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RESEARCH PAPER

Measurement of lipid transfer proteins in genetically engineered maize using liquid chromatography with tandem mass spectrometry (LC-MS/MS)

Ryan C. Hill,  Xiujuan Wang, Barry W. Schafer, Satyalinga Srinivas Gampala, and Rod A. Herman

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ABSTRACT. Endogenous allergenicity evaluation is a required part of the risk assessment for genetically engineered (GE) crops. Although maize is not considered a major allergenic food, a lipid transfer protein (Zea m 14) in maize grain has been identified as a potential IgE-mediated food allergen. Currently, the relationship between allergen exposure and risk of sensitization is not well understood. Hence, reliable quantitative methods are useful for determining the natural range and variability of allergen levels across multiple geographies and genetic backgrounds. A LC-MS/MS analytical method was developed and validated in our laboratory to quantify Zea m 14 in grain from 2 GE maize hybrids and 20 non-GE maize hybrids. The measured Zea m 14 levels in GE maize grain and conventional non-GE maize grain ranged from 146.87 to 574.93 ng/mg across 16 field sites located in the United States and Argentina. The method accurately quantified endogenous Zea m 14 from maize grain and results show Zea m 14 levels in the GE maize varieties were within the natural variation observed in traditionally bred non-GE maize.

KEYWORDS. allergen, biotechnology, isoform, LTP, LC-MS/MS, maize, mass spectrometry, surrogate peptide, Zea m 14

INTRODUCTION

Advances in biotechnology have led to the cultivation of genetically engineered (GE)

crops containing multiple beneficial traits (referred to as stacked trait products) to combat plant pests, weeds, and diseases. Novel genetically engineered crops are thoroughly

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evaluated for safety to humans and livestock during the product registration process. As part of the approval process, comprehensive composition and nutrient assessments are performed to compare GE crops with their conventional non-GE counterparts, termed comparative assessment (Lundry et al., 2013, Nicolai et al., 2013). When no meaningful differences in key nutrients, anti-nutrients, or metabolites are identified between the new GE crop and the conventional comparator, the GE crop is considered to be as safe as the non-GE crop for food and feed purposes. In addition, an allergenicity assessment may be performed on the GE crop to consider if unintended changes in endogenous allergen profiles have occurred with the insertion of the transgene relative to the conventional crop, in particular within crops considered to be major allergenic foods such as soybean (EFSA, 2010).

Although maize (*Zea mays*) is not considered to be a major allergenic food, a lipid transfer protein (Zea m 14) in maize grain has been identified as a potential IgE-mediated food allergen (Pastorello et al., 2000). Lipid transfer proteins are low molecular weight proteins ranging from 7–9 kDa and have been implicated in causing systemic allergic reactions from ingestion of various seeds and fruits (Pastorello et al., 2002, Pastorello et al., 2013). Two novel GE hybrid maize stacked products (breeding stacks) have recently been developed to provide farmers with multiple modes of action to combat maize pests and weeds and decrease yield loss due to plant damage and weed competition. The first hybrid maize stack, MON-89034–3 × DAS-01507–1 × SYN-IR162–4 × MON-00603–6, referred to as GE hybrid maize stack 1 for simplicity, expresses Cry1A.105 + Cry2Ab2 proteins (MON-89034–3), Cry1F + phosphinothricin acetyltransferase (PAT) proteins (DAS-01507–1), Vip3Aa20 + mannose-6-phosphate isomerase (PMI) proteins (SYN-IR162–4), and the 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) protein (MON-00603–6). The second hybrid maize stack, MON-87427–7 × MON-89034–3 × DAS-01507–1 × MON-87411–9 × DAS-59122–7 × DAS-40278–9, referred to as GE hybrid maize stack 2 for simplicity, expresses the CP4 EPSPS protein (MON-87427–7), Cry1A.105 + Cry2Ab2 proteins

(MON-89034–3), Cry1F + PAT proteins (DAS-01507–1), Cry3Bb1 + CP4 EPSPS proteins and DvSnf7 RNA (MON-87411–9), Cry34Ab1 + Cry35Ab1 + PAT proteins (DAS-59122–7), and the aryloxyalkanoate dioxygenase-1 (AAD-1) protein (DAS-40278–9). The Cry1A.105, Cry2Ab2, Cry1F, and Vip3Aa20 proteins provide plant protection against target lepidopteron pests, Cry3Bb1 provides protection against western corn rootworm, DvSnf7 RNA is an inverted repeat sequence designed to match that of western corn rootworm (*Diabrotica virgifera virgifera*) and provides protection against corn rootworm larval feeding, the Cry34Ab1 and Cry35Ab1 proteins protect against several target coleopteran pests, the PMI protein was used as a selectable marker in the development of event SYN-IR162–4, and the PAT, CP4 EPSPS, and AAD-1 proteins provide tolerance to the herbicides glufosinate, glyphosate, and aryloxyphenoxypropionate herbicides, respectively.

The purpose of this study was to evaluate endogenous Zea m 14 protein levels in maize grain from GE hybrid maize stack 1, GE hybrid maize stack 2, and 20 commercially available non-GE maize varieties grown in 16 field sites located in the United States and Argentina to assess the natural variation of Zea m 14 (including known isoforms) across different backgrounds, environments and growing practices.

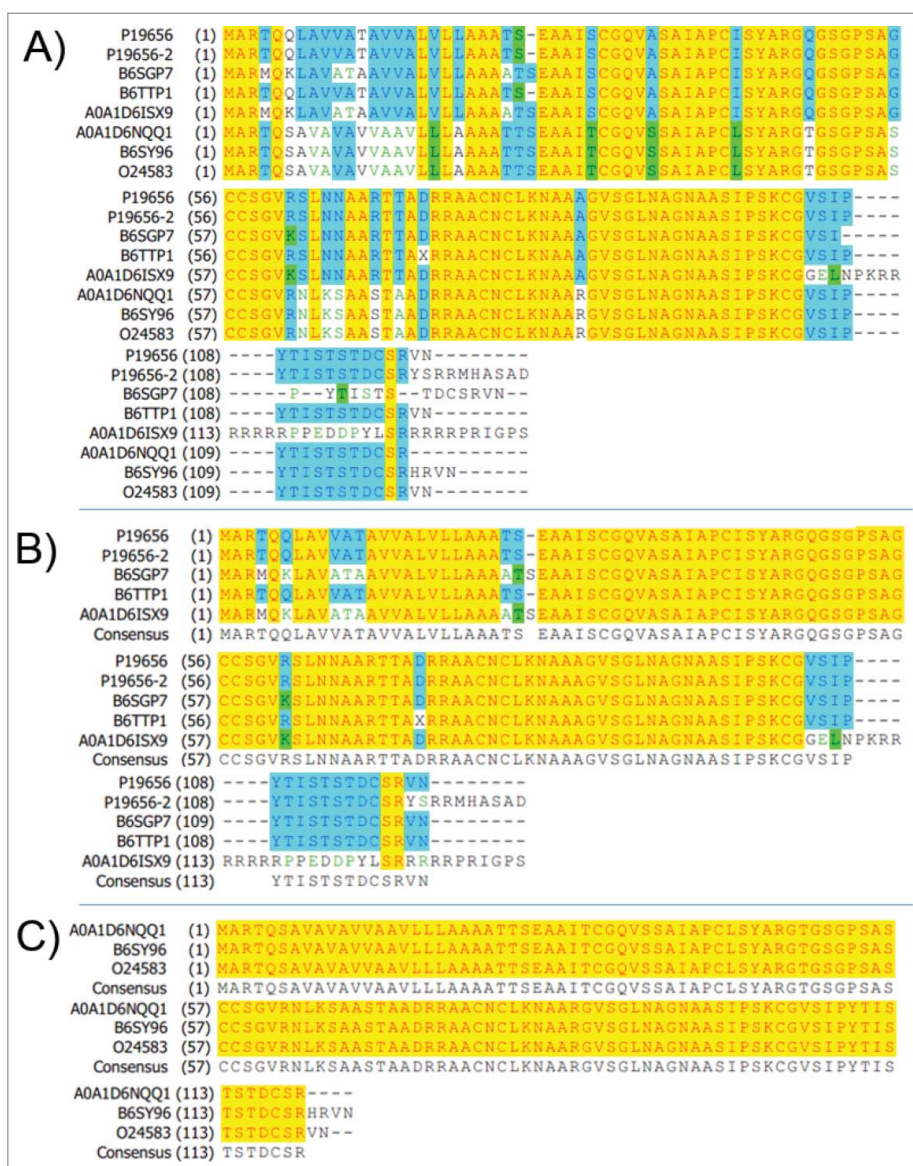
RESULTS

Method optimization

A multiplexing liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) assay was developed and validated to measure peptides as surrogates for endogenous Zea m 14 protein abundance in maize grain. In this application, trypsin enzyme is used to produce peptides with predicted sequence specific to Zea m 14, termed signature peptide, which are subsequently analyzed by tandem mass spectrometry. The ~12 kDa Zea m 14 protein sequence in maize (GeneBank accession P19656) contains a 27 amino acid signal peptide (Tchang et al., 1988) (~3 kDa) in addition to the 93 amino acid polypeptide in the mature

protein after processing (~9 kDa). The mature protein without signal peptide was used to obtain known isoforms from public databases such as UniProt, NCBI, and Phytozome for homology assessment. Sequence alignments were performed using a CLUSTAL W type alignment with Vector NTI Align X software (Fig. 1). The sequences were split into 2

FIGURE 1. Known isoforms for Zea m 14 (P19656) were obtained from public databases UniProt, NCBI, and Phytozome. Sequence alignments were performed using a CLUSTAL W type alignment in Vector NTI Align X software with conserved regions, identical regions, and blocks of similar residues identified in blue, yellow, and green respectively (A). The identified sequences were split into 2 groups based on homology and consensus sequences were generated to identify conserved polypeptide regions for broadest isoform coverage (B and C). The peptide NAAAGVSGLNAGNAA-SIPSK was selected to monitor the group identified as Isoform A and the GVSGLNAGNAA-SIPSK peptide was identified to monitor the group identified as Isoform B.



groups, Isoform A or Isoform B, based on the degree of homology and a consensus sequence was generated for each group to achieve the broadest isoform coverage. All possible tryptic peptides from the consensus sequences were considered from a theoretical digestion. Peptide selection criteria included stability from an overnight digestion, conserved peptide sequence among isoforms, no modifiable residues such as cysteine or methionine, and a length of between 6–20 amino acids for increased specificity. A previous study measuring specific isoforms of Zea m 14 in non-GE maize varieties (Stevenson et al., 2015) performed phylogenetic analysis to assess isoform homology and identified a less abundant variant (Isoform C) which was also included in method development. The NAAAGVSGLNAGNAASIPSK, GVSGLNAGNAASIPSK, and LGGGVSMANANIPSK peptides were identified to monitor Isoform A, Isoform B, and Isoform C, respectively (Table 1). The sum of all 3 peptides were used to report total Zea m 14 results for comparison of GE and non-GE maize varieties.

The optimal extraction procedure consisted of a 100 mg ground and lyophilized maize grain sample extracted for 2 hours in 1 mL of 125 mM ammonium bicarbonate buffer using a thermomixer set to 22°C and 1100 rpm. The extraction buffer was adapted from published literature (Kuppannan et al., 2011) quantifying

intact Zea m 14 using liquid chromatography with ultraviolet and mass spectrometry detection (LC-UV/MS). The final extraction procedure was designed to be simple with minimal sample preparation to support high-throughput analysis as well as ease of method transfer to multiple laboratories in an effort to standardize the approach. In addition, the choice in extraction procedure allowed for a more direct comparison of peptide results to the referenced intact Zea m 14 protein results measured by LC-UV/MS without confounding quantification with extraction efficiency.

Several quantitation methodologies were investigated for the measurement of Zea m 14, including Absolute Quantification (termed AQUA) (Gerber et al., 2003, Stevenson et al., 2010) and standard addition (Hu & Owens, 2011). Although AQUA and standard addition-measurements are scientifically valid in proteomic studies, the choice of methodology to use will depend on the objective of the study. AQUA measurements are very precise, however the measurements are based on the signal intensity of the heavy isotope labeled peptide relative to its natural abundance peptide in the unknown sample. This can complicate the interpretation of results across different studies or laboratories. Standard addition measurements are very accurate; however, natural abundance peptide standards fortified into unknown samples may be challenging to

TABLE 1. Zea m 14 sequence and surrogate peptide information.

