

Model-Based Tolerance Intervals Derived from Cumulative Historical Composition Data: Application for Substantial Equivalence Assessment of a Genetically Modified Crop

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Supporting Information

ABSTRACT: Compositional analysis is a requisite component of the substantial equivalence framework utilized to assess genetically modified (GM) crop safety. Statistical differences in composition data between GM and non-GM crops require a context in which to determine biological relevance. This context is provided by surveying the natural variation of key nutrient and antinutrient levels within the crop population with a history of safe use. Data accumulated from various genotypes with a history of safe use cultivated in relevant commercial crop-growing environments over multiple seasons are discussed as the appropriate data representative of this natural variation. A model-based parametric tolerance interval approach, which accounts for the correlated and unbalanced data structure of cumulative historical data collected from multisite field studies conducted over multiple seasons, is presented. This paper promotes the application of this tolerance interval approach to generate reference ranges for evaluation of the biological relevance of statistical differences identified during substantial equivalence assessment of a GM crop.

KEYWORDS: *composition, genetically modified (GM), linear mixed model, safety assessment, statistical analysis, substantial equivalence, tolerance interval, unbalanced data*

INTRODUCTION

Evaluation of compositional equivalence is a fundamental aspect of genetically modified (GM) crop safety assessments required for registration and commercialization of a candidate GM crop.^{1,2} Substantial equivalence has been adopted globally as the framework for this evaluation and embodies a comparative approach focusing on the determination of compositional similarities and potential differences between the GM crop and its conventional counterpart.³ Generally, the GM crop is grown per regulatory guidelines that require a multisite field experiment with a near-isogenic (parental) comparator line to which levels of key nutrients, antinutrients, and toxicants are statistically compared.^{4,5} This approach has been promoted by numerous international regulatory bodies.^{6–8} Statistically significant differences from these pairwise comparisons may occur and could be misinterpreted to indicate an unintended effect of the genetic modification. These differences are understood in a broader biological context when compared to the natural variation observed within the population of conventional crop varieties with an established history of safe use.⁸ The ultimate conclusion derived from a safety assessment is whether the GM crop is as safe as the conventional crop with a history of safe use grown under a range of environmental conditions and management practices, rather than the identification of potential differences between

the GM crop and a near-isogenic comparator line grown under a limited number of identical conditions. Therefore, although it is important to conduct field experiments for GM crops, it is as important to compile composition data to represent natural variation. A consensus is lacking on how to represent this natural variation.

The impact of genetic background, environment, management practices, and other factors on natural variation of crop composition has been demonstrated previously.^{9–13} Consequently, it is essential to take these established critical factors into consideration when data that represent natural variation are compiled. The intent is to capture and estimate the total variation contributed by various factors, not to characterize or quantify variances due to individual factors. Samples collected from various genotypes with a history of safe use grown in relevant commercial crop-growing environments over multiple seasons are necessary to obtain adequate representation of natural variation of the crop population.¹⁴ The data acquired from compositional analyses of such a sample set, referred to here as cumulative historical data, provide the basis of a

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comprehensive approach to evaluating the biological context of statistically significant differences.

A statistical tolerance interval is derived from a sample distribution to estimate the population range (Figure 1). The

