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Multiplexed Protein Quantification in Maize Leaves by Liquid Chromatography Coupled with Tandem Mass Spectrometry: An Alternative Tool to Immunoassays for Target Protein Analysis in Genetically Engineered Crops

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S *Supporting Information*

ABSTRACT: A multiplexing liquid chromatography coupled with tandem mass spectrometry (LC−MS/MS) method to quantify three proteins in maize leaves was developed and validated. For each protein, a hybrid Q-TRAP mass spectrometer was operated in the information-dependent acquisition (IDA) mode to select optimal potential signature peptides. The respective signature peptides were then further optimized and quantified as protein surrogates by multiple reaction monitoring (MRM). Leaf crude extracts were subject to microwave-assisted trypsin digestion for 30 min and then injected directly onto a high-performance liquid chromatography (HPLC) column without further separation or enrichment. The minimum sample process enabled us to achieve high recovery and good reproducibility, with a throughput of 200 samples per day. Using recombinant proteins as standards, a linear dynamic quantitative range of 2 orders of magnitude was obtained (correlation coefficient > 0.997) with good accuracy (deviation from nominal concentration < 15%) for all three proteins. Our study demonstrates that LC−MS/MS can be used as an alternative to immunoassays to quantify multiple low abundant proteins in genetically engineered crops.

KEYWORDS: Liquid chromatography, mass spectrometry, protein quantification, multiplex, transgene analysis

INTRODUCTION

Accurate quantification of target proteins in complex biological samples requires a robust analytical technique with good sensitivity and specificity. Since the commercialization of the first genetically engineered (GE) crop in the mid-1990s, characterization of transgene expression at the protein level in GE crops has been achieved almost exclusively by immunoassays, such as enzyme-linked immunosorbent assays (ELISAs), because of its high specificity, sensitivity, and throughput.[1,2] However, immunoassays rely heavily on high-quality antibodies that are not always obtainable and, therefore, are not suitable for weakly or non-immunoreactive antigens. Membrane and/or membrane-associated proteins present great challenges to immunoassays, especially ELISAs. Additionally, the development of an immunoassay is time-consuming and usually takes several months or even longer. Considering the large number of genes of interest being tested in field trials in both academic and industrial institutes, we believe it is important to develop an alternative protein detection/quantification technique for which method development can be accomplished in a shorter time.

For decades, liquid chromatography coupled with tandem mass spectrometry (LC−MS/MS) has been an indispensible bioanalytical tool to quantify small molecules, including pharmaceuticals[3] and pesticide residues,[4] for high-throughput sample analysis (hundreds of samples per day). In the recent literature, LC−MS/MS was applied to protein quantifications in protein biomarker research, but very few if any studies provide a fully validated bioanalytical method suitable for similar high-throughput sample analysis.[5−9] Furthermore, the majority of the literature still focuses on relative quantification using isotope-labeling techniques,[10−13] and absolute protein quantification, if performed, was accomplished by either isotope dilution without a calibration curve or using a calibration curve generated with peptide standards.[14−17] In both cases, it is assumed that proteolysis will produce equal molar amounts of signature peptides and peptides are stable. These underlying assumptions, however, must be validated.[18−22]

We report the first method development and validation of LC−MS/MS for quantifying three proteins expressed in transgenic maize leaves, namely, gene-shuffled glyphosate acetyltransferase variant (GAT4621),[23] a highly resistant allele mutant of maize acetolactate synthase (ALS) (zmHRA),[24] and phosphinothricin acetyltransferase (PAT).[25] These three proteins are co-expressed in maize to achieve multiple herbicide resistance against single or combinations of herbicides containing glyphosate, ALS inhibitors (sulfonylureas), and glufosinate as active ingredients. The major objective of the study is to develop and validate an alternative analytical technique for plant transgene protein analysis. In this study, well-established validation protocols for LC−MS/MS quantification of small molecules, which include small peptides, were adapted and recombinant proteins were used as reference standards. We show that LC−MS/MS

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can be used to quantify multiple low abundant proteins in transgenic crops with high sensitivity and specificity, as well as excellent precision and accuracy.

■ MATERIALS AND METHODS

Materials. All chemicals were purchased from Sigma-Aldrich unless indicated otherwise. The extraction buffer PBST contained phosphate-buffered saline (PBS) and 0.05% Tween 20 (both from EMD Chemicals). The digestion buffer contained 50 mM ammonium bicarbonate (ABC) without adjusting pH. TNT-4 buffer used for ELISA contained 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 75 mM KCl, 75 mM NaCl, 0.05% Tween-20, 1% polyethylene glycol (PEG), 10% ethylene glycol, and 0.01% thimerosal. Two protease inhibitor cocktails (CalBiochem Mix III and Roche Complete EDTA free) were ordered from VWR International.

Maize plants were grown locally in the greenhouse of Pioneer Hi-Bred International. The maize leaves harvested at stage V6 or R1 were ground after lyophilization. Recombinant protein standards were expressed and purified in *Escherichia coli* internally (Protein Core Facility, Pioneer Hi-Bred International), and aliquots were stored at −80 °C for single usage. The protein purities (>95%) were assessed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), with protein concentrations measured by amino acid analysis (Keck Facility, Yale University). Stable isotope-labeled peptides (^{13}C and ^{15}N isotope purities >95%) were ordered from Elim Biopharmaceuticals (Hayward, CA).