Protein ID	Protein Sequence ^{a,b}	Peptide MW (Da)	Protein MW (Da)
Isoform A	MARTQQLAVVATAVVALVLLAAATSEAAISCG QVASAIAPCISYARGQGSGPSAGCCSGVRSLNNA RTTADRRRAACNCLKNAAAGVSGLNAGNAASIPSK CGVSIPYTISTSTDCSRNV	1769.91	11705.32
Isoform B	MARTQSAVAVAVVAVLLAAAATTSEAAITCG QVSSAIAPCLSYARGTGSGPSASCCSGVRNLKSAA STAADRRRAACNCLKNAARGVSGLNAGNAASIPSK CGVSIPYTISTSTDCSR	1442.76	11513.10
Isoform C	MAARSSSSQQLVAAAVALAALLLAAGAGTA SAVSCGEVTSSVAPCLGYAMGSAASPSAACCSG VRLNSRASSADRQATCNCLKSMTGRLGGGVSM ANANIPSKCGVSVGPISPTVDCTKYVGPAPAP CMSS	1486.77	13227.22

^aConsensus sequence used for peptide selection and protein conversion calculations.

^bMonitored signature peptide underlined in the protein sequence.

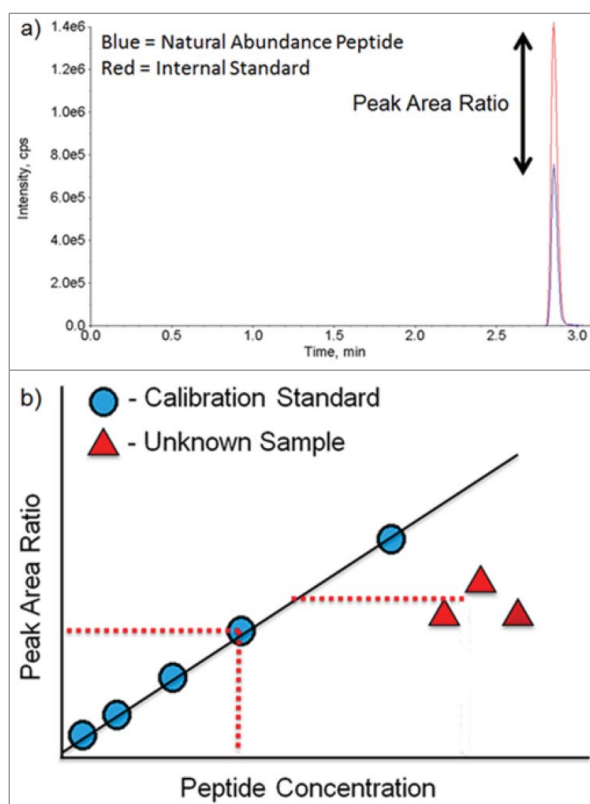
explain in a regulatory landscape where discrete sample identification is important. Due to the need to generate Zea m 14 data across multiple studies, laboratories, or years, reference calibration curves using synthetic natural abundance and heavy isotope labeled peptides were chosen to quantify Zea m 14 in this assay. In this application, the heavy isotope labeled peptide is used as an internal standard to normalize all unknown samples and calibration standards. The resulting peak area ratio between natural abundance peptide and heavy isotope labeled peptide in unknown samples (Fig. 2a) is interpolated to the peak area ratios produced from the reference standard curve (Fig. 2b). The use of peak area ratios to normalize all samples allows for high confidence in data generated

between different analyses and reduces the impact of matrix effects (signal suppression or enhancement) as the measurement is not based strictly on signal intensity.

Method validation

The analytical method was validated in terms of accuracy, precision, ruggedness, specificity, selectivity, linearity, extraction efficiency, digestion efficiency, and limits of detection (LOD) and quantitation (LOQ) for the measurement of Zea m 14 in maize grain following accepted practices adapted to endogenous analytes (Hill et al., 2015, Settlege et al., 2017). The 2 most abundant MS/MS

FIGURE 2. a) Chromatogram showing coelution of natural abundance peptide and heavy isotope labeled internal standard peptide for GVSGLNAGNAASIPSK. The difference between the natural abundance peptide and the heavy isotope labeled internal standard peptide peak area produces a measureable peak area ratio. b) The peak area ratio between natural abundance peptide and heavy isotope labeled peptide is interpolated to the peak area ratios produced from the reference standard curve to normalize all samples.



fragmentation ions (Jenkins et al., 2015) (considering charge state) were selected for each peptide (Table 2). One MS/MS ion was monitored as the quantitation ion (reported result) while the second distinct MS/MS ion was used to confirm the result during method validation and was held to the same validation criteria as the quantitation ion. An additional advantage to validating 2 MS/MS ions allows the use of the second MS/MS ion as the reported result if isobaric interferences not observed in method development are present when analyzing real time samples which may have a slightly different matrix background.

The extraction efficiency was evaluated by serially extracting maize grain 5 times and comparing the detected peptide levels in the first extraction with the sum from all 5 extractions. The extraction was effective with mean extraction efficiencies ranging from 83–85% for Isoform A, and 85–86% for Isoform B. Ideally, purified intact protein standards fortified into buffer and control tissue would be used to evaluate digestion completeness as well as accuracy, precision, and ruggedness. Due to the complexity of purifying endogenous Zea m 14 protein from maize grain, an alternative approach was necessary. Fortunately, the

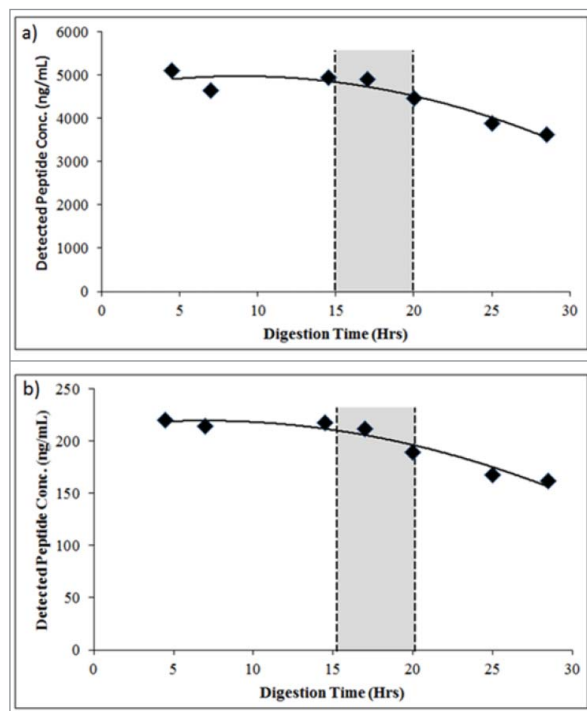
abundance of Zea m 14 allowed for an evaluation of digestion with a time-course experiment on extracted maize grain (Lowenthal et al., 2014, Bronsema et al., 2013). Three replicate aliquots of the maize grain were extracted, digested, and quenched with acid to stop the digestion at approximately 4, 8, 14, 18, 20, 25, and 30 hour durations. The signature peptide levels produced were plotted against digestion time (Fig. 3). No increase in peptide response was observed after 20 hours and the detected signature peptide response was consistent between 15–20 hours (the end of the indicated digestion interval). The results indicate that the peptides are stable over the course of the digestion and the selected length of duration (17 hours) is suitable for complete digestion of Zea m 14 and its possible isoforms. Isoform C was not detected in the tissues used for extraction efficiency or digestion efficiency; as a minor isoform (43% identical to P19656) its measurement was included in the method after meeting further validation acceptance criteria in terms of accuracy and precision from fortification experiments and minimal overall contribution to total Zea m 14 concentration.

The accuracy, precision, and ruggedness of the method were evaluated over multiple

TABLE 2. Zea m 14 accuracy/precision/ruggedness results.

Protein	Peptide Spike (ng/mL)	Mean Recovery (ng/mL)	Recovery Range (%)	Overall Mean (%)	Std Dev (%)	CV (%)	LOQ (3X, 10X)
NAAAGVSGLNAGNAASIPSK	7.81	7.18	88.39–96.72	91.91	2.86	3.11	0.01 (0.03, 0.09)
+3y6 (m/z = 590.64/602.35)	15.63	14.32	89.28–94.09	91.62	1.58	1.72	
Quantitation Ion	500.00	489.47	95.48–101.89	97.89	1.95	2.00	
NAAAGVSGLNAGNAASIPSK	7.81	7.27	89.96–97.73	93.05	2.62	2.82	0.01 (0.04, 0.13)
+3y9 (m/z = 590.64/844.45)	15.63	14.43	89.65–96.21	92.37	2.24	2.43	
Confirmation Ion	500.00	493.8	95.92–102.72	98.76	2.40	2.43	
GVSGLNAGNAASIPSK	7.81	7.67	96.08–100.69	98.12	1.74	1.78	0.01 (0.04, 0.13)
+2y9 (m/z = 721.88/844.45)	15.63	15.62	96.99–101.66	99.99	1.55	1.55	
Quantitation Ion	500.00	509.90	99.19–105.33	101.98	2.12	2.08	
GVSGLNAGNAASIPSK	7.81	7.87	96.79–106.99	100.71	4.28	4.25	0.01 (0.04, 0.13)
+2y11 (m/z = 721.88/1029.53)	15.63	16.62	100.53–111.30	106.36	4.48	4.21	
Confirmation Ion	500.00	539.24	104.38–109.96	107.85	1.92	1.78	
LGGGVSMANAANIPSK	7.81	6.54	72.37–97.86	83.66	8.21	9.81	0.01 (0.04, 0.13)
+3y6 (m/z = 496.26/629.36)	15.63	13.99	86.98–92.83	89.55	1.86	2.08	
Quantitation Ion	500.00	491.05	95.08–101.15	98.21	1.93	1.97	
LGGGVSMANAANIPSK	7.81	8.27	92.30–116.25	105.89	9.29	8.78	
+3y7 (m/z = 496.26/700.40)	15.63	16.11	96.34–110.06	103.09	4.92	4.77	
Confirmation Ion	500.00	507.07	97.74–104.77	101.41	2.37	2.34	

FIGURE 3. The completeness of the digestion for Zea m 14 and its possible isoforms was evaluated with a time course experiment. Replicate aliquots of the maize grain extract were prepared, digested, and quenched with acid to stop the digestion at approximately 4, 8, 14, 18, 20, 25, and 30 hour durations (Isoform A – (a), Isoform B – (b)).



analyses with natural abundance peptide standard fortified into digested maize grain. Maize grain was extracted, digested, and pooled to create a uniform digested sample. Triplicate aliquots of the pooled digested sample (unfortified) were analyzed to establish the baseline response for Zea m 14 peptides and this baseline was subtracted from the same tissue fortified with natural

were determined to be 1.84 ng/mg and 3.69 ng/mg dry weight, respectively (Fig. 3). The LOD and LOQ were verified by statistical approaches using the standard deviation from recovery results of the respective proposed LOQ (Table 2) (Keith et al., 1983).

The linear range of the calibration standards spanned 4 orders of magnitude (7 ppb to

$$\text{Recovery (\%)} = \text{Gross Detected Peptide} \times \left(\frac{\text{Fortified Peptide Level}}{\text{Fortified Peptide Level} + \text{Detected Mean Baseline}} \right) \times 100$$

abundance peptide standard using the following equation (Jones, et al., 2012):

The average recoveries at each fortification level fell within the range of 70 to 110% with a CV < 20% for both quantitation and confirmation MS/MS ions representing each peptide (Table 2). The target LOD and LOQ for total Zea m 14 (sum of Zea m 14 possible isoforms)

10 ppm surrogate peptide) with correlation coefficients greater than 0.9995 for all linear regression analyses. The detection limits can go much lower, however due to the concentrations of Zea m 14 typically found in maize grain, and considering linearity, emphasis was placed on the upper limits of quantitation. Dilution linearity of endogenous Zea m 14 protein

was evaluated with 3 replicate serial dilutions prepared from a single maize grain extract due to possible high abundance of the protein. The samples were serially diluted, digested, and the detected level of natural abundance peptides was interpolated from the reference standard curve. The detected peptide CV was $\leq 20\%$ across dilutions 2–16X for all Zea m 14 isoforms.

Matrix effects were evaluated by comparing the response of heavy isotope labeled peptide internal standard fortified into buffer with the response of heavy isotope labeled peptide internal standard fortified into digested maize grain (matrix). In general, a difference of $\geq 20\%$ in the observed peak area between the heavy isotope labeled peptide internal standards fortified into buffer compared with matrix could be considered indicative of a potential matrix effect on the natural abundance peptide. A negative value for the matrix effect indicates matrix suppression and a positive value for matrix effect indicates matrix enhancement. The matrix effects on each heavy isotope labeled peptide internal standard were within $\pm 20\%$ indicating matrix effects on surrogate peptides representing each isoform of Zea m 14 were acceptable. Additionally, it is important to note that through the use of stable isotope internal standards, the matrix effects are normalized resulting in accurate quantitative results.