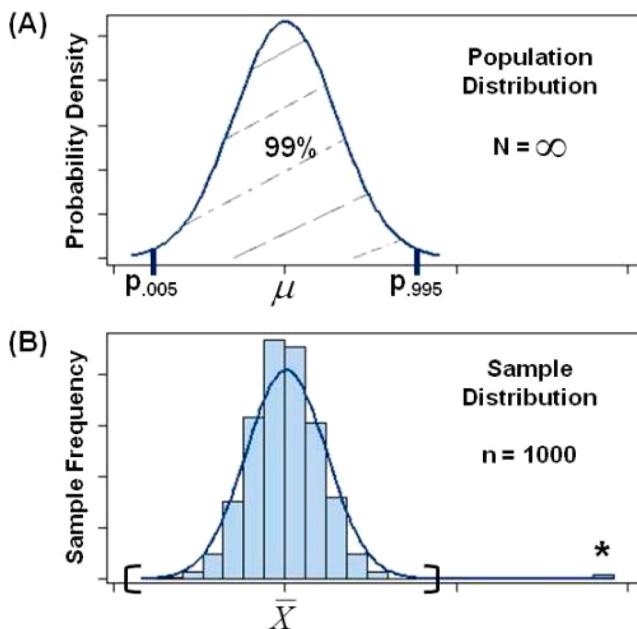


Figure 1. Conceptual illustration of a 99%-content, 95%-confidence tolerance interval. (A) Theoretical population distribution where $p_{.005}$ and $p_{.995}$ represent the 0.5th and 99.5th percentile points, respectively. Between $p_{.005}$ and $p_{.995}$ lie 99% of the population values. The population distribution is based on an infinite number of population values (i.e., $N = \infty$), where μ represents the center of the distribution or the population mean. (B) Sample distribution is based on a finite number of sample values (e.g., $n = 1000$), where \bar{X} represents the center of the distribution or the sample mean. The 99%-content, 95%-confidence tolerance interval, denoted by the brackets on the x -axis, is generated on the basis of the sample distribution to infer the range between the 0.5th and 99.5th percentile points of the population distribution. In practice, there can be extreme sample values, also known as outliers. The asterisk (*) denotes an example of an extreme value, which is not included in the tolerance interval.

better the sampling of natural variability associated with a crop population, the better the true population can be modeled using a tolerance interval. For the compositional equivalence evaluation, a statistical tolerance interval for each analyte can be computed from the cumulative historical data to infer the natural range of analyte values from the crop population with a history of safe use. A tolerance interval is a statistically constructed interval that is expected to contain at least a specified proportion (i.e., percent content) of the population, with a specified confidence level.^{15,16} For example, a 99%-content tolerance interval with 95% confidence for a specific analyte is intended to contain at least 99% of the population values for this analyte, with a confidence level of 95%. Figure 1 illustrates the concept of a 99%-content, 95%-confidence tolerance interval. The tolerance interval is generated using the sample data distribution to infer the range between the 0.5th and 99.5th percentiles of the population distribution.

There are varied approaches to the computation of tolerance intervals. Both the classical parametric approach^{17,18} and the nonparametric approach¹⁹ have been used to compute tolerance intervals for composition data. These two approaches

assume data values are identically and independently distributed (*iid*).^{15,16} However, data values from multisite field studies are not independent from each other and tend to be highly correlated. Additionally, data sets from multisite field studies are typically unbalanced due to unequal allocation of genotypes to environments. These tolerance interval approaches failed to consider the data structure when applied to composition data.

In this paper, we describe a model-based parametric approach to derive a statistical tolerance interval from cumulative historical data. A linear mixed model, which has been widely used to statistically analyze data from agricultural experiments involving multisite studies,²⁰ is applied to estimate parameter values that are subsequently needed for the tolerance interval computation. The theoretical basis underlying this tolerance interval approach is presented and compared to other currently employed approaches used to represent natural variation observed in conventional crop composition. An example of the use of this tolerance interval approach is demonstrated using maize grain cumulative historical data, both as an overall composition data set and in the context of two specific analytes.

MATERIALS AND METHODS

Maize Grain Samples from Multisite Field Studies for Compositional Analyses. Maize grain samples were collected from 61 unique Pioneer brand non-GM commercial hybrid maize lines, which ranged in comparative relative maturity (Pioneer CRM) rating from 75 to 119 (Figure 2). Commercial launch of these hybrid

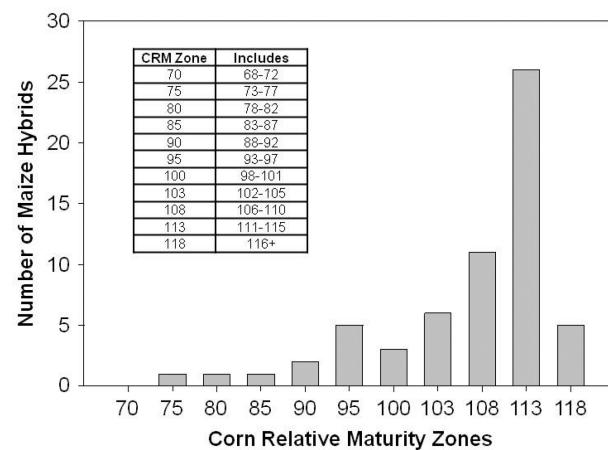


Figure 2. Distribution of corn relative maturity zones of the 61 Pioneer brand non-genetically modified commercial maize hybrids used to generate tolerance intervals. Comparative relative maturity (CRM) ratings of individual maize lines are grouped into zones as shown.