Sample Preparation. A total of 600 μL of PBST buffer was added per 10 mg leaf tissue, weighed into 1.2 mL of micro titertubes (Quality Scientific Plastics). Samples were mixed in a Spex Certiprep 2000 GenoGrinder at a setting of 1600 strokes/min for 30 s and then centrifuged (4 °C, 3900*g*) for 10 min. The supernatant collected was kept on ice, and total extracted proteins (TEPs) were measured with a Bradford assay if necessary. A total of 50 μL of supernatant was added to 110 μL of digestion buffer in polymerase chain reaction (PCR) tubes. An appropriate amount of internal standard (IS) and sequencing grade modified trypsin (Promega) was added (trypsin/TEP ratio ∼ 1:15). Samples were mixed briefly and spun in a microcentrifuge. Samples were then placed in a homemade sample holder fitted into a CEM Discover Proteomics System (Matthews, NC). Digestion lasted for 30 min (45 °C, 50 W) unless indicated otherwise. For overnight digestion, samples were placed in at an oven at a temperature of 37 °C (∼18 h). After acidification with 10 μL of 10% (v/v) formic acid, samples were subject to LC−MS/MS analysis. For leaf punch samples, 10 punches instead of 10 mg of leaf tissue were used for each sample. Each sample extract was normalized with PBST to 1 μg/μL TEP, and 50 μL of extract was used for digestion. The two protease inhibitor cocktails (CalBiochem Mix III and Roche Complete EDTA free) were used according to the product specification sheets. For example, one Roche Complete EDTA-free tablet was dissolved in 2 mL of HPLC-grade water to prepare 25× stock solution, which was then diluted into extraction or digestion buffer.

LC−MS/MS. The LC−MS/MS system included an AB Sciex 4000 Q-TRAP with a Turbo ion-spray source and Waters Acquity ultra-performance liquid chromatography (UPLC). The autosampler temperature was kept at 5 °C during analysis. A total of 20 μL was injected onto an Aquasil, 100 × 2.1 mm, 3 μm, C18 column (ThermoFisher), which was kept at 60 °C to decrease backpressure. LC was performed at a flow rate of 0.6 mL/min, unless otherwise specified. Mobile phases consisted of 0.1% formic acid (MPA) and 0.1% formic acid in acetonitrile (MPB). The LC run started at 15% MPB for 0.2 min, followed by a 2.5 min linear gradient to 35% MPB. This was followed by 1 min of backwash of 0.1% formic acid in 90% acetonitrile (0.8 mL/min) using a separate PerkinElmer Series 275 Micropump and a high-pressure 6-port

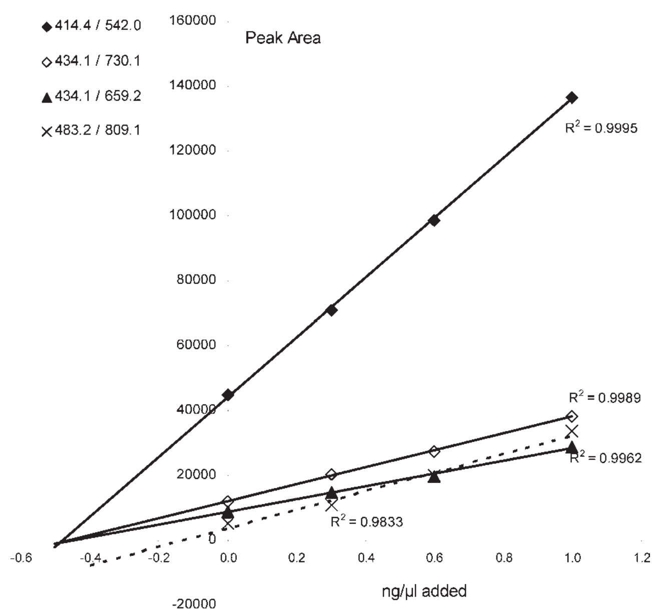

Figure 1. Example of quantification of GAT4621 in a transgenic maize leaf crude extract using standard addition with three different signature peptides. One MRM transition was chosen for SAFHLGGFYGGK (414.4/542.0) and GVATLEGYR (483.2/809.1), and two transitions were chosen for peptide HAEEILR (434.1/730.1 and 434.1/659.2). Protein concentrations in the original samples were calculated by linear extrapolation to the baseline using Microsoft Excel.

2-position valve (VICI-Valco) to reverse the flow on the column. During the backwash, the UPLC flow rate was reduced to 0.1 mL/min and diverted to waste. After the backwash, the valve was switched back and the column was washed further by UPLC with 90% MPB (0.8 mL/min) for 1 min, followed by column equilibrium for 1 min. The total run time for each injection was ∼6 min. The backflush was needed to reduce carry-over of zmHRA peptide VFVLNNQHLGMVVQLEDR from 5−8% to less than 0.5%.