Extracted sample storage-stability (to account for possible sample reanalysis) was evaluated over an extended duration. Three replicate samples were initially extracted, digested, and analyzed by LC-MS/MS. The sample extracts were stored at approximately 4°C, redigested the following day, and analyzed by LC-MS/MS. The difference in results from the day 0 and day 2 were $>20\%$ for Zea m 14 indicating samples must be freshly extracted for accurate results. Additionally, digested sample and reference standard storage stability was evaluated over an extended period of time to incorporate instrumentation/analysis issues. An analytical set including reference standards, unfortified digested maize grain, and digested maize grain fortified at the LOD, LOQ, and high-range natural-abundance peptide concentrations was analyzed. The sample extracts

were then stored at approximately 4°C (temperature of the autosampler in the analytical method parameters) and reanalyzed after 3 d. The results were within $\pm 20\%$ of the original analysis indicating sample integrity for the storage interval.

Zea m 14 field site analysis

A total of 320 samples were measured by surrogate peptide LC-MS/MS for Zea m 14 abundance in 2 GE maize varieties and 20 non-GE maize varieties harvested from 16 field sites in the United States and Argentina (Table 3). The range of endogenous Zea m 14 expression in the GE hybrid maize stack 1 (327.54–551.22 ng/mg) was within the range observed in the 7 non-GE maize varieties (146.87–574.93 ng/mg) in the Argentina field sites. The range of Zea m 14 levels in the GE hybrid maize stack 2 (158.89–338.41 ng/mg) was also within the range found in 13 non-GE maize varieties (155.27–536.91 ng/mg) in the United States field trials. The overall mean Zea m 14 ranged 2-fold for conventional maize in Argentina and 3-fold in the United States field trials.

Discussion

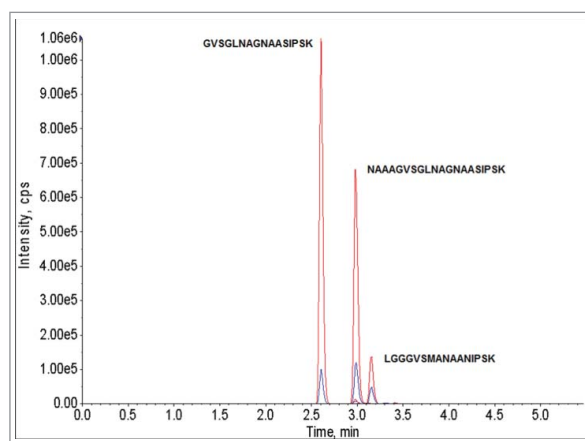
Historically, immunochemistry techniques such as ELISA (Geng et al., 2015, Gu et al., 2017) have been used to quantitatively measure transgenic proteins in plant matrices. The methodology is highly accurate, however requires a substantial resource investment to generate antibodies that are selective and specific to the target analyte. This is further complicated with endogenous analytes present in multiple isomeric forms which may not be distinguished by ELISA, lack of control tissue to confidently assess antibody quality, and the need to purify high quality protein standards. Measuring peptides as surrogates for target protein analyzed by tandem mass spectrometry is a suitable alternative for endogenous allergen quantitation. The methodology is multiplexing (Fig. 4), has a short development time, and is able to selectively detect different isoforms of endogenous allergens. Furthermore, the simplicity of the

TABLE 3. Zea m 14 levels in 2 GE maize stacks and 20 non-GE maize varieties.

Maize Variety	Zea m 14			Country
	Mean ^a (ng/mg)	Range ^a (ng/mg)	Std Dev ^a (ng/mg)	
GE hybrid maize stack 1	419.23	327.54–551.22	54.28	Argentina
Internal Conventional Maize Hybrid 1	235.91	159.57–303.94	35.76	Argentina
Conventional Maize Hybrid 31D06	364.62	249.99–475.45	61.88	Argentina
Conventional Maize Hybrid IMPERIO	330.96	247.98–448.80	53.99	Argentina
Conventional Maize Hybrid KM4321	417.41	242.50–574.93	111.73	Argentina
Conventional Maize Hybrid MILL527	351.18	278.47–430.28	53.13	Argentina
Conventional Maize Hybrid NT 525	197.90	146.87–295.00	40.50	Argentina
Conventional Maize Hybrid PAN 5E-203	377.44	270.65–493.79	65.10	Argentina
GE hybrid maize stack 2	253.51	158.89–338.41	50.98	United States
Internal Conventional Maize Hybrid 2	368.49	271.45–536.91	63.33	United States
Conventional Maize Hybrid: AgVenture 7844	253.81	191.14–324.65	37.41	United States
Conventional Maize Hybrid: Beck's 5509	206.63	185.69–230.15	15.50	United States
Conventional Maize Hybrid: Beck's 5828	299.88	281.94–311.05	13.43	United States
Conventional Maize Hybrid: Beck's 6158	285.60	228.91–362.46	47.97	United States
Conventional Maize Hybrid: Beck's 6175	303.45	223.36–381.75	62.59	United States
Conventional Maize Hybrid: Beck's 6272	317.82	280.70–358.38	31.78	United States
Conventional Maize Hybrid: Dairyland DS-1811	239.23	221.84–247.22	11.70	United States
Conventional Maize Hybrid: Master's Choice MC6060	421.66	324.30–476.31	63.02	United States
Conventional Maize Hybrid: Master's Choice MC6150	160.90	155.27–173.58	8.56	United States
Conventional Maize Hybrid: Mycogen 2H721	325.71	234.01–396.76	64.01	United States
Conventional Maize Hybrid: Pfister 2874	278.92	241.92–310.60	22.31	United States
Conventional Maize Hybrid: Viking 52–11N	182.03	163.10–233.58	21.77	United States

^aResults represent sum of all isoforms of Zea m 14.^bMON-89034–3 × DAS-01507–1 × SYN-IR162–4 × MON-00603–6.^cMON-87427–7 × MON-89034–3 × DAS-01507–1 × MON-87411–9 × DAS-59122–7 × DAS-40278–9.

FIGURE 4. Typical chromatogram for Limit of Quantitation (LOQ) sample depicting heavy labeled internal standard in red and natural abundance peptide in blue for each isoform of Zea m 14.

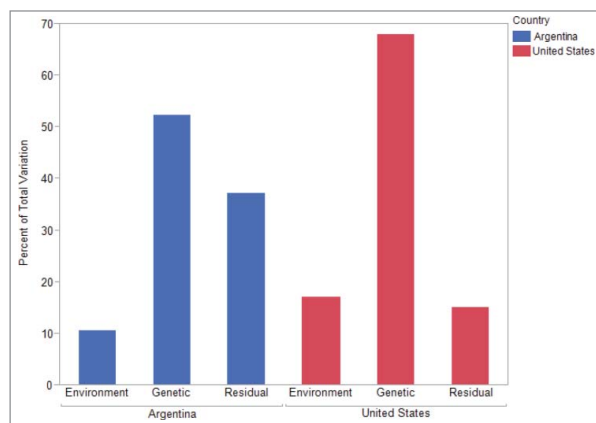


LC-MS/MS method allowed for rapid generation of data from field samples (approximately 160 samples over a 2-day period from extraction through generation of data). The Zea m 14 results from this study using the validated peptide LC-MS/MS (146.87–574.93 ng/mg) method were comparable to previously published literature using ELISA (Gu et al., 2017) (171–865 ng/mg) or LC-UV/MS (Kuppannan et al., 2011) (341–781 ng/mg) despite the variation attributed to genetic factors, environmental factors, and difference in reference standards used between the 3 analyses. The results between the 3 analytical techniques also inform on the natural range of Zea m 14 in maize grain.

The intent of the allergenicity assessment for GE crops is to compare endogenous allergen levels in the new GE crop to that of non-GE varieties. To identify biologically meaningful differences in endogenous allergens between GE maize and conventional non-GE maize, variation across different genetic backgrounds and locations needs to be well understood. To estimate the natural variability in endogenous Zea m 14, 20 non-GE maize varieties representing different genetic backgrounds were planted alongside 2 GE varieties in 16 field sites

located in the major maize growing regions of the United States and Argentina representing different environments and growing practices. The endogenous Zea m 14 levels for both GE stacks (158.89–551.22 ng/mg) fell within the range observed for non-GE varieties (146.87–574.93 ng/mg) in the 320 samples tested. Having demonstrated that the allergen profile in the GE stacks is similar to the non-GE varieties, a variance component analysis was performed to determine whether environment or genetics contributed more to the 3-fold difference in Zea m 14 levels in the non-GE varieties. The Zea m 14 results for Argentina were observed to be higher than in the United States indicating environment contributions play a role in Zea m 14 expression (Table 3). However, the results from variance component analysis showed that Zea m 14 levels are highly different among different conventional maize varieties (52.4% and 68.0% of total variation in Argentina and United States, respectively), while environmental factors only contribute 10.5% and 17.0% of total variation in Argentina and United States, respectively (Fig. 5). Although environmental factors have been demonstrated to have a large impact on soybean allergen abundance (Geng et al., 2016, Stevenson et al., 2012), this

FIGURE 5. Variance component analysis was used to determine contribution of environment or genetics to Zea m 14 levels in non-GE maize varieties. The Zea m 14 results for Argentina were observed to be higher than in the United States indicating environmental factors largely contribute to Zea m 14 levels. However, the results from variance component analysis showed that Zea m 14 levels are variable among different conventional maize varieties (52.4% and 68.0% of total variation in Argentina and United States respectively), while environmental factors only contribute to 10.5% and 17.0% of total variation in Argentina and United States respectively.



study agrees with reported results that maize genetics have a large impact on Zea m 14 levels (Gu et al., 2017). Additionally, a recent study examined the natural variation in non-GE maize using a similar LC-MS/MS methodology (Stevenson et al., 2015). Although a direct comparison can't be made (reported results in that study were in μg Zea m 14/mg protein rather than ng/mg of grain sample), the variation of Zea m 14 observed between non-GE varieties is in agreement with findings in this study.

In summary, a high throughput surrogate peptide LC-MS/MS method was developed and validated to measure levels of the Zea m 14 and its known isoforms in maize grain. The levels detected by the surrogate peptide LC-MS/MS methodology were in agreement with published literature for ELISA (Gu et al., 2017) and intact protein LC-UV/MS (Kuppanan et al., 2011) indicating that all 3 methodologies are suitable for protein quantitation. The advantage of the surrogate peptide methodology for endogenous allergen quantitation is that it is highly specific (capable of monitoring different isoforms), selective (internal standards resolve non-target endogenous components), multiplexing (including a range of higher molecular weight proteins) (Houston et al., 2011), capable of quantitation over a large linear range, and does not require antibodies or purified intact proteins. This method was used to quantify Zea m 14 in grain from 2 field trials in North America and Argentina across 2 GE maize hybrids and 20 non-GE maize hybrids to further characterize the natural variation of Zea m 14. The surrogate peptide results showed that Zea m 14 levels in both GE hybrids was within the range observed in the non-GE hybrids. A further evaluation of environmental and genetic factors in the 20 non-GE hybrids showed that while a 3-fold difference was observed between locations, the genetic background more significantly contributed to Zea m 14 levels in this study. The results demonstrate that variation resulting from introducing a trait through transgenesis does not influence Zea m 14 levels in a manner inconsistent with the variation introduced by traditional breeding. While the value of measuring endogenous allergen levels in GE

crops has been questioned (Herman & Ladics, 2011, Graf et al., 2014, Ladics et al., 2014, Panda et al., 2012), it is useful to document the natural variability of endogenous allergens such as Zea m 14 to understand variability within maize grain.

MATERIALS AND METHODS

Materials

Maize was grown to the R6 (maturity) growth stage for grain harvesting. Maize grain was ground, lyophilized, and stored at -80°C until analysis. Synthetic peptides representative of natural abundance peptides from each protein and corresponding heavy isotope labeled peptide internal standards (L-Lysine- $^{13}\text{C}_6$, $^{15}\text{N}_2$ or L-Arginine- $^{13}\text{C}_6$, $^{15}\text{N}_4$ depending on protein sequence, New England Peptide). Sequencing grade trypsin was purchased frozen (Promega Corporation, V5111).