lines occurred between 1989 and 2011 (Figure 3). The maize grain samples were collected from eight multisite field studies between 2003 and 2011. These field sites consisted of 47 unique environments representative of commercial maize-growing regions of the United States, Canada, Chile, and Argentina (Table 1). Each field site, season, and planting date combination represents one unique environment. The experimental design utilized at each field site was a randomized complete block design with either three or four blocks.

Maize production at each field site was conducted using standard agronomic practices in accordance with local practices and recommendations. A summary of monthly temperature, precipitation, and irrigation data from each location is provided in Supporting Information (Supplementary Tables 1–5). Grain samples were

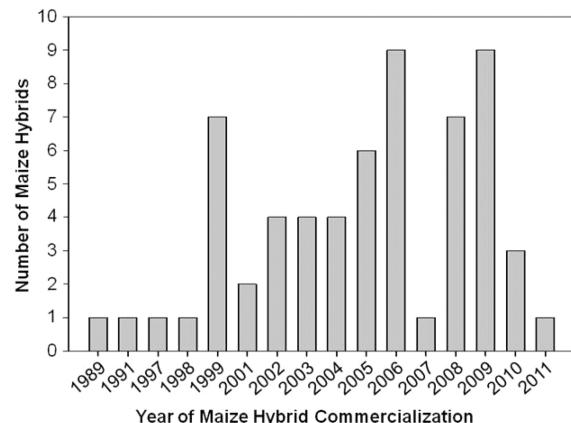


Figure 3. Distribution of commercialization year of the 61 Pioneer brand non-genetically modified commercial maize hybrids used to generate tolerance intervals.

collected after reaching physiological maturity (R6 growth stage),²¹ where five previously self-pollinated ears per plot (one ear per plant) were husked, shelled, and pooled to make one grain sample per plot. Grain samples were shipped frozen to EPL Bio Analytical Services (EPL BAS, Niantic, IL, USA). Samples were stored frozen prior to and after grinding.

Table 1. Summary of Location, Planting Date, and Soil Texture for the 47 Unique Environments from Which Maize Grain Samples Were Collected for Compositional Analyses

year	location ^a	planting date	soil texture	year	location ^a	planting date	soil texture
2003	Chula, Georgia, USA	May 30	sandy loam		Deerfield, Michigan, USA	June 7	silty clay loam
	Bagley, Iowa, USA	June 5	loam		Geneva (1), Minnesota, USA	May 17	clay loam
	Larned, Kansas, USA	May 31	sandy loam		Geneva (2), Minnesota, USA	May 26	clay loam
	York, Nebraska, USA	May 30	silt loam		Brunswick, Nebraska, USA	May 5	loamy fine sand
	New Holland, Ohio, USA	June 10	silt loam		York (1), Nebraska, USA	April 28	silt loam
	Hereford, Pennsylvania, USA	June 17	loam		York (2), Nebraska, USA	May 4	silt loam
2007	Quitman, Georgia, USA	May 18	fine sandy loam		York (3), Nebraska, USA	May 27	silt loam
	Richland, Iowa, USA	May 11	loam		Hinton, Oklahoma, USA	May 12	sandy loam
	Larned, Kansas, USA	May 18	loam		Branchton (1), Ontario, Canada	May 7	silt loam
	York, Nebraska, USA	May 11	silt loam		Branchton (2), Ontario, Canada	May 28	silt loam
	Branchton, Ontario, Canada	May 14	silt loam		Thorndale, Ontario, Canada	May 19	loam
	Germansville, Pennsylvania, USA	May 24	loam		Germansville, Pennsylvania, USA	June 4	loam
2009–2010	Peumo, Cachapoal, Chile	December 2	silt loam		Groom (1), Texas, USA	May 4	silty clay loam
	Colina, Chacabuco, Chile	November 19	loam		Groom (2), Texas, USA	June 15	clay loam
	Buin, Maipo, Chile	November 23	loam		Verona, Wisconsin, USA	May 24	silt loam
	Loreto, Maipo, Chile	November 27	loam		2010–2011	Gahan, Buenos Aires, Argentina	October 24
2010	Stewardson, Illinois, USA	May 25	silt loam		Inés Indart, Buenos Aires, Argentina	October 23	silt loam
	Wyoming, Illinois, USA	May 25	silt loam		Tacuarí, Buenos Aires, Argentina	October 24	silt loam
	Rockville, Indiana, USA	May 6	silty clay loam		Olivar, Cachapoal, Chile	December 2	sandy loam
	Sheridan, Indiana, USA	May 6	silt loam		Popeta, Cachapoal, Chile	December 7	clay loam
	Atlantic, Iowa, USA	May 24	clay loam		Colina, Chacabuco, Chile	December 14	loam
	Bagley, Iowa, USA	May 21	loam				
	Dana, Iowa, USA	May 20	clay loam				
	Lime Springs, Iowa, USA	May 19	clay loam				
	Richland, Iowa, USA	May 6	silt loam				
	Larned, Kansas, USA	May 24	loamy sand				