The mass spectrometer was operated in both multiple reaction monitoring (MRM) and linear ion-trap mode to select signature peptides. A complete list of MRM transitions was generated using MRM-initiated detection and sequencing (MIDAS) (AB Sciex) software for all tryptic peptides with an appropriate length (8−20 amino acids). Details of MIDAS applications were described in the literature.[26] The digested recombinant protein was analyzed using MRM-triggered information-dependent acquisition (IDA) to obtain both MRM chromatograms and MS/MS spectra, with the latter facilitating selection of the product ions with the highest sensitivity. The mass spectrometer was run in MRM mode at unit-mass resolution in both Q1 and Q3. The following electrospray ionization source parameters were used: dwell time, 30 ms for all MRM transitions; ion-spray voltage, 5500 V; ion source temperature, 600 °C; curtain gas (CUR), 30; both ion source gas 1 (GS1) and ion source gas 2 (GS2), 80; collision gas (CAD), high. The MRM transitions of 414.4/542.0, 704.5/933.1, and 762.0/784.3 were monitored for peptides SAFH<u>L</u>GGFYGGK (triply charged), VFVLNNQHLGMVVQ<u>L</u>EDR (triply charged), and SVVAVIG<u>L</u>PNDPSVR (doubly charged), respectively. For IS, where <u>L</u> was labeled with stable isotopes ^{13}C and ^{15}N (total mass difference of 7 Da), the MRM transitions of 416.7/545.5, 706.8/936.6, and 765.5/784.3 were monitored, respectively. The isotope purity of the IS was checked by monitoring nonlabeled transitions. Chromatograms were integrated using AB Sciex software Analyst 1.4.2 with a Classic algorithm. Peak area ratios to the corresponding IS were plotted against protein concentrations. A linear regression with $1/x^2$ (where x = concentration) weighting was used for calibration curve fitting.

Table 1. Quantifications (ng/μL) of GAT4621 and PAT in Four Transgenic Maize Leaf Crude Extracts Using Standard Addition by Monitoring Multiple Peptides[a]

peptides monitored (GAT4621)		SAFHLGGFYGGK	HAEEILR	HAEEILR	GVATLEGYR		
MRM transitions		414.4/542.0	434.1/730.1	434.1/659.2	483.2/809.1	mean[b]	CV[b] (%)
TGEX1	concentration (R^2)	0.371(0.9956)	0.459(0.9986)	0.420(0.9967)	3.271(0.7321)	0.417	10.6
TGEX2	concentration (R^2)	0.477(0.9995)	0.466(0.9989)	0.446(0.9962)	0.134(0.9833)	0.463	3.3
TGEX3	concentration (R^2)	0.597(0.9985)	0.588(0.9992)	0.582(0.9994)	−15.983(0.0786)	0.589	1.3
TGEX4	concentration (R^2)	0.523(0.9972)	0.549(0.9804)	0.603(0.9816)	1.077(0.9929)	0.559	7.3
peptides monitored (PAT)		**SVVAVIGLPNDPSVR**	**LGLGSTLYTHLLK**	**SMEAQGFK**	**LHEALGYTAR**		
MRM transitions		762.0/784.3	472.6/651.8	449.1/679.2	377.5/621.1	mean[b]	CV[b] (%)
TGEX1	concentration (R^2)	1.589(0.9997)	1.689(0.9980)	1.731(0.9943)	1.415(0.9950)	1.606	4.6
TGEX2	concentration (R^2)	0.655(0.9974)	0.603(0.9985)	0.616(0.9971)	0.652(0.9963)	0.631	4.3
TGEX3	concentration (R^2)	0.758(1.0000)	0.793(0.9941)	0.736(0.9995)	0.694(0.9993)	0.745	3.9
TGEX4	concentration (R^2)	0.736(0.9963)	0.675(0.9974)	0.692(0.9996)	0.656(0.9999)	0.690	4.5

[a] Protein concentrations in the original samples were calculated by linear extrapolation to the baseline using Microsoft Excel. No interference was found for any of these MRM transitions when four nontransgenic isoline samples were examined. [b] Data from peptide GVATLEGYR were excluded in statistics because of poor stability during digestion (see the text).

Standard Addition. Standard addition experiments were carried out for GAT4621 and PAT with four pre-extracted transgenic samples (1 μg/μL TEP). For each sample, different amounts of recombinant protein standards were spiked into 50 μL aliquots of extracts at final concentrations of 0, 0.3, 0.6, and 1 ng/μL. Protein concentrations in the original samples were calculated by linear extrapolation to the baseline using Microsoft Excel, with one example shown in Figure 1.

Extraction Efficiency and Spike Recovery. Extraction efficiency was evaluated through serial extractions of four transgenic samples. A total of 10 mg of leaf tissues was extracted with 600 μL of PBST, and 50 μL of extracts (in triplicate) was analyzed. The pellet from the first extraction was treated the same way as the original 10 mg leaf tissue sample, namely, extracted with 600 μL of extraction buffer for another round of trypsin digestion. The same amount of IS was added to all digested samples before LC−MS/MS analysis. Peak areas (normalized by IS) of the two extractions were used to calculate the extraction efficiency that is $[A_{\text{first}}/(A_{\text{first}} + A_{\text{second}})] \times 100\%$.

For spike recovery experiments, 600 μL of PBST buffers fortified with protein standards at the lowest (0.04 ng/μL GAT4621 and PAT and 0.08 ng/μL zmHRA), medium (0.4 ng/μL GAT4621 and PAT and 0.8 ng/μL zmHRA) and highest (4 ng/μL GAT4621 and PAT and 8 ng/μL zmHRA) concentrations of the standard curves was used to extract 10 mg of tissues. These three samples were treated as unknown samples and were analyzed in triplicate. Spike recoveries, expressed as a percentage, were determined by comparing the measured concentrations to the fortified concentrations.