Method overview

One-hundred milligram ground and lyophilized maize grain samples were defatted twice with hexane and dried before extracting for 2 hours with 125 mM ammonium bicarbonate extraction buffer in a thermomixer set at 22°C and 1100 rpm. The samples were centrifuged for >3000 rpm for 10 minutes at 4°C . A $100\ \mu\text{L}$ aliquot of supernatant was denatured at 95°C and reduced with dithiothreitol at final concentration of 15 mM for 30 minutes followed by cooling at 4°C for 10 minutes. The denatured extracts were incubated overnight (approximately 17 hours) at 37°C with $5\ \mu\text{g}$ trypsin enzyme. After the addition of heavy isotope labeled peptide internal standards, the digestion reaction was quenched with formic acid/water (50/50, v/v) and centrifuged at >3000 rpm for 10 minutes at 4°C . Aliquots of digested extracts were analyzed along with calibration standards prepared in buffer containing 0.1% bovine serum albumin by LC-MS/MS. The LC-MS/MS system included an AB Sciex 6500 QTRAP with turbo ion-spray source and Waters Acquity I-Class UPLC. A total of

10 μ L of sample was injected onto an Acquity UPLC BEH130 C18 1.7 μ m (2.1 $\times$ 50 mm) column kept at 50°C. The reverse phase analysis was performed using acetonitrile containing 0.1% formic acid (MPB) and water containing 0.1% formic acid (MPA). The LC flow rate was 0.5 mL/min with a linear gradient from 5.0% MPB to 15% MPB over 3 minutes, followed by 100% MPB for 1 minute to wash the column, and 5.0% MPB for 1 minute to re-equilibrate the column. The needle wash and seal wash contained 50/50 methanol/water and the auto-sampler temperature was kept at 4°C during analysis. The mass spectrometer was operated in positive ion mode with ionspray voltage of 5500 V and temperature set at 550°C. The MS/MS transitions and further transition-specific parameters can be found in Table 4.

Zea m 14 analysis

Four replicates each of GE hybrid maize stack 1 and non-GE maize (31D06, IMPERIO, KM4321, MILL527, NT 525, and PAN 5E-203) were harvested from 8 field sites located in the major maize growing regions of Argentina (Berdier, Carmen de Areco, El Crisol, Inés Indart, Los Indios, San Patricio, San Pedro, and Tacuarí). Three of the 6 non-GE hybrids were randomly chosen to be planted at each of the 8 field sites.

Four replicates each of GE hybrid maize stack 2 and non-GE maize (AgVenture 7844, Beck's 5509, Beck's 5828, Beck's 6158, Beck's 6175, Beck's 6272, Dairyland DS-1811, Master's Choice MC6060, Master's

Choice MC6150, Mycogen 2H721, Pfister 2874, and Viking 52–11N) were harvested from 8 field sites located in the major maize growing regions of the United States (Iowa - 3 sites, Illinois, Indiana, Missouri, Nebraska, and Pennsylvania). Three of the 12 non-GE hybrids were randomly chosen to be planted at each of the 8 field sites.

Zea m 14 and 3 possible isoforms were monitored in the isoform multiplexed LC-MS/MS analysis. Peptides specific to each isoform of Zea m 14 were measured and converted into protein concentrations expressed on a dry tissue weight basis (ng/mg) as the sum of all isoforms for each sample.

Variance component analysis

To estimate the variance component from different sources, the Zea m 14 levels were analyzed by fitting a linear regression model:

$$Y = \mu + G + E + \varepsilon$$

Where Y is the observed Zea m 14 level, μ is the overall mean, G is the genetic effect from Maize variety, E is the environmental effect from various locations, and ε is the error. Both genetic and environmental factors were considered as random effects. The variance components were calculated using the SAS procedure PROC MIXED (SAS 9.3, SAS Institute, Cary NC) using estimation method REML. The proportion of variance for genetic effect and environmental effect were calculated by dividing

TABLE 4. Zea m 14 MS/MS transition and mass spectrometer conditions.

Q1	Q3	ID ^a	DP (V)	CE (V)	CXP (V)
496.26	629.36	LGGGVSMANAANIPSK.+3y6_Q	55	16	14
496.26	700.40	LGGGVSMANAANIPSK.+3y7_C	55	33	14
498.93	637.38	HIS.LGGGVSMANAANIPSK.+3y6	55	33	14
590.64	602.35	NAAAGVSGLNAGNAASIPSK.+3y6_Q	55	22	11
590.64	844.45	NAAAGVSGLNAGNAASIPSK.+3y9_C	55	35	20
593.31	610.37	HIS.NAAAGVSGLNAGNAASIPSK.+3y6	55	22	11
721.88	844.45	GVSGLNAGNAASIPSK.+2y9_Q	85	33	17
721.88	1029.53	GVSGLNAGNAASIPSK.+2y11_C	85	41	19
725.89	852.47	HIS.GVSGLNAGNAASIPSK.+2y9	85	33	17

^aQ = Quantitation Transition, C = Confirmation Transition, HIS = Heavy Isotope Labeled Internal Standard Peptide Transition

the variance component of genetic factor (G) and environmental factor (E) by total variance, respectively. The data from US and Argentina were modeled separately since there are no Maize varieties shared between the 2 countries in this study.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors are employees of Dow Agro-Sciences LLC, a wholly owned subsidiary of The Dow Chemical Company, which develops transgenic crops and produces insecticides, herbicides, and fungicides for agricultural applications.

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Measurement of Lipid Transfer Proteins in Genetically Engineered Maize using Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS)

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Supplementary Material

Supporting Information S1: Recovery of Zea m 14 (NAAAGVSGLNAGNAASIPSK Peptide, Quantitation Ion, m/z 590.64/602.35, +3y6)

Analytical Set ID	Sample ID	Date of Analysis ^a	Spike Conc. (ng/mL) ^{b,f}	Total Conc. (ng/mL) ^c	Calc. Conc. (ng/mL) ^d	Corrected Conc. (ng/mL) ^{e,f}	Accuracy (%) ^f	Mean (%) ^f	Std Dev (%) ^f	CV (%) ^f
Set 1	Control R1	Day 1	N/A	177.95		N/A	N/A	N/A	N/A	N/A
Set 1	Control R2	Day 1	N/A	182.97	183.19	N/A	N/A	N/A	N/A	N/A
Set 1	Control R3	Day 1	N/A	188.66		N/A	N/A	N/A	N/A	N/A
Set 1	LOD Rep 1	Day 1	7.81	173.49	191.01	7.10	90.83			
Set 1	LOD Rep 2	Day 1	7.81	182.04	191.01	7.45	95.30			
Set 1	LOD Rep 3	Day 1	7.81	171.74	191.01	7.02	89.91	92.54	3.23	3.49
Set 1	LOD Rep 4	Day 1	7.81	171.79	191.01	7.03	89.94			
Set 1	LOD Rep 5	Day 1	7.81	184.74	191.01	7.56	96.72			
Set 1	LOQ Rep 1	Day 1	15.63	185.31	198.82	14.56	93.20			
Set 1	LOQ Rep 2	Day 1	15.63	183.73	198.82	14.44	92.41			
Set 1	LOQ Rep 3	Day 1	15.63	177.51	198.82	13.95	89.28	91.49	1.48	1.62
Set 1	LOQ Rep 4	Day 1	15.63	181.17	198.82	14.24	91.12			
Set 1	LOQ Rep 5	Day 1	15.63	181.82	198.82	14.29	91.45			
Set 1	High Rep 1	Day 1	500.00	673.16	683.19	492.65	98.53			
Set 1	High Rep 2	Day 1	500.00	696.10	683.19	509.45	101.89			
Set 1	High Rep 3	Day 1	500.00	665.19	683.19	486.83	97.37	98.59	2.03	2.06
Set 1	High Rep 4	Day 1	500.00	673.70	683.19	493.05	98.61			
Set 1	High Rep 5	Day 1	500.00	659.70	683.19	482.81	96.56			
Set 2	Control R1	Day 2	N/A	273.32		N/A	N/A	N/A	N/A	N/A
Set 2	Control R2	Day 2	N/A	274.00	270.68	N/A	N/A	N/A	N/A	N/A
Set 2	Control R3	Day 2	N/A	264.72		N/A	N/A	N/A	N/A	N/A
Set 2	LOD Rep 1	Day 2	7.81	246.16	278.49	6.91	88.39			
Set 2	LOD Rep 2	Day 2	7.81	254.60	278.49	7.14	91.42	90.86	2.25	2.47
Set 2	LOD Rep 3	Day 2	7.81	258.37	278.49	7.25	92.77			
Set 2	LOQ Rep 1	Day 2	15.63	261.35	286.30	14.26	91.29			
Set 2	LOQ Rep 2	Day 2	15.63	269.40	286.30	14.70	94.09	91.83	2.05	2.24
Set 2	LOQ Rep 3	Day 2	15.63	257.95	286.30	14.08	90.10			
Set 2	High Rep 1	Day 2	500.00	756.71	770.68	490.94	98.19			
Set 2	High Rep 2	Day 2	500.00	735.81	770.68	477.38	95.48	96.73	1.37	1.41
Set 2	High Rep 3	Day 2	500.00	743.97	770.68	482.67	96.53			

^aThe 'Date of Analysis' indicates the date the samples were extracted.^bConcentration of peptide reference standard fortified into tissue containing endogenous Zea m 14 protein.^cTotal detected peptide level (endogenous Zea m 14 + reference standard fortification).^dTheoretical total concentration of peptide = peptide spike + average of detected peptide in "control" replicates without reference standard addition.^eCorrected Conc. (ng/mL) = Calc. Conc. × Accuracy (%)^fN/A = Not applicable.

Supporting Information S2: Recovery of Zea m 14 (GVSGLNAGNAASIPSK Peptide, Quantitation Ion, m/z 721.88/844.45, +2y9)

Analytical Set ID	Sample ID	Date of Analysis ^a	Spike Conc. (ng/mL) ^{b,f}	Total Conc. (ng/mL) ^c	Calc. Conc. (ng/mL) ^d	Corrected Conc. (ng/mL) ^{e,f}	Accuracy (%) ^f	Mean (%) ^f	Std Dev (%) ^f	CV (%) ^f
Set 1	Control R1	Day 1	N/A	6.58		N/A	N/A	N/A	N/A	N/A
Set 1	Control R2	Day 1	N/A	6.60	6.57	N/A	N/A	N/A	N/A	N/A
Set 1	Control R3	Day 1	N/A	6.54		N/A	N/A	N/A	N/A	N/A
Set 1	LOD Rep 1	Day 1	7.81	14.49	14.39	7.87	100.69	97.76	2.11	2.15
Set 1	LOD Rep 2	Day 1	7.81	13.87	14.39	7.53	96.43			
Set 1	LOD Rep 3	Day 1	7.81	13.85	14.39	7.52	96.30			
Set 1	LOD Rep 4	Day 1	7.81	13.82	14.39	7.51	96.08			
Set 1	LOD Rep 5	Day 1	7.81	14.29	14.39	7.76	99.32			
Set 1	LOQ Rep 1	Day 1	15.63	22.57	22.20	15.88	101.66	100.48	1.04	1.03
Set 1	LOQ Rep 2	Day 1	15.63	22.32	22.20	15.71	100.55			
Set 1	LOQ Rep 3	Day 1	15.63	21.95	22.20	15.45	98.90			
Set 1	LOQ Rep 4	Day 1	15.63	22.44	22.20	15.79	101.08			
Set 1	LOQ Rep 5	Day 1	15.63	22.25	22.20	15.66	100.24			
Set 1	High Rep 1	Day 1	500.00	517.49	506.57	510.78	102.16	100.73	1.40	1.39
Set 1	High Rep 2	Day 1	500.00	516.90	506.57	510.19	102.04			
Set 1	High Rep 3	Day 1	500.00	510.65	506.57	504.03	100.81			
Set 1	High Rep 4	Day 1	500.00	503.73	506.57	497.20	99.44			
Set 1	High Rep 5	Day 1	500.00	502.46	506.57	495.94	99.19			
Set 2	Control R1	Day 2	N/A	9.35		N/A	N/A	N/A	N/A	N/A
Set 2	Control R2	Day 2	N/A	9.07	9.13	N/A	N/A	N/A	N/A	N/A
Set 2	Control R3	Day 2	N/A	8.96		N/A	N/A	N/A	N/A	N/A
Set 2	LOD Rep 1	Day 2	7.81	16.88	16.94	7.78	99.62	98.71	0.97	0.98
Set 2	LOD Rep 2	Day 2	7.81	16.74	16.94	7.72	98.83			
Set 2	LOD Rep 3	Day 2	7.81	16.55	16.94	7.63	97.69			
Set 2	LOQ Rep 1	Day 2	15.63	25.07	24.75	15.83	101.30	99.17	2.16	2.17
Set 2	LOQ Rep 2	Day 2	15.63	24.01	24.75	15.15	96.99			
Set 2	LOQ Rep 3	Day 2	15.63	24.56	24.75	15.50	99.22			
Set 2	High Rep 1	Day 2	500.00	524.76	509.13	515.35	103.07	104.07	1.15	1.11
Set 2	High Rep 2	Day 2	500.00	528.54	509.13	519.05	103.81			
Set 2	High Rep 3	Day 2	500.00	536.27	509.13	526.65	105.33			

^aThe 'Date of Analysis' indicates the date the samples were extracted.^bConcentration of peptide reference standard fortified into tissue containing endogenous Zea m 14 protein.^cTotal detected peptide level (endogenous Zea m 14 + reference standard fortification).^dTheoretical total concentration of peptide = peptide spike + average of detected peptide in "control" replicates without reference standard addition.^eCorrected Conc. (ng/mL) = Calc. Conc. × Accuracy (%)^fN/A = Not applicable.