Compositional Analyses of Maize Grain Samples. Compositional analyses of processed samples were conducted in accordance with guidance from the Organisation for Economic Co-operation and Development (OECD) maize composition consensus document²² and included an assessment of proximate, fiber, fatty acid, amino acid, mineral, vitamin, secondary metabolite, and antinutrient analyte concentrations. For an in-depth illustration of substantial equivalence assessment of composition data, discussion herein is limited to the two individual analytes tryptophan and oleic acid.

Compositional analyses of the processed samples were conducted by EPL BAS using validated methods. For tryptophan analysis, samples were hydrolyzed with lithium hydroxide and quantified using reverse phase high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection (HPLC-UV).²³ The HPLC-UV instrument was calibrated using external standards. For oleic acid analysis, ether extraction and base hydrolysis were conducted on the samples, followed by conversion of free fatty acids to fatty acid methyl esters (FAME). FAME concentrations were quantified using gas chromatography with flame ionization detection (GC-FID). The GC-FID instrument was calibrated using external standards.^{24–26} A molecular weight conversion was performed to convert the FAME concentration value to a fatty acid concentration value.

Construction of Statistical Tolerance Intervals for Composition Data Derived from Multisite Field Studies. For a given composition analyte, the response value y_{ijk} from the i th environment, j th genotype, and k th block was modeled as

^aLocation is the closest town to the field trial site. Locations followed by numbers in parentheses differentiate between unique environments.

$$y_{ijk} = \mu + \text{environment}_i + \text{genotype}_j + (\text{genotype} \times \text{environment})_{ji} + \text{block}(\text{environment})_{ki} + \epsilon_{ijk}$$

where μ represents the grand mean; random effect environment_i is identically and independently distributed (*iid*) $N(0, \sigma_E^2)$; random effect genotype_j is *iid* $N(0, \sigma_G^2)$; interaction effect (genotype \times environment)_{ji} is *iid* $N(0, \sigma_{G \times E}^2)$; random effect block(environment)_{ki} is *iid* $N(0, \sigma_B^2)$; and the plot error term ϵ_{ijk} is *iid* $N(0, \sigma_e^2)$. Other than the grand mean being a fixed effect, all other terms in the model are random effects. This linear mixed model (LMM) is also known as a random effects model. On the basis of the LMM configuration described above, the variance of y_{ijk} is the sum of variance components from all identified sources in the model, denoted as

$$\text{Var}(y_{ijk}) = \sigma_E^2 + \sigma_G^2 + \sigma_{G \times E}^2 + \sigma_B^2 + \sigma_e^2 \equiv \sigma_{\text{total}}^2$$