Calibration Curves and Quality Control (QC) Samples. A calibration curve was prepared by spiking the recombinant protein mixture at eight concentrations (0, 0.04, 0.08, 0.2, 0.5, 1, 2, 3.2, and 4 ng/μL for analytes GAT4621 and PAT and 0, 0.08, 0.16, 0.4, 1, 2, 4, 6.4, and 8 ng/μL for zmHRA) into 50 μL of negative leaf extract. Fortified QC samples were prepared in a similar way at three different levels (0.12, 1.2, and 3.0 ng/μL for analytes GAT4621 and PAT and 0.24, 2.4, and 6.0 ng/μL for zmHRA). Acceptable standard and QC values, which were back-calculated from a calibration curve, should fall within ±15% of theoretical values, except for the lower limit of quantitation (LLOQ) (±20%). A calibration curve was rejected if more than two standards or two adjacent standards in the run sequence failed these criteria. A calibration curve was also rejected if the LLOQ or upper limit of quantitation (ULOQ) failed.

ELISA Sample Analysis. The ELISA analysis for GAT4621, zmHRA, and PAT was performed by the Pioneer Hi-Bred immunoassay group. Briefly, a typical sandwich-type ELISA[1] was developed for the quantification of each target protein in maize leaf extracts. Because of immunoreactivity and protein conformation, it was necessary to use two different extraction buffers for the ELISA analysis: TNT-4 buffer for GAT4621 and zmHRA and PBST buffer for PAT. In these assays, standards (triplicate wells) and samples (duplicate wells) were incubated in stabilized plates precoated with an antibody specific for the protein of interest. After 1 h of incubation, unbound substances were washed from the plate and the bound protein was incubated with a different protein-specific antibody conjugated to the enzyme horseradish peroxidase (HRP). The quantification of the bound complex was accomplished through the addition of the HRP substrate solution. The reaction was stopped with sulfuric acid, and the optical density of each well was determined using a Molecular Devices plate reader at a wavelength of 450 nm. Softmax Pro software was used to perform the calculations with quadratic fitting for the calibration curves. To compare LC−MS/MS and ELISA methods, 10 lyophilized leaf samples expressing the three proteins were analyzed 2−3 times (depending upon sample availability) across multiple days using both methods.

RESULTS

Signature Peptide Selection. All tryptic peptides of the target proteins with appropriate length (8−20 amino acids) were surveyed using tryptically digested recombinant proteins. Three GAT4621 peptides selected for highest MS response and ease of chromatography were chosen as potential signature peptides for further optimization and validation. One best MRM transition was chosen for each peptide, except HAEEILR, for which two transitions were chosen (Table 1 and Figure 1). These four MRM transitions of the three peptides were monitored using standard addition to analyze four individual transgenic samples. The standard addition method had the advantage of eliminating matrix effects among samples without using isotope-labeled IS because all of the samples used for the measurements were from the same original sample and had the same background. As an example shown in Figure 1, the GAT4621 concentration was calculated by linear extrapolation to the baseline and consistent results were obtained with three different MRM transitions of two peptides.

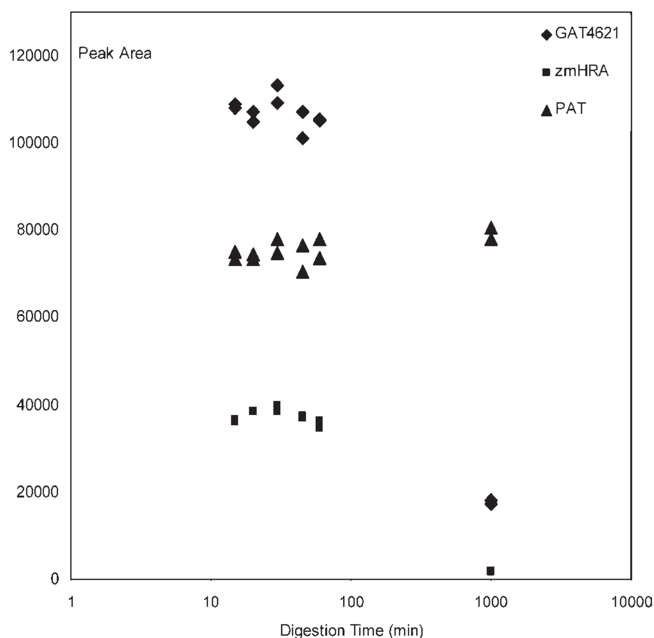

Figure 2. Time courses (15, 20, 30, 45, and 60 min) for microwave-assisted digestion of a fortified QC sample (3 ng/μL for GAT4621 and PAT, and 6 ng/μL for zmHRA) by monitoring the corresponding signature peptides (SAFHLGGFYGGK, VFVLNNQHLGMVVQLEDR, and SVVAVIGLPNDPSVR). Intensities of the three peptides from overnight digestion of the same sample are also included for comparison. The respective ISs were also measured, and the ISs for both GAT4621 and zmHRA were nondetectable following overnight digestion (data not shown).