Supporting Information S3: Recovery of Zea m 14 (LGGVSMANAAANIPSK Peptide, Quantitation Ion, m/z 496.26/629.36, +3y6)

Analytical Set ID	Sample ID	Date of Analysis ^a	Spike Conc. (ng/mL) ^{b,f}	Total Conc. (ng/mL) ^{c,f}	Calc. Conc. (ng/mL) ^d	Corrected Conc. (ng/mL) ^{e,f}	Accuracy (%) ^f	Mean (%) ^f	Std Dev (%) ^f	CV (%) ^f
Set 1	Control R1	Day 1	N/A	ND		N/A	N/A	N/A	N/A	N/A
Set 1	Control R2	Day 1	N/A	ND	N/A	N/A	N/A	N/A	N/A	N/A
Set 1	Control R3	Day 1	N/A	ND		N/A	N/A	N/A	N/A	N/A
Set 1	LOD Rep 1	Day 1	7.81	6.25	7.81	6.25	79.98	78.58	4.36	5.55
Set 1	LOD Rep 2	Day 1	7.81	5.96	7.81	5.96	76.28			
Set 1	LOD Rep 3	Day 1	7.81	5.65	7.81	5.65	72.37			
Set 1	LOD Rep 4	Day 1	7.81	6.54	7.81	6.54	83.71			
Set 1	LOD Rep 5	Day 1	7.81	6.29	7.81	6.29	80.55			
Set 1	LOQ Rep 1	Day 1	15.63	13.72	15.63	13.72	87.81	88.51	1.07	1.21
Set 1	LOQ Rep 2	Day 1	15.63	13.94	15.63	13.94	89.20			
Set 1	LOQ Rep 3	Day 1	15.63	13.92	15.63	13.92	89.10			
Set 1	LOQ Rep 4	Day 1	15.63	13.98	15.63	13.98	89.49			
Set 1	LOQ Rep 5	Day 1	15.63	13.59	15.63	13.59	86.98			
Set 1	High Rep 1	Day 1	500.00	489.26	500.00	489.26	97.85	97.88	2.16	2.20
Set 1	High Rep 2	Day 1	500.00	505.74	500.00	505.75	101.15			
Set 1	High Rep 3	Day 1	500.00	488.51	500.00	488.51	97.70			
Set 1	High Rep 4	Day 1	500.00	488.12	500.00	488.12	97.62			
Set 1	High Rep 5	Day 1	500.00	475.41	500.00	475.41	95.08			
Set 2	Control R1	Day 2	N/A	ND		N/A	N/A	N/A	N/A	N/A
Set 2	Control R2	Day 2	N/A	ND	N/A	N/A	N/A	N/A	N/A	N/A
Set 2	Control R3	Day 2	N/A	ND		N/A	N/A	N/A	N/A	N/A
Set 2	LOD Rep 1	Day 2	7.81	7.65	7.81	7.65	97.86	92.13	5.06	5.49
Set 2	LOD Rep 2	Day 2	7.81	7.05	7.81	7.05	90.26			
Set 2	LOD Rep 3	Day 2	7.81	6.90	7.81	6.90	88.28			
Set 2	LOQ Rep 1	Day 2	15.63	14.29	15.63	14.29	91.44	91.27	1.65	1.80
Set 2	LOQ Rep 2	Day 2	15.63	13.99	15.63	13.99	89.55			
Set 2	LOQ Rep 3	Day 2	15.63	14.51	15.63	14.51	92.83			
Set 2	High Rep 1	Day 2	500.00	483.96	500.00	483.96	96.79	98.76	1.74	1.77
Set 2	High Rep 2	Day 2	500.00	496.80	500.00	496.80	99.36			
Set 2	High Rep 3	Day 2	500.00	500.61	500.00	500.61	100.12			

^aThe 'Date of Analysis' indicates the date the samples were extracted.^bConcentration of peptide reference standard fortified into tissue containing endogenous Zea m 14 protein.^cTotal detected peptide level (endogenous Zea m 14 + reference standard fortification).^dTheoretical total concentration of peptide = peptide spike + average of detected peptide in "control" replicates without reference standard addition.^eCorrected Conc. (ng/mL) = Calc. Conc. × Accuracy (%)^fND = No Detection, below the limit of detection, N/A = Not applicable.

Supporting Information S4: Recovery of Zea m 14 (NAAAGVSGLNAGNAASIPSK Peptide, Confirmation Ion, m/z 590.64/844.45, +3y9)

Analytical Set ID	Sample ID	Date of Analysis ^a	Spike Conc. (ng/mL) ^{b,f}	Total Conc. (ng/mL) ^c	Calc. Conc. (ng/mL) ^d	Corrected Conc. (ng/mL) ^{e,f}	Accuracy (%) ^f	Mean (%) ^f	Std Dev (%) ^f	CV (%) ^f
Set 1	Control R1	Day 1	N/A	151.32		N/A	N/A	N/A	N/A	N/A
Set 1	Control R2	Day 1	N/A	158.56	158.62	N/A	N/A	N/A	N/A	N/A
Set 1	Control R3	Day 1	N/A	165.98		N/A	N/A	N/A	N/A	N/A
Set 1	LOD Rep 1	Day 1	7.81	149.72	166.43	7.03	89.96	93.32	2.97	3.19
Set 1	LOD Rep 2	Day 1	7.81	162.66	166.43	7.64	97.73			
Set 1	LOD Rep 3	Day 1	7.81	154.50	166.43	7.25	92.83			
Set 1	LOD Rep 4	Day 1	7.81	152.44	166.43	7.16	91.59			
Set 1	LOD Rep 5	Day 1	7.81	157.27	166.43	7.38	94.49			
Set 1	LOQ Rep 1	Day 1	15.63	156.79	174.25	14.06	89.98	92.03	2.73	2.97
Set 1	LOQ Rep 2	Day 1	15.63	162.53	174.25	14.57	93.27			
Set 1	LOQ Rep 3	Day 1	15.63	156.21	174.25	14.01	89.65			
Set 1	LOQ Rep 4	Day 1	15.63	167.63	174.25	15.03	96.21			
Set 1	LOQ Rep 5	Day 1	15.63	158.67	174.25	14.23	91.06			
Set 1	High Rep 1	Day 1	500.00	664.11	658.62	504.17	100.83	99.99	2.17	2.17
Set 1	High Rep 2	Day 1	500.00	676.52	658.62	513.59	102.72			
Set 1	High Rep 3	Day 1	500.00	658.43	658.62	499.85	99.97			
Set 1	High Rep 4	Day 1	500.00	656.82	658.62	498.63	99.73			
Set 1	High Rep 5	Day 1	500.00	637.06	658.62	483.63	96.73			
Set 2	Control R1	Day 2	N/A	254.94		N/A	N/A	N/A	N/A	N/A
Set 2	Control R2	Day 2	N/A	246.05	245.36	N/A	N/A	N/A	N/A	N/A
Set 2	Control R3	Day 2	N/A	235.10		N/A	N/A	N/A	N/A	N/A
Set 2	LOD Rep 1	Day 2	7.81	232.99	253.18	7.19	92.03	92.60	2.42	2.62
Set 2	LOD Rep 2	Day 2	7.81	241.18	253.18	7.44	95.26			
Set 2	LOD Rep 3	Day 2	7.81	229.16	253.18	7.07	90.51			
Set 2	LOQ Rep 1	Day 2	15.63	245.96	260.99	14.73	94.24	92.93	1.39	1.50
Set 2	LOQ Rep 2	Day 2	15.63	242.90	260.99	14.54	93.07			
Set 2	LOQ Rep 3	Day 2	15.63	238.73	260.99	14.29	91.47			
Set 2	High Rep 1	Day 2	500.00	714.97	745.36	479.61	95.92	96.70	0.74	0.76
Set 2	High Rep 2	Day 2	500.00	725.89	745.36	486.94	97.39			
Set 2	High Rep 3	Day 2	500.00	721.48	745.36	483.98	96.80			

^aThe 'Date of Analysis' indicates the date the samples were extracted.^bConcentration of peptide reference standard fortified into tissue containing endogenous Zea m 14 protein.^cTotal detected peptide level (endogenous Zea m 14 + reference standard fortification).^dTheoretical total concentration of peptide = peptide spike + average of detected peptide in "control" replicates without reference standard addition.^eCorrected Conc. (ng/mL) = Calc. Conc. × Accuracy (%)^fN/A = Not applicable.

Supporting Information S5: Recovery of Zea m 14 (GVSGLNAGNAASIPSK Peptide, Confirmation Ion, m/z 721.88/1029.53, +2y11)

Analytical Set ID	Sample ID	Date of Analysis ^a	Spike Conc. (ng/mL) ^{b,f}	Total Conc. (ng/mL) ^c	Calc. Conc. (ng/mL) ^d	Corrected Conc. (ng/mL) ^{e,f}	Accuracy (%) ^f	Mean (%) ^f	Std Dev (%) ^f	CV (%) ^f
Set 1	Control R1	Day 1	N/A	6.91		N/A	N/A	N/A	N/A	N/A
Set 1	Control R2	Day 1	N/A	7.11	7.04	N/A	N/A	N/A	N/A	N/A
Set 1	Control R3	Day 1	N/A	7.11		N/A	N/A	N/A	N/A	N/A
Set 1	LOD Rep 1	Day 1	7.81	14.44	14.85	7.59	97.21	102.80	4.16	4.05
Set 1	LOD Rep 2	Day 1	7.81	14.86	14.85	7.82	100.03			
Set 1	LOD Rep 3	Day 1	7.81	15.36	14.85	8.08	103.41			
Set 1	LOD Rep 4	Day 1	7.81	15.80	14.85	8.31	106.34			
Set 1	LOD Rep 5	Day 1	7.81	15.89	14.85	8.36	106.99			
Set 1	LOQ Rep 1	Day 1	15.63	24.40	22.67	16.82	107.64	109.45	1.70	1.56
Set 1	LOQ Rep 2	Day 1	15.63	24.40	22.67	16.82	107.63			
Set 1	LOQ Rep 3	Day 1	15.63	25.00	22.67	17.24	110.32			
Set 1	LOQ Rep 4	Day 1	15.63	25.02	22.67	17.25	110.37			
Set 1	LOQ Rep 5	Day 1	15.63	25.23	22.67	17.39	111.30			
Set 1	High Rep 1	Day 1	500.00	555.13	507.04	547.43	109.49	108.95	0.89	0.82
Set 1	High Rep 2	Day 1	500.00	553.42	507.04	545.73	109.15			
Set 1	High Rep 3	Day 1	500.00	550.10	507.04	542.46	108.49			
Set 1	High Rep 4	Day 1	500.00	557.53	507.04	549.79	109.96			
Set 1	High Rep 5	Day 1	500.00	545.99	507.04	538.41	107.68			
Set 2	Control R1	Day 2	N/A	8.82		N/A	N/A	N/A	N/A	N/A
Set 2	Control R2	Day 2	N/A	9.03	8.84	N/A	N/A	N/A	N/A	N/A
Set 2	Control R3	Day 2	N/A	8.67		N/A	N/A	N/A	N/A	N/A
Set 2	LOD Rep 1	Day 2	7.81	16.33	16.65	7.66	98.07	97.25	0.72	0.74
Set 2	LOD Rep 2	Day 2	7.81	16.12	16.65	7.56	96.79			
Set 2	LOD Rep 3	Day 2	7.81	16.13	16.65	7.57	96.88			
Set 2	LOQ Rep 1	Day 2	15.63	24.59	24.46	15.71	100.53	101.20	0.87	0.86
Set 2	LOQ Rep 2	Day 2	15.63	24.68	24.46	15.76	100.89			
Set 2	LOQ Rep 3	Day 2	15.63	25.00	24.46	15.97	102.19			
Set 2	High Rep 1	Day 2	500.00	531.11	508.84	521.89	104.38	106.01	1.79	1.68
Set 2	High Rep 2	Day 2	500.00	537.97	508.84	528.62	105.72			
Set 2	High Rep 3	Day 2	500.00	549.12	508.84	539.58	107.92			

^aThe 'Date of Analysis' indicates the date the samples were extracted.^bConcentration of peptide reference standard fortified into tissue containing endogenous Zea m 14 protein.^cTotal detected peptide level (endogenous Zea m 14 + reference standard fortification).^dTheoretical total concentration of peptide = peptide spike + average of detected peptide in "control" replicates without reference standard addition.^eCorrected Conc. (ng/mL) = Calc. Conc. × Accuracy (%)^fN/A = Not applicable.

