need to be carefully evaluated to determine the most suitable approach. For some challenging proteins, such as intractable proteins, purification, or enrichment can be difficult due to their low expression or poor solubility. In some cases, an immunoassays cleanup step is even incorporated into an LC-MS/MS assay to overcome technical challenges. When it is difficult to produce protein standards and furthermore to generate antibodies, immunoassay can be a challenging for this situation and MS is likely the best choice for quantifying the target protein, especially when it is in a complex matrix, such as membrane bound proteins. Peptide standards unique to the protein(s) of interest can easily be synthesized and quantitated when peptides are soluble and stable, while interfering proteins and matrix components can be removed or digested prior to separation by liquid chromatography.

Validation. Analytical methods must be validated to demonstrate method performance and ensure quality. Guidelines on analytical method validation, including both immunoassays and MS assays, have been published in various sources for different applications.[65−69] Most method validation parameters, such as accuracy, precision, specificity, limits of quantitation, linearity, quantitative range, extraction efficiency, and stability, are common for both techniques. However, there are technique specific parameters as well, such as carry-over and digestion time courses in MS methods, and antibody specificity and cross-reactivity testing for immunoassays. MS method validation can be complex and time-consuming compared to ELISA, and other multiplexing immunoassays could also be complex due to additional technique specific parameters, method complexity and serial analysis.

Regulatory Acceptance. Regulatory authorities do not typically dictate the technique of choice for regulatory data collection. Although LC−MS/MS methods have gained increased recognition over the past decade, immunoassays are still preferred techniques of choice in many geographic regions due to its easy implementation, lower requirements for analyst training, relatively low cost, and ease of transfer. Protein expression data packages containing quantitative immunoassay analysis have been accepted by many global regulatory agencies, including United States Department of Agriculture (USDA), Food and Drug Association (FDA), Environmental Protection Agency (EPA), and European Food Safety Authority (EFSA).[70] MS-based protein quantification has been used in the pharmaceutical industry and accepted by the FDA. The FDA guidance for Bioanalytical Method Validation Guidance for Industry[71] which is based on the guidance document drafted by the International Council for Harmonization (ICH),[65] also listed the suggestion for the usage of LC−MS/MS methods for quantitative analysis. In the guidance on allergenicity assessment of GM plants, EFSA clearly stated that "either enzyme-linked immunosorbent assay (ELISA) or mass spectrometry (MS) approaches are appropriate methods for the quantification of endogenous allergens, both allowing the specific detection and quantification of single known allergens.[72]

With the fast-growing global population and the increasing concerns about food security, modern agriculture has embraced biotechnology as a promising solution. Looking ahead to the future application perspectives emphasize the critical need for well-developed and validated quantitative technologies to support the development of genetically modified crops, ensuring high specificity, sensitivity, multiplexing, and accurate quantitation. The application perspectives underscore that both immunoassays and MS-based methods are crucial for all stages of trait development, including early development and regulatory studies of trait protein quantitation in support of GM product registration. As we move forward, the application of these techniques will be

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pivotal in addressing scientific questions and specific requirements related to the protein being quantified. The future application will carefully require consideration of factors such as targeted measurement, sample preparation, cost, personnel expertise, method development time, multiplexing, and throughput when choosing between immunoassay or LC−MS/MS for protein quantitation. Ultimately, the future application of these techniques will be driven by the specific circumstances and needs of end users, ushering in an era of advanced quantitative technologies that will play a crucial role in modern agriculture, addressing food security concerns for the expanding global population.

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Y.W. and M.B. outlined the review plan. M.B., Y.W., T.G., and K.K. contributed to the introduction. M.C., T.G., K.K., and S.C. contributed to immunoassay description, M.C. made the immunoassay figures. Y.W., N.H., J.S., and D.G. contributed to mass spectrometry description, Y.W. made the mass spectrometry figures. S.C., J.S., and D.G. contributed to discussion. T.G. contributed to references. C.A., Z.L., and J.M. reviewed and edited the manuscript. Y.W. and M.B. contributed to conclusion. Y.W. and M.B. led manuscript optimization. All authors read and approved the final manuscript.

Notes

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ABBREVIATIONS USED

GM,genetically modified; ELISA,enzyme-linked immunosorbent assay; MSD,meso scale discovery; MS,mass spectrometry; LC−MS/MS,liquid chromatography coupled to tandem mass spectrometers; IS,internal standard

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