As summarized in Table 1, the GAT4621 concentrations measured from the first three different MRM transitions were practically the same, indicating that any one of the three transitions can be used to quantify the protein. However, GAT4621 concentrations based on peptide GVATLEGYR were very different. Poor linearity with both microwave-assisted digestion (Table 1) and overnight digestion indicates that this peptide cannot be produced consistently in the leaf extract.

Similarly, four MRM transitions from four PAT signature peptides were monitored to analyze the same transgenic extract samples using standard addition experiments. Consistent results (see also Table 1) were obtained from four selected peptides, indicating that any of the four peptides can be used to quantify protein PAT expressed in maize leaf extract.

zmHRA is a mutant form of endogenous enzyme ALS with two amino acid mutations.[24] Analysis of *in silica* tryptic digestion shows that only one peptide (VFVLNNQHLGMVVQLEDR) associated with one mutation could possibly be used to quantify zmHRA specifically without interference from ALS and the other peptide associated with the other mutation is too large for LC−MS/MS analysis.

Peptide Stability and Microwave-Assisted Trypsin Digestion. Microwave-assisted proteolysis and overnight digestion (37 °C) were compared for their digestion efficiencies and peptide stability to fortified QC and transgenic samples. With overnight digestion, significant losses were observed for some of the peptides selected above, including spiked ISs, namely, synthetic isotope-labeled peptides. Specifically, near 100% of both the zmHRA peptide of choice and its IS were lost with overnight digestion of fortified QC and transgenic samples (Figure 2), making microwave-assisted digestion a superior option. The addition of two commercial protease inhibitor cocktails failed to prevent the loss completely. For example, both cocktails decreased the loss of zmHRA IS from ∼100 to ∼50%. It is worth noting, however, that the two different digestions of a neat zmHRA solution generated the same results quantitatively. With this, three peptides, SAFHLGGFYGGK, VFVLNNQHLGMVVQLEDR, and SVVAVIGLPNDPSVR, were selected as the final signature peptides to quantify three proteins, GAT4621, zmHRA, and PAT, respectively.

Microwave-assisted digestion was optimized by changing the digestion time and protein/enzyme ratios. A total of 30 min was chosen as the optimum digestion time because the cleavage appeared complete for all three peptides (Figure 2), and longer time might result in more peptide loss because of instability. A protein/trypsin ratio of 1:15 was chosen because more trypsin did not produce more peptides. These selected peptides were stable for at least 24 h after acidification (see below).

Extraction Efficiency and Spike Recovery. Extraction efficiency was evaluated through serial extractions of four different transgenic leaf samples using extraction buffer PBST. Extraction efficiencies were 88% (GAT4621), 87% (zmHRA), and 89% (PAT), with precision [coefficient of variation (CV), %] of 1% (GAT4621), 2% (zmHRA), and 1% (PAT), respectively. Spike recovery was investigated at three different concentrations (the lowest, medium, and highest standard concentrations). Mean recoveries were 111% (GAT4621), 95% (zmHRA), and 111% (PAT), with precision (CV, %) of 9% (GAT4621), 16% (zmHRA), and 7% (PAT), respectively.

Calibration Curves and Linearity. Five independent calibration curves were prepared and analyzed on five different days, with one typical calibration curve shown in Figure 3. Good linearity was demonstrated over 2 orders of magnitude (0.04−4 ng/μL for GAT4621 and PAT and 0.08−8 ng/μL for zmHRA). Regression analyses yielded mean correlation coefficients of 0.9971, 0.9975, and 0.9980 for GAT4621, zmHRA, and PAT, respectively, with the minimum correlation coefficient of 0.9959 for all proteins (Table S1 of the Supporting Information).

Sensitivity and Specificity. Defined by the lower limits of the linearity curves, the LLOQ is 0.04, 0.08, and 0.04 ng/μL for proteins GAT4621, zmHRA and PAT, respectively, which equates to 2.4 ng of GAT4621, 4.8 ng of zmHRA, and 2.4 ng of PAT per milligram [parts per million (ppm)] of lyophilized leaf tissue in consideration of 600 μL of buffer used to extract 10 mg of tissue. As shown in Figure S1 of the Supporting Information, no interfering chromatography peaks were found at the LLOQ levels when four nontransgenic isoline samples were analyzed.

Precision and Accuracy. Interbatch precision and accuracy for the standards were assessed with the above five calibration curves, and intrabatch precision and accuracy were also assessed with fortified quality control samples at three different levels (Tables S2 and S3 of the Supporting Information). Because extraction efficiency cannot be tracked with fortified QC samples, four well-mixed ground transgenic samples were analyzed for further intrabatch (Table S4 of the Supporting Information) and interbatch (Table 2 and Table S2 of the Supporting Information) precision analysis. Precision and accuracy were satisfactory for all three proteins with both accuracy (deviation from nominal concentration) and precision (CV, %) within ±15%.