Supporting Information S6: Recovery of Zea m 14 (LGGVSMANAAANIPSK Peptide, Confirmation Ion, m/z 496.26/700.40, +3y7)

Analytical Set ID	Sample ID	Date of Analysis ^a	Spike Conc. (ng/mL) ^{b,f}	Total Conc. (ng/mL) ^{c,f}	Calc. Conc. (ng/mL) ^d	Corrected Conc. (ng/mL) ^{e,f}	Accuracy (%) ^f	Mean (%) ^f	Std Dev (%) ^f	CV (%) ^f
Set 1	Control R1	Day 1	N/A	ND		N/A	N/A	N/A	N/A	N/A
Set 1	Control R2	Day 1	N/A	ND	N/A	N/A	N/A	N/A	N/A	N/A
Set 1	Control R3	Day 1	N/A	ND		N/A	N/A	N/A	N/A	N/A
Set 1	LOD Rep 1	Day 1	7.81	8.37	7.81	8.37	107.18	112.08	4.28	3.82
Set 1	LOD Rep 2	Day 1	7.81	8.95	7.81	8.95	114.57			
Set 1	LOD Rep 3	Day 1	7.81	8.42	7.81	8.42	107.72			
Set 1	LOD Rep 4	Day 1	7.81	9.08	7.81	9.08	116.25			
Set 1	LOD Rep 5	Day 1	7.81	8.96	7.81	8.96	114.68			
Set 1	LOQ Rep 1	Day 1	15.63	16.38	15.63	16.38	104.85	105.32	4.10	3.89
Set 1	LOQ Rep 2	Day 1	15.63	17.20	15.63	17.20	110.06			
Set 1	LOQ Rep 3	Day 1	15.63	15.96	15.63	15.96	102.13			
Set 1	LOQ Rep 4	Day 1	15.63	17.02	15.63	17.02	108.90			
Set 1	LOQ Rep 5	Day 1	15.63	15.73	15.63	15.73	100.69			
Set 1	High Rep 1	Day 1	500.00	508.16	500.00	508.16	101.63	101.30	2.64	2.60
Set 1	High Rep 2	Day 1	500.00	523.85	500.00	523.85	104.77			
Set 1	High Rep 3	Day 1	500.00	499.79	500.00	499.79	99.96			
Set 1	High Rep 4	Day 1	500.00	511.91	500.00	511.91	102.38			
Set 1	High Rep 5	Day 1	500.00	488.68	500.00	488.68	97.74			
Set 2	Control R1	Day 2	N/A	ND		N/A	N/A	N/A	N/A	N/A
Set 2	Control R2	Day 2	N/A	ND	N/A	N/A	N/A	N/A	N/A	N/A
Set 2	Control R3	Day 2	N/A	ND		N/A	N/A	N/A	N/A	N/A
Set 2	LOD Rep 1	Day 2	7.81	7.21	7.81	7.21	92.30	95.57	3.20	3.35
Set 2	LOD Rep 2	Day 2	7.81	7.71	7.81	7.71	98.69			
Set 2	LOD Rep 3	Day 2	7.81	7.48	7.81	7.48	95.72			
Set 2	LOQ Rep 1	Day 2	15.63	15.05	15.63	15.05	96.34	99.37	4.24	4.26
Set 2	LOQ Rep 2	Day 2	15.63	15.24	15.63	15.24	97.56			
Set 2	LOQ Rep 3	Day 2	15.63	16.28	15.63	16.28	104.21			
Set 2	High Rep 1	Day 2	500.00	495.85	500.00	495.85	99.17	101.61	2.39	2.35
Set 2	High Rep 2	Day 2	500.00	519.69	500.00	519.69	103.94			
Set 2	High Rep 3	Day 2	500.00	508.61	500.00	508.61	101.72			

^aThe 'Date of Analysis' indicates the date the samples were extracted.^bConcentration of peptide reference standard fortified into tissue containing endogenous Zea m 14 protein.^cTotal detected peptide level (endogenous Zea m 14 + reference standard fortification).^dTheoretical total concentration of peptide = peptide spike + average of detected peptide in "control" replicates without reference standard addition.^eCorrected Conc. (ng/mL) = Calc. Conc. × Accuracy (%)^fND = No Detection, below the limit of detection, N/A = Not applicable.

Supporting Information S7: Zea m 14 Extraction Efficiency Results

Protein	Peptide Monitored ^a	Mean Extraction Efficiency (%)	Extraction Efficiency Range (%)	Std Dev (%)	CV (%)	N ^b	Extractions ^c
Zea m 14/Isoform A	NAAAGVSGLNAGNAASIPSK	83.82	82.73-84.56	0.96	1.14	3	3
Isoform B	GVSGLNAGNAASIPSK	85.66	85.34-86.14	0.42	0.49	3	3
Isoform C	LGGGVSMANAANIPSK	ND ^d	N/A ^e	N/A ^e	N/A ^e	3	3

^aResults represent quantitation ions for each peptide.^bNumber of 100 mg corn seed replicate samples.^cNumber of serial extractions performed on each replicate sample.^dND = Not Detected; Below the limit of detection.^eN/A = Not Applicable.

Supporting Information S8: Matrix Effects on Zea m 14 Peptides

Sample ID	NAAAGVSGLNAGNAASIPSK Heavy Internal Standard Peak Area	GVSGLNAGNAASIPSK Heavy Internal Standard Peak Area	LGGGVSMANAANIPSK Heavy Internal Standard Peak Area
Buffer + HIS Rep 1	1734380	1939560	304466
Buffer + HIS Rep 2	1833460	2153530	348268
Buffer + HIS Rep 3	1723620	2113910	333257
Buffer + HIS Rep 4	1744190	2251520	322558
Buffer + HIS Rep 5	1755080	2259980	341075
Mean Peak Area	1758146	2143700	329925
Matrix + HIS Rep 1	1739860	2372320	348866
Matrix + HIS Rep 2	1794340	2370610	364777
Matrix + HIS Rep 3	1777970	2415490	344962
Matrix + HIS Rep 4	1812360	2448240	362566
Matrix + HIS Rep 5	1793750	2414170	369893
Mean Peak Area	1783656	2404166	358213
Matrix Effect (%)	1.45	12.15	8.57

Supporting Information S9: Total Zea m 14 Protein (ng/mg) in Non-Transgenic Control (GE Hybrid Maize Stack I Study)

DAS Sample Number	Site ID	Description	Total Zea m 14 Protein (ng/mg) ^a	Mean (ng/mg)	Std Dev (ng/mg)
Replicate 1	Site 1	Control	298.72	317.19	38.97
Replicate 2	Site 1	Control	354.82		
Replicate 3	Site 1	Control	271.45		
Replicate 4	Site 1	Control	343.77		
Replicate 1	Site 2	Control	357.47	356.08	56.89
Replicate 2	Site 2	Control	430.09		
Replicate 3	Site 2	Control	292.06		
Replicate 4	Site 2	Control	344.70		
Replicate 1	Site 3	Control	327.92	364.51	38.65
Replicate 2	Site 3	Control	350.89		
Replicate 3	Site 3	Control	360.48		
Replicate 4	Site 3	Control	418.75		
Replicate 1	Site 4	Control	460.28	486.71	48.76
Replicate 2	Site 4	Control	517.42		
Replicate 3	Site 4	Control	536.91		
Replicate 4	Site 4	Control	432.21		
Replicate 1	Site 5	Control	333.23	358.14	40.53
Replicate 2	Site 5	Control	364.22		
Replicate 3	Site 5	Control	322.30		
Replicate 4	Site 5	Control	412.80		
Replicate 1	Site 6	Control	321.49	361.09	60.67
Replicate 2	Site 6	Control	314.38		
Replicate 3	Site 6	Control	446.49		
Replicate 4	Site 6	Control	362.00		
Replicate 1	Site 7	Control	338.55	375.49	41.60
Replicate 2	Site 7	Control	348.83		
Replicate 3	Site 7	Control	383.91		
Replicate 4	Site 7	Control	430.68		
Replicate 1	Site 8	Control	288.54	328.68	31.71
Replicate 2	Site 8	Control	322.65		
Replicate 3	Site 8	Control	339.46		
Replicate 4	Site 8	Control	364.09		

^aSum of Zeam 14 and 3 possible isomers.

Overall Mean (ng/mg): 368.49
 Overall Min (ng/mg): 271.45
 Overall Max (ng/mg): 536.91
 Overall Std Dev (ng/mg): 63.33

Supporting Information S10: Total Zea m 14 Protein (ng/mg) in MON-89Ø34-3 × DAS-Ø15Ø7-1 × SYN-IR162-4 × MON-ØØ6Ø3-6 (GE Hybrid Maize Stack I Study)

DAS Sample Number	Site ID	Description	Total Zea m 14 (ng/mg) ^a	Mean (ng/mg)	Std Dev (ng/mg)
Replicate 1	Site 1	Stack I	459.90	369.91	62.36
Replicate 2	Site 1	Stack I	327.54		
Replicate 3	Site 1	Stack I	363.98		
Replicate 4	Site 1	Stack I	328.20		
Replicate 1	Site 2	Stack I	384.56	448.51	56.56
Replicate 2	Site 2	Stack I	417.29		
Replicate 3	Site 2	Stack I	495.13		
Replicate 4	Site 2	Stack I	497.08		
Replicate 1	Site 3	Stack I	428.72	439.28	27.34
Replicate 2	Site 3	Stack I	405.71		
Replicate 3	Site 3	Stack I	457.43		
Replicate 4	Site 3	Stack I	465.26		
Replicate 1	Site 4	Stack I	352.20	428.51	87.77
Replicate 2	Site 4	Stack I	381.29		
Replicate 3	Site 4	Stack I	429.34		
Replicate 4	Site 4	Stack I	551.22		
Replicate 1	Site 5	Stack I	484.28	444.50	28.41
Replicate 2	Site 5	Stack I	428.18		
Replicate 3	Site 5	Stack I	420.58		
Replicate 4	Site 5	Stack I	444.97		
Replicate 1	Site 6	Stack I	406.72	442.80	43.65
Replicate 2	Site 6	Stack I	436.03		
Replicate 3	Site 6	Stack I	422.71		
Replicate 4	Site 6	Stack I	505.76		
Replicate 1	Site 7	Stack I	336.26	397.42	51.64
Replicate 2	Site 7	Stack I	411.92		
Replicate 3	Site 7	Stack I	382.27		
Replicate 4	Site 7	Stack I	459.23		
Replicate 1	Site 8	Stack I	361.72	382.93	22.40
Replicate 2	Site 8	Stack I	392.89		
Replicate 3	Site 8	Stack I	409.68		
Replicate 4	Site 8	Stack I	367.42		
^a Sum of Zeam 14 and 3 possible isomers			Overall Mean (ng/mg):	419.23	
			Overall Min (ng/mg):	327.54	
			Overall Max (ng/mg):	551.22	
			Overall Std Dev (ng/mg):	54.28	

Supporting Information S11: Total Zea m 14 Protein (ng/mg) in 31D06 Maize Reference Line (GE Hybrid Maize Stack I Study)

DAS Sample Number	Site ID	Description	Total Zea m 14 (ng/mg) ^a	Mean (ng/mg)	Std Dev (ng/mg)
Replicate 1	Site 1	31D06	464.69	445.26	34.81
Replicate 2	Site 1	31D06	396.84		
Replicate 3	Site 1	31D06	475.45		
Replicate 4	Site 1	31D06	444.06		
Replicate 1	Site 2	31D06	365.20	377.75	23.96
Replicate 2	Site 2	31D06	395.19		
Replicate 3	Site 2	31D06	400.30		
Replicate 4	Site 2	31D06	350.32		
Replicate 1	Site 3	31D06	309.89	306.13	18.56
Replicate 2	Site 3	31D06	329.92		
Replicate 3	Site 3	31D06	286.31		
Replicate 4	Site 3	31D06	298.38		
Replicate 1	Site 5	31D06	310.12	381.98	49.47
Replicate 2	Site 5	31D06	417.31		
Replicate 3	Site 5	31D06	411.67		
Replicate 4	Site 5	31D06	388.84		
Replicate 1	Site 7	31D06	249.99	311.99	46.03
Replicate 2	Site 7	31D06	360.52		
Replicate 3	Site 7	31D06	312.93		
Replicate 4	Site 7	31D06	324.51		
^a Sum of Zeam 14 and 3 possible isomers			Overall Mean (ng/mg):	364.62	
			Overall Min (ng/mg):	249.99	
			Overall Max (ng/mg):	475.45	
			Overall Std Dev (ng/mg):	61.88	

Supporting Information S12: Total Zea m 14 Protein (ng/mg) in IMPERIO Maize Reference Line (GE Hybrid Maize Stack I Study)