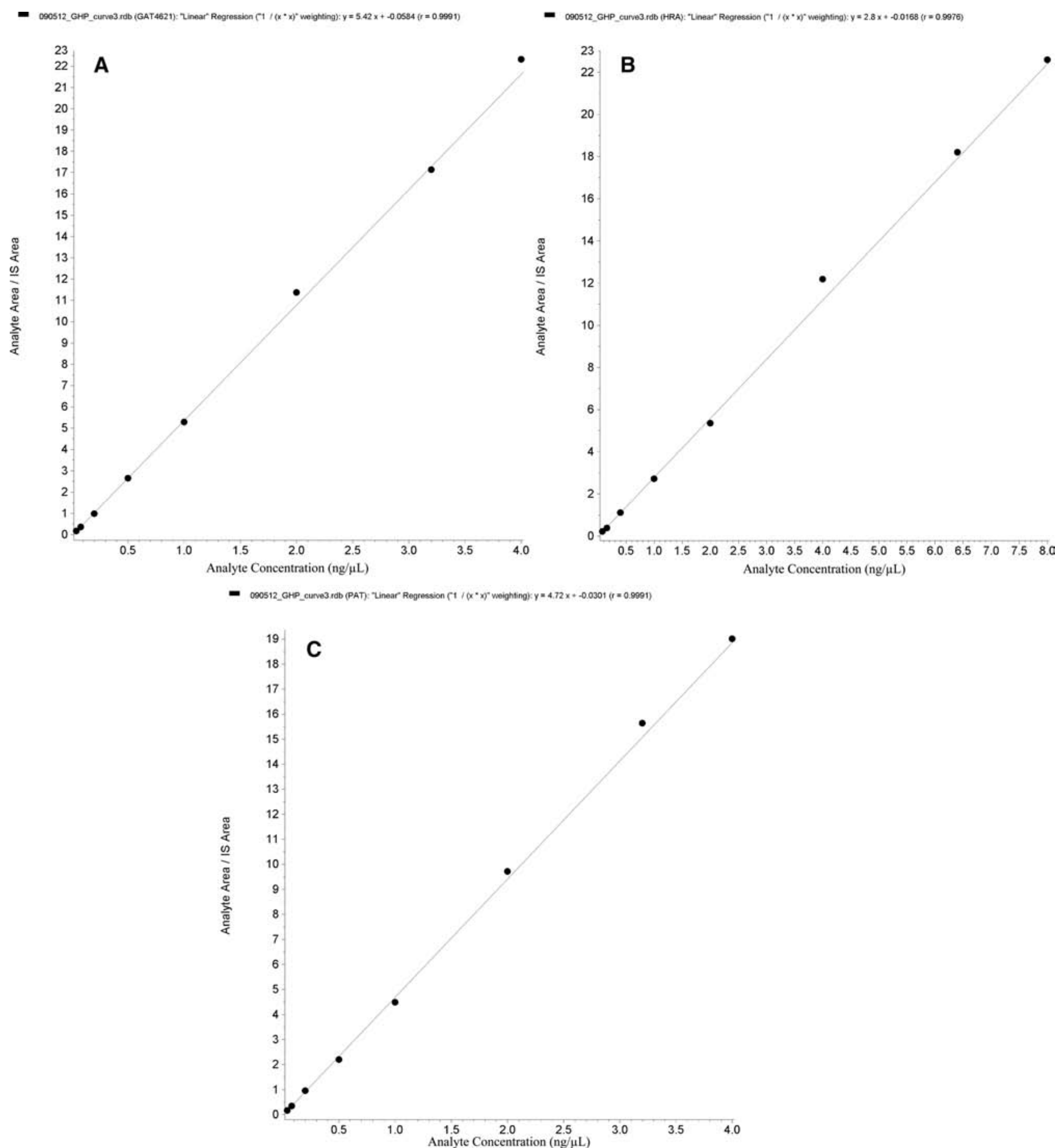

Figure 3. Examples of calibration curves of three proteins in maize leaf crude extract: A, GAT4621 (0.04−4 ng/μL); B, zmHRA (0.08−8 ng/μL); and C, PAT (0.04−4 ng/μL).

The stability of processed samples was evaluated by reinjecting samples left on the chilled autosampler (5 °C) 24 h after initial injection. Consistent peak areas and calculated results (Table 2) indicate that the three selected peptides were stable for at least 24 h at 5 °C after acidification with formic acid.

Comparison to ELISAs. The analysis of 10 transgenic leaf samples were repeated by both LC−MS/MS and ELISAs across multiple days (Figure 4). Overall, it is evident that a less day-to-day variation was observed with LC−MS/MS results. Statistical analysis showed that, for GAT4621 and zmHRA, all LC−MS/MS data points fell within ELISA ± 3 standard deviation (SD) limits, with most data points within ELISA ± 2 SD limits. For PAT, although LC−MS/MS results were consistently higher relative to ELISA values (see the Discussion for a possible explanation), the majority of LC−MS/MS data points (78%) was still within ELISA ± 2 SD limits. The fact that most of LC−MS/MS data points fell within ELISA ± 2 SD limits indicates that two independent analytical methods generated comparable results.

Analysis of Leaf Punch Samples. When thousands of leaf punch samples comprising a few punches each were analyzed, it is

Table 2. Interbatch Precision of Four Transgenic Samples (ng/μL, in Triplicate)

sample	leaf TG1			leaf TG2			leaf TG3			leaf TG4		
analyte	GAT4621	zmHRA	PAT	GAT4621	zmHRA	PAT	GAT4621	zmHRA	PAT	GAT4621	zmHRA	PAT
	0.511	0.176	1.791	0.500	0.191	0.649	0.600	0.208	0.709	0.601	0.184	0.689
day 2	0.478	0.167	1.736	0.529	0.209	0.682	0.587	0.189	0.673	0.626	0.193	0.718
	0.502	0.211	1.879	0.504	0.235	0.706	0.553	0.220	0.744	0.609	0.199	0.744
	0.472	0.184	1.758	0.512	0.165	0.647	0.620	0.185	0.751	0.631	0.172	0.690
day 2−3[a]	0.476	0.158	1.757	0.531	0.198	0.682	0.564	0.188	0.736	0.657	0.198	0.714
	0.511	0.191	1.937	0.514	0.235	0.673	0.613	0.226	0.755	0.588	0.208	0.763
	0.432	0.183	1.804	0.479	0.174	0.705	0.469	0.147	0.636	0.575	0.164	0.734
day 3	0.423	0.153	1.857	0.442	0.157	0.686	0.526	0.168	0.723	0.556	0.166	0.673
	0.452	0.150	1.877	0.429	0.179	0.631	0.521	0.192	0.732	0.597	0.183	0.749
	0.514	0.183	1.843	0.527	0.202	0.714	0.618	0.201	0.754	0.702	0.194	0.719
day 7	0.476	0.162	1.768	0.526	0.188	0.678	0.626	0.185	0.737	0.601	0.175	0.725
	0.500	0.160	1.841	0.533	0.170	0.696	0.557	0.158	0.677	0.638	0.165	0.755
mean	0.479	0.173	1.821	0.502	0.192	0.679	0.571	0.189	0.719	0.615	0.183	0.723
CV (%)	6.42	10.41	3.37	6.96	13.21	3.78	8.51	12.41	5.28	6.35	8.19	3.90

[a] Samples processed on day 2 were reinjected on day 3.

more convenient to normalize data by TEPs rather than by sample weight. Because it is impractical to obtain many homogeneous leaf punches, interbatch reproducibility was tracked by repeated analysis of aliquots of pre-extracted transgenic samples (stored at −80 °C) included in each of the 16 batches. The precision (CV, %) obtained over a period of 3 weeks was 9, 14, and 6% for GAT4621, zmHRA, and PAT, respectively.

DISCUSSION

For more than 3 decades, LC−MS/MS has been successfully used to quantify small molecules, including small peptides,[27] in complex biological samples. When proteins are quantified by LC−MS/MS, it is signature peptides that are quantified as intact protein surrogates. The central question is how to generate and quantify representative signature peptides from target proteins. Therefore, signature peptide selection and optimization are the most important parts of method development. It should be validated that the selected peptides represent intact proteins quantitatively, requiring that proteolysis be complete or at least consistent among samples and selected peptides be stable chemically and enzymatically.[18,19] We believe that the digestion was complete under our optimized conditions because more trypsin or longer digestion time did not increase the selected peptides. In addition, excellent linearity was observed over a range of 2 orders of magnitude with recombinant proteins (not peptides) used as reference standards (Figure 3 and Table S1 of the Supporting Information). Excellent linearity would not be expected if digestion is not complete because trypsin digestion is nondiscriminatory for all extracted proteins and the analyte concentrations are quite low compared to TEP (0.004−0.4% for GAT4621 and PAT and 0.008−0.8% for zmHRA). It is also critical to choose the best peptides to achieve high sensitivity and specificity, because different peptides typically exhibit different MS responses and offer different specificities. Because it is challenging to predict MS response and other relevant physical chemistry properties,[28] all possible tryptic peptides of target proteins were investigated to find the best candidates. In this study, several such peptides were used to quantify each protein when possible (GAT4621 and PAT). Consistent results obtained for a few typical transgenic samples (Table 1) validate our choice of signature peptides.

While it is convenient to use constituent peptides as reference standards,[14−17] its validity relies on the assumptions that proteolysis is complete and selected peptides are stable.[18] When recombinant protein standards are used, proteolysis completeness and peptide stability will have reduced impact on assay accuracy,[22] although complete digestion and stable peptides are preferred to achieve the highest sensitivity and assay reliability. Recombinant protein standards also facilitated direct comparison to ELISAs, where recombinant protein standards are often used.[2] We are aware that a subtle difference between native and recombinant proteins may exist because of possible post-translational modifications (PTMs). However, if results of a few typical transgenic samples (not fortified samples with recombinant proteins) determined from several signature peptides are consistent, chances for biased measurements are slight if not impossible.

The high specificity of LC−MS/MS is demonstrated by its ability to quantify zmHRA without any interference from endogenous ALS. We took advantage of the fact that the three proteins are foreign to maize, and specificity could be confirmed easily with readily available negative control samples. When negative samples are not available (e.g., quantification of an endogenous protein), multiple signature peptides are necessary for cross-validation because interference cannot be predicted with confidence.[6] The absence of interference can be inferred from consistent results derived from multiple peptides. Although bioinformatic tools, such as BLAST, can be used,[7] the absence of homology in BLAST searches for selected peptides does not necessarily guarantee no interference because rearrangement of the amino acids does not necessarily change all MRM transitions. For example, isomeric peptides HAEEILR and HLIEEAR, which apparently lack homology, will have the same MRM transition of 434.1/730.1 because their product ions (y_6) have the same m/z Therefore, unless all y ions are monitored (assuming y ions are the most abundant ions), which is not practical, it might not be safe to use the absence of homology in BLAST as the sole evidence for specificity. On the other hand, homology in BLAST results only indicates potential interference, but it may not