DAS Sample Number	Site ID	Description	Total Zea m 14 (ng/mg) ^a	Mean (ng/mg)	Std Dev (ng/mg)
Replicate 1	Site 3	IMPERIO	247.98	321.48	50.07
Replicate 2	Site 3	IMPERIO	343.42		
Replicate 3	Site 3	IMPERIO	359.65		
Replicate 4	Site 3	IMPERIO	334.87		
Replicate 1	Site 4	IMPERIO	261.08	326.07	47.82
Replicate 2	Site 4	IMPERIO	358.34		
Replicate 3	Site 4	IMPERIO	365.44		
Replicate 4	Site 4	IMPERIO	319.41		
Replicate 1	Site 6	IMPERIO	290.95	333.20	42.62
Replicate 2	Site 6	IMPERIO	373.92		
Replicate 3	Site 6	IMPERIO	365.62		
Replicate 4	Site 6	IMPERIO	302.30		
Replicate 1	Site 7	IMPERIO	264.18	374.75	85.54
Replicate 2	Site 7	IMPERIO	350.74		
Replicate 3	Site 7	IMPERIO	435.30		
Replicate 4	Site 7	IMPERIO	448.80		
Replicate 1	Site 8	IMPERIO	309.11	299.31	22.05
Replicate 2	Site 8	IMPERIO	325.81		
Replicate 3	Site 8	IMPERIO	280.11		
Replicate 4	Site 8	IMPERIO	282.23		
^a Sum of Zeam 14 and 3 possible isomers			Overall Mean (ng/mg):	330.96	
			Overall Min (ng/mg):	247.98	
			Overall Max (ng/mg):	448.80	
			Overall Std Dev (ng/mg):	53.99	

Supporting Information S13: Total Zea m 14 Protein (ng/mg) in KM4321 Maize Reference Line (GE Hybrid Maize Stack I Study)

DAS Sample Number	Site ID	Description	Total Zea m 14 (ng/mg) ^a	Mean (ng/mg)	Std Dev (ng/mg)
Replicate 1	Site 1	KM4321	300.33	275.70	24.66
Replicate 2	Site 1	KM4321	273.70		
Replicate 3	Site 1	KM4321	286.28		
Replicate 4	Site 1	KM4321	242.50		
Replicate 1	Site 5	KM4321	429.79	461.93	68.16
Replicate 2	Site 5	KM4321	540.67		
Replicate 3	Site 5	KM4321	385.63		
Replicate 4	Site 5	KM4321	491.63		
Replicate 1	Site 6	KM4321	537.65	550.03	17.02
Replicate 2	Site 6	KM4321	540.79		
Replicate 3	Site 6	KM4321	574.93		
Replicate 4	Site 6	KM4321	546.75		
Replicate 1	Site 8	KM4321	449.45	381.99	48.35
Replicate 2	Site 8	KM4321	341.13		
Replicate 3	Site 8	KM4321	383.50		
Replicate 4	Site 8	KM4321	353.89		
^a Sum of Zeam 14 and 3 possible isomers			Overall Mean (ng/mg):	417.41	
			Overall Min (ng/mg):	242.50	
			Overall Max (ng/mg):	574.93	
			Overall Std Dev (ng/mg):	111.73	

Supporting Information S14: Total Zea m 14 Protein (ng/mg) in MILL527 Maize Reference Line (GE Hybrid Maize Stack I Study)

DAS Sample Number	Site ID	Description	Total Zea m 14 (ng/mg) ^a	Mean (ng/mg)	Std Dev (ng/mg)
Replicate 1	Site 2	MILL527	332.70	344.74	62.20
Replicate 2	Site 2	MILL527	334.85		
Replicate 3	Site 2	MILL527	281.13		
Replicate 4	Site 2	MILL527	430.28		
Replicate 1	Site 3	MILL527	280.96	318.10	50.17
Replicate 2	Site 3	MILL527	278.47		
Replicate 3	Site 3	MILL527	327.74		
Replicate 4	Site 3	MILL527	385.24		
Replicate 1	Site 6	MILL527	399.95	390.71	19.15
Replicate 2	Site 6	MILL527	390.92		
Replicate 3	Site 6	MILL527	408.01		
Replicate 4	Site 6	MILL527	363.96		
^a Sum of Zeam 14 and 3 possible isomers			Overall Mean (ng/mg):		351.18
			Overall Min (ng/mg):		278.47
			Overall Max (ng/mg):		430.28
			Overall Std Dev (ng/mg):		53.13

Supporting Information S15: Total Zea m 14 Protein (ng/mg) in NT 525 Maize Reference Line (GE Hybrid Maize Stack I Study)

DAS Sample Number	Site ID	Description	Total Zea m 14 (ng/mg) ^a	Mean (ng/mg)	Std Dev (ng/mg)
Replicate 1	Site 2	NT 525	233.58	194.29	38.15
Replicate 2	Site 2	NT 525	162.24		
Replicate 3	Site 2	NT 525	220.41		
Replicate 4	Site 2	NT 525	160.93		
Replicate 1	Site 4	NT 525	232.74	248.48	31.26
Replicate 2	Site 4	NT 525	228.27		
Replicate 3	Site 4	NT 525	237.92		
Replicate 4	Site 4	NT 525	295.00		
Replicate 1	Site 5	NT 525	167.73	186.17	17.10
Replicate 2	Site 5	NT 525	208.98		
Replicate 3	Site 5	NT 525	185.84		
Replicate 4	Site 5	NT 525	182.14		
Replicate 1	Site 8	NT 525	175.71	162.68	14.46
Replicate 2	Site 8	NT 525	174.15		
Replicate 3	Site 8	NT 525	153.97		
Replicate 4	Site 8	NT 525	146.87		
^a Sum of Zeam 14 and 3 possible isomers			Overall Mean (ng/mg):	197.90	
			Overall Min (ng/mg):	146.87	
			Overall Max (ng/mg):	295.00	
			Overall Std Dev (ng/mg):	40.50	

Supporting Information S16: Total Zea m 14 Protein (ng/mg) in PAN 5E-203 Maize Reference Line (GE Hybrid Maize Stack I Study)

DAS Sample Number	Site ID	Description	Total Zea m 14 (ng/mg) ^a	Mean (ng/mg)	Std Dev (ng/mg)
Replicate 1	Site 1	PAN 5E-203	270.65	316.21	44.93
Replicate 2	Site 1	PAN 5E-203	338.64		
Replicate 3	Site 1	PAN 5E-203	287.72		
Replicate 4	Site 1	PAN 5E-203	367.83		
Replicate 1	Site 4	PAN 5E-203	493.79	403.63	66.93
Replicate 2	Site 4	PAN 5E-203	345.32		
Replicate 3	Site 4	PAN 5E-203	414.20		
Replicate 4	Site 4	PAN 5E-203	361.21		
Replicate 1	Site 7	PAN 5E-203	424.80	412.48	38.59
Replicate 2	Site 7	PAN 5E-203	457.99		
Replicate 3	Site 7	PAN 5E-203	400.47		
Replicate 4	Site 7	PAN 5E-203	366.65		
^a Sum of Zeam 14 and 3 possible isomers			Overall Mean (ng/mg):	377.44	
			Overall Min (ng/mg):	270.65	
			Overall Max (ng/mg):	493.79	
			Overall Std Dev (ng/mg):	65.10	

Supporting Information S17: Total Zea m 14 Protein (ng/mg) in Non-Transgenic Control (GE Hybrid Maize Stack II Study)

DAS Sample Number	Site ID	Description	Total Zea m 14 (ng/mg) ^a	Mean Zea m 14 (ng/mg) ^a	Std Dev (ng/mg) ^a
Replicate 1	Site 1	Control	159.57	189.33	31.24
Replicate 2	Site 1	Control	177.93		
Replicate 3	Site 1	Control	186.83		
Replicate 4	Site 1	Control	232.99		
Replicate 1	Site 2	Control	261.94	236.74	17.10
Replicate 2	Site 2	Control	231.68		
Replicate 3	Site 2	Control	229.28		
Replicate 4	Site 2	Control	224.05		
Replicate 1	Site 3	Control	216.98	230.83	10.61
Replicate 2	Site 3	Control	230.55		
Replicate 3	Site 3	Control	242.70		
Replicate 4	Site 3	Control	233.09		
Replicate 1	Site 4	Control	N/A ^b	209.91	11.02
Replicate 2	Site 4	Control	222.45		
Replicate 3	Site 4	Control	201.75		
Replicate 4	Site 4	Control	205.54		
Replicate 1	Site 5	Control	N/A ^b	211.30	15.23
Replicate 2	Site 5	Control	224.21		
Replicate 3	Site 5	Control	215.20		
Replicate 4	Site 5	Control	194.50		
Replicate 1	Site 6	Control	303.94	254.21	34.36
Replicate 2	Site 6	Control	244.01		
Replicate 3	Site 6	Control	244.04		
Replicate 4	Site 6	Control	224.87		
Replicate 1	Site 7	Control	277.75	254.81	23.29
Replicate 2	Site 7	Control	241.41		
Replicate 3	Site 7	Control	229.12		
Replicate 4	Site 7	Control	270.98		
Replicate 1	Site 8	Control	302.02	287.46	18.56
Replicate 2	Site 8	Control	263.92		
Replicate 3	Site 8	Control	302.61		
Replicate 4	Site 8	Control	281.29		
^a Sum of Zeam 14 and 3 possible isomers.			Overall Mean (ng/mg):	235.91	
^b N/A = Not Applicable, low sample volume.			Overall Min (ng/mg):	159.57	
			Overall Max (ng/mg):	303.94	
			Overall Std Dev (ng/mg):	35.76	

Supporting Information S18: Total Zea m 14 Protein (ng/mg) in MON 87427 × MON 89034 × TC1507 × MON 87411 × DAS-59122-7 × DAS-40278-9 (GE Hybrid Maize Stack II Study)

DAS Sample Number	Site ID	Description	Total Zea m 14 (ng/mg) ^a	Mean Zea m 14 (ng/mg) ^a	Std Dev (ng/mg) ^a
Replicate 1	Site 1	Stack II	201.50	209.94	19.84
Replicate 2	Site 1	Stack II	188.68		
Replicate 3	Site 1	Stack II	235.20		
Replicate 4	Site 1	Stack II	214.39		
Replicate 1	Site 2	Stack II	222.93	240.98	22.90
Replicate 2	Site 2	Stack II	220.67		
Replicate 3	Site 2	Stack II	267.17		
Replicate 4	Site 2	Stack II	253.16		
Replicate 1	Site 3	Stack II	329.68	299.01	32.99
Replicate 2	Site 3	Stack II	269.78		
Replicate 3	Site 3	Stack II	325.39		
Replicate 4	Site 3	Stack II	271.20		
Replicate 1	Site 4	Stack II	314.96	308.31	13.87
Replicate 2	Site 4	Stack II	315.72		
Replicate 3	Site 4	Stack II	315.04		
Replicate 4	Site 4	Stack II	287.50		
Replicate 1	Site 5	Stack II	204.14	199.62	13.10
Replicate 2	Site 5	Stack II	208.84		
Replicate 3	Site 5	Stack II	205.31		
Replicate 4	Site 5	Stack II	180.21		
Replicate 1	Site 6	Stack II	241.36	205.05	35.82
Replicate 2	Site 6	Stack II	223.23		
Replicate 3	Site 6	Stack II	158.89		
Replicate 4	Site 6	Stack II	196.73		
Replicate 1	Site 7	Stack II	256.51	243.41	10.74
Replicate 2	Site 7	Stack II	236.36		
Replicate 3	Site 7	Stack II	233.10		
Replicate 4	Site 7	Stack II	247.68		
Replicate 1	Site 8	Stack II	301.14	321.75	15.40
Replicate 2	Site 8	Stack II	324.43		
Replicate 3	Site 8	Stack II	338.41		
Replicate 4	Site 8	Stack II	323.04		
^a Sum of Zeam 14 and 3 possible isomers.			Overall Mean (ng/mg):	253.51	
			Overall Min (ng/mg):	158.89	
			Overall Max (ng/mg):	338.41	
			Overall Std Dev (ng/mg):	50.98	

Supporting Information S19: Total Zea m 14 Protein (ng/mg) in AgVenture 7844 Maize Reference Line (GE Hybrid Maize Stack II Study)

DAS Sample Number	Site ID	Description	Total Zea m 14 (ng/mg) ^a	Mean Zea m 14 (ng/mg) ^a	Std Dev (ng/mg) ^a
Replicate 1	Site 2	AgVenture 7844	262.33	271.89	35.63
Replicate 2	Site 2	AgVenture 7844	249.77		
Replicate 3	Site 2	AgVenture 7844	250.8		
Replicate 4	Site 2	AgVenture 7844	324.65		
Replicate 1	Site 3	AgVenture 7844	308.23	272.28	29.69
Replicate 2	Site 3	AgVenture 7844	238.73		
Replicate 3	Site 3	AgVenture 7844	260.49		
Replicate 4	Site 3	AgVenture 7844	281.66		
Replicate 1	Site 4	AgVenture 7844	191.14	217.26	17.53
Replicate 2	Site 4	AgVenture 7844	228.79		
Replicate 3	Site 4	AgVenture 7844	223.93		
Replicate 4	Site 4	AgVenture 7844	225.18		
^a Sum of Zeam 14 and 3 possible isomers.			Overall Mean (ng/mg):		253.81
			Overall Min (ng/mg):		191.14
			Overall Max (ng/mg):		324.65
			Overall Std Dev (ng/mg):		37.41