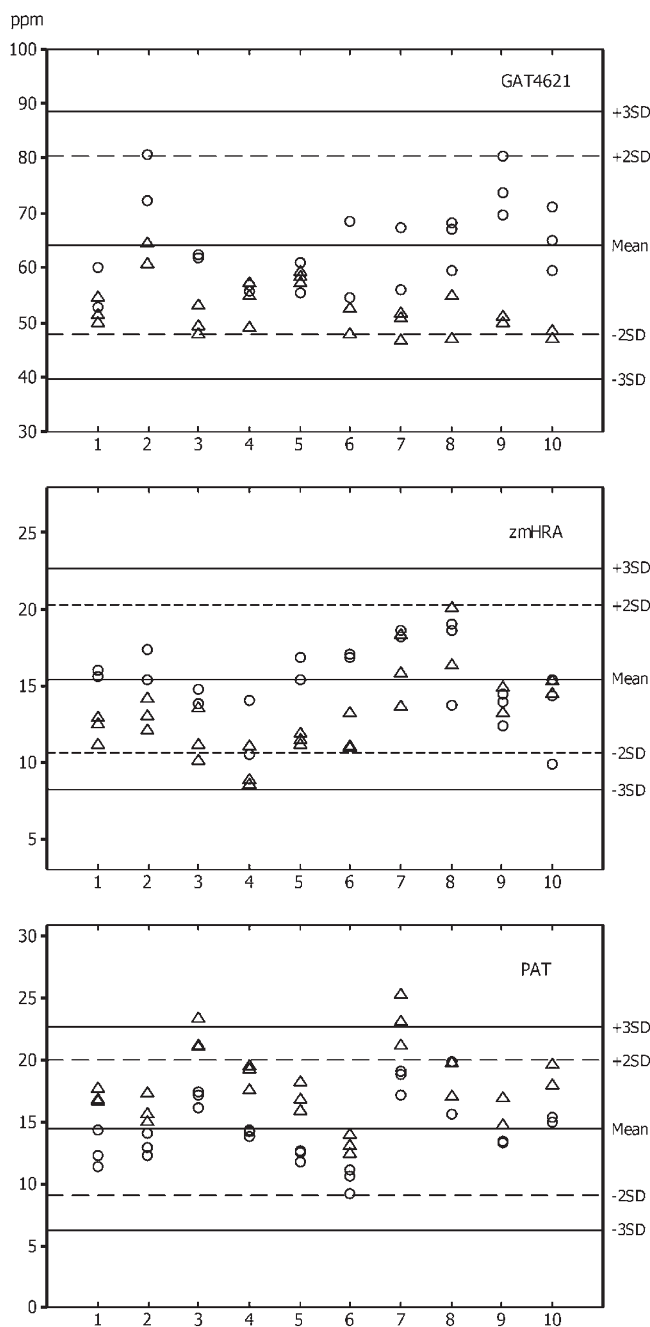

Figure 4. Comparison of LC−MS/MS (△) and ELISA (○) results (ppm on a dry weight basis) of 3 proteins (GAT4621, zmHRA, and PAT) in 10 transgenic leaf samples. ELISA data were used to construct the mean ± 2 and ± 3 SD control limits.

present an actual problem because the interfering signals could be below experimental LLOQs. Another important advantage of LC−MS/MS is that a different product ion of the same peptide often offers different specificity.

Microwave-assisted protein digestion can significantly reduce digestion time and sometimes increases peptide coverage in protein identification research.[29−31] Our results showed that some tryptic peptides could not survive overnight digestion, suggesting that some peptides were further digested by other endogenous proteases, modified or simply unstable under overnight digestion conditions. Fast and efficient peptide cleavage with microwave-assisted and high-pressure trypsin digestions resulted in much less or negligible loss, making it possible to choose otherwise unusable signature peptides and enabling more peptide coverage[31,32] during the qualitative study of protein identification.

As discussed previously, protein quantification by LC−MS/MS is based on protein primary structures (signature peptides) and target proteins will be quantified regardless of conformation changes. On the other hand, ELISAs measure immunoreactivity, and protein conformation changes might have a significant impact on quantification results. Realistically, we believe a perfect agreement between these two very different analytical techniques is ideal but should not always be expected. A direct comparison between the two different analytical techniques indicates that the LC−MS/MS and ELISA results are overall comparable for all three proteins (Figure 4). For PAT, the higher LC−MS/MS results measured could be due to lower spike recovery in ELISA (75%) than in LC−MS/MS (111%). It is important to note that the LC−MS/MS results for GAT4621 and PAT were further confirmed by measurements of different signature peptides (data not shown).

While advantages of LC−MS/MS for protein quantifications are well-discussed in the literature,[8,26] it is worth emphasizing that typical method development can be accomplished in a short time (days) and multiplexing is easily achievable. In contrast, ELISAs usually take longer (months) to develop, and typically, a separate assay is needed for each target protein. Therefore, we strongly believe that LC−MS/MS will become a very important analytical tool for target protein analysis in GE crops and other biological samples.

ASSOCIATED CONTENT

Supporting Information. Correlation coefficients of calibration curves, interbatch precision and accuracy of calibration standards, intrabatch precision and accuracy of fortified QC samples, and intrabatch precision of four transgenic samples (Tables S1−S4) and no interference from the null matrix for the three proteins at LLOQ levels (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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