Supporting Information S20: Total Zea m 14 Protein (ng/mg) in Beck's 5509 Maize Reference Line (GE Hybrid Maize Stack II Study)

DAS Sample Number	Site ID	Description	Total Zea m 14 (ng/mg) ^a	Mean Zea m 14 (ng/mg) ^a	Std Dev (ng/mg) ^a
Replicate 1	Site 1	Beck's 5509	198.85	197.45	9.04
Replicate 2	Site 1	Beck's 5509	207.68		
Replicate 3	Site 1	Beck's 5509	197.58		
Replicate 4	Site 1	Beck's 5509	185.69		
Replicate 1	Site 2	Beck's 5509	230.15	223.21	7.96
Replicate 2	Site 2	Beck's 5509	213.26		
Replicate 3	Site 2	Beck's 5509	220.31		
Replicate 4	Site 2	Beck's 5509	229.11		
Replicate 1	Site 7	Beck's 5509	199.95	199.23	13.56
Replicate 2	Site 7	Beck's 5509	190.80		
Replicate 3	Site 7	Beck's 5509	188.07		
Replicate 4	Site 7	Beck's 5509	218.09		
^a Sum of Zeam 14 and 3 possible isomers.			Overall Mean (ng/mg):		206.63
			Overall Min (ng/mg):		185.69
			Overall Max (ng/mg):		230.15
			Overall Std Dev (ng/mg):		15.5

Supporting Information S21: Total Zea m 14 Protein (ng/mg) in Beck's 5828 Maize Reference Line (GE Hybrid Maize Stack II Study)

DAS Sample Number	Site ID	Description	Total Zea m 14 (ng/mg) ^a	Mean Zea m 14 (ng/mg) ^a	Std Dev (ng/mg) ^a
Replicate 1	Site 7	Beck's 5828	311.05	299.88	13.43
Replicate 2	Site 7	Beck's 5828	309.23		
Replicate 3	Site 7	Beck's 5828	297.29		
Replicate 4	Site 7	Beck's 5828	281.94		
^a Sum of Zeam 14 and 3 possible isomers.			Overall Mean (ng/mg):	299.88	
			Overall Min (ng/mg):	281.94	
			Overall Max (ng/mg):	311.05	
			Overall Std Dev (ng/mg):	13.43	

Supporting Information S22: Total Zea m 14 Protein (ng/mg) in Beck's 6158 Maize Reference Line (GE Hybrid Maize Stack II Study)

DAS Sample Number	Site ID	Description	Total Zea m 14 (ng/mg) ^a	Mean Zea m 14 (ng/mg) ^a	Std Dev (ng/mg) ^a
Replicate 1	Site 1	Beck's 6158	235.42	239.54	9.35
Replicate 2	Site 1	Beck's 6158	243.48		
Replicate 3	Site 1	Beck's 6158	250.34		
Replicate 4	Site 1	Beck's 6158	228.91		
Replicate 1	Site 3	Beck's 6158	347.41	339.79	22.50
Replicate 2	Site 3	Beck's 6158	362.46		
Replicate 3	Site 3	Beck's 6158	340.26		
Replicate 4	Site 3	Beck's 6158	309.03		
Replicate 1	Site 6	Beck's 6158	309.03	277.48	31.79
Replicate 2	Site 6	Beck's 6158	238.52		
Replicate 3	Site 6	Beck's 6158	265.51		
Replicate 4	Site 6	Beck's 6158	296.85		
^a Sum of Zeam 14 and 3 possible isomers.			Overall Mean (ng/mg):	285.60	
			Overall Min (ng/mg):	228.91	
			Overall Max (ng/mg):	362.46	
			Overall Std Dev (ng/mg):	47.97	

Supporting Information S23: Total Zea m 14 Protein (ng/mg) in Beck's 6175 Maize Reference Line (GE Hybrid Maize Stack II Study)

DAS Sample Number	Site ID	Description	Total Zea m 14 (ng/mg) ^a	Mean Zea m 14 (ng/mg) ^a	Std Dev (ng/mg) ^a
Replicate 1	Site 6	Beck's 6175	223.36	247.02	17.08
Replicate 2	Site 6	Beck's 6175	263.61		
Replicate 3	Site 6	Beck's 6175	247.84		
Replicate 4	Site 6	Beck's 6175	253.28		
Replicate 1	Site 8	Beck's 6175	355.39	359.88	18.93
Replicate 2	Site 8	Beck's 6175	365.78		
Replicate 3	Site 8	Beck's 6175	381.75		
Replicate 4	Site 8	Beck's 6175	336.60		
^a Sum of Zeam 14 and 3 possible isomers.			Overall Mean (ng/mg):		303.45
			Overall Min (ng/mg):		223.36
			Overall Max (ng/mg):		381.75
			Overall Std Dev (ng/mg):		62.59

Supporting Information 24: Total Zea m 14 Protein (ng/mg) in Beck's 6272 Maize Reference Line (GE Hybrid Maize Stack II Study)

DAS Sample Number	Site ID	Description	Total Zea m 14 (ng/mg) ^a	Mean Zea m 14 (ng/mg) ^a	Std Dev (ng/mg) ^a
Replicate 1	Site 7	Beck's 6272	358.38	317.82	31.78
Replicate 2	Site 7	Beck's 6272	280.70		
Replicate 3	Site 7	Beck's 6272	315.81		
Replicate 4	Site 7	Beck's 6272	316.39		
^a Sum of Zeam 14 and 3 possible isomers.			Overall Mean (ng/mg):		317.82
			Overall Min (ng/mg):		280.70
			Overall Max (ng/mg):		358.38
			Overall Std Dev (ng/mg):		31.78

Supporting Information S25: Total Zea m 14 Protein (ng/mg) in Dairyland DS-1811 Maize Reference Line (GE Hybrid Maize Stack II Study)

DAS Sample Number	Site ID	Description	Total Zea m 14 (ng/mg) ^a	Mean Zea m 14 (ng/mg) ^a	Std Dev (ng/mg) ^a
Replicate 1	Site 8	Dairyland DS-1811	247.22	239.23	11.70
Replicate 2	Site 8	Dairyland DS-1811	243.57		
Replicate 3	Site 8	Dairyland DS-1811	244.29		
Replicate 4	Site 8	Dairyland DS-1811	221.84		
^a Sum of Zeam 14 and 3 possible isomers.			Overall Mean (ng/mg):		239.23
			Overall Min (ng/mg):		221.84
			Overall Max (ng/mg):		247.22
			Overall Std Dev (ng/mg):		11.70

Supporting Information S26: Total Zea m 14 Protein (ng/mg) in Master's Choice MC6060 Maize Reference Line (GE Hybrid Maize Stack II Study)

DAS Sample Number	Site ID	Description	Total Zea m 14 (ng/mg) ^a	Mean Zea m 14 (ng/mg) ^a	Std Dev (ng/mg) ^a
Replicate 1	Site 2	Master's Choice MC6060	324.30	380.19	67.97
Replicate 2	Site 2	Master's Choice MC6060	341.51		
Replicate 3	Site 2	Master's Choice MC6060	378.64		
Replicate 4	Site 2	Master's Choice MC6060	476.31		
Replicate 1	Site 8	Master's Choice MC6060	457.11	463.13	7.86
Replicate 2	Site 8	Master's Choice MC6060	471.11		
Replicate 3	Site 8	Master's Choice MC6060	455.68		
Replicate 4	Site 8	Master's Choice MC6060	468.60		
^a Sum of Zeam 14 and 3 possible isomers.			Overall Mean (ng/mg):	421.66	
			Overall Min (ng/mg):	324.30	
			Overall Max (ng/mg):	476.31	
			Overall Std Dev (ng/mg):	63.02	

Supporting Information S27: Total Zea m 14 Protein (ng/mg) in Master's Choice MC6150 Maize Reference Line (GE Hybrid Maize Stack II Study)

DAS Sample Number	Site ID	Description	Total Zea m 14 (ng/mg) ^a	Mean Zea m 14 (ng/mg) ^a	Std Dev (ng/mg) ^a
Replicate 1	Site 4	Master's Choice MC6150	155.27	160.90	8.56
Replicate 2	Site 4	Master's Choice MC6150	158.42		
Replicate 3	Site 4	Master's Choice MC6150	173.58		
Replicate 4	Site 4	Master's Choice MC6150	156.32		
^a Sum of Zeam 14 and 3 possible isomers.			Overall Mean (ng/mg):	160.90	
			Overall Min (ng/mg):	155.27	
			Overall Max (ng/mg):	173.58	
			Overall Std Dev (ng/mg):	8.56	

Supporting Information S28: Total Zea m 14 Protein (ng/mg) in Mycogen 2H721 Maize Reference Line (GE Hybrid Maize Stack II Study)

DAS Sample Number	Site ID	Description	Total Zea m 14 (ng/mg) ^a	Mean Zea m 14 (ng/mg) ^a	Std Dev (ng/mg) ^a
Replicate 1	Site 4	Mycogen 2H721	326.06	368.93	33.31
Replicate 2	Site 4	Mycogen 2H721	359.08		
Replicate 3	Site 4	Mycogen 2H721	396.76		
Replicate 4	Site 4	Mycogen 2H721	393.80		
Replicate 1	Site 5	Mycogen 2H721	364.37	282.49	58.92
Replicate 2	Site 5	Mycogen 2H721	235.01		
Replicate 3	Site 5	Mycogen 2H721	286.08		
Replicate 4	Site 5	Mycogen 2H721	244.52		
^a Sum of Zeam 14 and 3 possible isomers.			Overall Mean (ng/mg):	325.71	
			Overall Min (ng/mg):	234.01	
			Overall Max (ng/mg):	396.76	
			Overall Std Dev (ng/mg):	64.01	

Supporting Information S29: Total Zea m 14 Protein (ng/mg) in Pfister 2874 Maize Reference Line (GE Hybrid Maize Stack II Study)

DAS Sample Number	Site ID	Description	Total Zea m 14 (ng/mg) ^a	Mean Zea m 14 (ng/mg) ^a	Std Dev (ng/mg) ^a
Replicate 1	Site 1	Pfister 2874	266.11	280.92	12.62
Replicate 2	Site 1	Pfister 2874	275.90		
Replicate 3	Site 1	Pfister 2874	286.52		
Replicate 4	Site 1	Pfister 2874	295.14		
Replicate 1	Site 5	Pfister 2874	305.04	281.31	20.16
Replicate 2	Site 5	Pfister 2874	255.74		
Replicate 3	Site 5	Pfister 2874	281.57		
Replicate 4	Site 5	Pfister 2874	282.88		
Replicate 1	Site 6	Pfister 2874	310.60	274.55	34.94
Replicate 2	Site 6	Pfister 2874	298.27		
Replicate 3	Site 6	Pfister 2874	241.92		
Replicate 4	Site 6	Pfister 2874	247.41		
^a Sum of Zeam 14 and 3 possible isomers.			Overall Mean (ng/mg):	278.92	
			Overall Min (ng/mg):	241.92	
			Overall Max (ng/mg):	310.60	
			Overall Std Dev (ng/mg):	22.31	

Supporting Information S30: Total Zea m 14 Protein (ng/mg) in Viking 52-11N Maize Reference Line (GE Hybrid Maize Stack II Study)

DAS Sample Number	Site ID	Description	Total Zea m 14 (ng/mg) ^a	Mean Zea m 14 (ng/mg) ^a	Std Dev (ng/mg) ^a
Replicate 1	Site 3	Viking 52-11N	181.44	189.82	30.41
Replicate 2	Site 3	Viking 52-11N	233.58		
Replicate 3	Site 3	Viking 52-11N	163.10		
Replicate 4	Site 3	Viking 52-11N	181.17		
Replicate 1	Site 5	Viking 52-11N	168.95	174.23	4.42
Replicate 2	Site 5	Viking 52-11N	175.22		
Replicate 3	Site 5	Viking 52-11N	173.18		
Replicate 4	Site 5	Viking 52-11N	179.58		
^a Sum of Zeam 14 and 3 possible isomers.			Overall Mean (ng/mg):	182.03	
			Overall Min (ng/mg):	163.10	
			Overall Max (ng/mg):	233.58	
			Overall Std Dev (ng/mg):	21.77	