A two-sided 99%-content tolerance interval was constructed with 95% confidence for the distribution of $N(\mu, \sigma_{\text{total}}^2)$. With $\hat{\mu}$, $\hat{\sigma}_E^2$, $\hat{\sigma}_G^2$, $\hat{\sigma}_{G \times E}^2$, $\hat{\sigma}_B^2$, and $\hat{\sigma}_e^2$ denoted as the restricted maximum likelihood estimates of the grand mean and the variance components of the LMM, the estimate of total variance $\hat{\sigma}_{\text{total}}^2$ was computed as

$$\hat{\sigma}_{\text{total}}^2 = \hat{\sigma}_E^2 + \hat{\sigma}_G^2 + \hat{\sigma}_{G \times E}^2 + \hat{\sigma}_B^2 + \hat{\sigma}_e^2$$

Let R_0 represent the ratio of σ_{total}^2 over the variance of $\hat{\mu}$ and assume that R_0 is known. It follows from sections 2.3.1 and 4.5.1 of Krishnamoorthy and Mathew²⁷ that the lower and upper tolerance limits (*LTL* and *UTL*, respectively) for the two-sided 100(1- γ)-content, 100(1- α)-confidence tolerance interval are

$$\begin{aligned} LTL &= \hat{\mu} - \hat{\sigma}_{\text{total}} \sqrt{\frac{m \cdot \chi_{1,1-\gamma}^2 (1/R_0)}{\chi_{m;\alpha}^2(0)}} \\ UTL &= \hat{\mu} + \hat{\sigma}_{\text{total}} \sqrt{\frac{m \cdot \chi_{1,1-\gamma}^2 (1/R_0)}{\chi_{m;\alpha}^2(0)}} \end{aligned} \quad (1.0)$$

where

$$m = 2 \frac{(\hat{\sigma}_{\text{total}}^2)^2}{J \hat{\Delta} J'}$$

Here $\chi_{1,1-\gamma}^2 (1/R_0)$ represents the 100(1 - γ)th percentile of a noncentral chi-square distribution with the degree of freedom being 1 and the noncentrality parameter being $1/R_0 \cdot \chi_{m;\alpha}^2(0)$ represents the 100 α th percentile of a central chi-square distribution with m degrees of freedom; m originates from the Satterthwaite approximation²⁸ to the degrees of freedom of $\hat{\sigma}_{\text{total}}^2$; $\hat{\Delta}$ is the estimated variance-covariance matrix of $(\hat{\sigma}_E^2, \hat{\sigma}_G^2, \hat{\sigma}_{G \times E}^2, \hat{\sigma}_B^2, \hat{\sigma}_e^2)$; J is a row vector of 1; and J' is a column vector of 1.

Following the approach taken by Bagui, Bhaumick, and Parnes,²⁹ here the unknown parameter R_0 (the ratio of the variance of the population over the variance of the grand mean estimator $\hat{\mu}$) was approximated by its point estimate $\hat{R}_0 = \hat{\sigma}_{\text{total}}^2 / \hat{\text{Var}}(\hat{\mu})$, with $\hat{\text{Var}}(\hat{\mu})$ being the estimated variance of the grand mean. This results in a two-sided tolerance interval that is narrower than the true two-sided 100(1- γ)-content, 100(1- α)-confidence tolerance interval.

The SAS (SAS Institute Inc., Cary, NC, USA) code for constructing the tolerance interval is provided in the Supporting Information (Supplementary Method).

RESULTS AND DISCUSSION

Justification for Model-Based Tolerance Interval

Approach. The parametric tolerance interval approach, which incorporates the linear mixed model described here, is superior to a classical parametric or nonparametric approach for unbalanced data generated from multisite field studies. Both the classical parametric and nonparametric approaches assume data values are identically and independently distributed (*iid*).

However, data values from multisite field studies are not *iid* and tend to be highly correlated. Data from multisite field studies are correlated on several levels: data values collected from the same genotype at the same field site are apt to be highly correlated, data values collected from different genotypes at the same field site are more likely correlated than those grown at different field sites, and data values collected from the same genotype at different field sites are likely correlated as well. Data from multisite field studies conducted over multiple seasons (referred to as cumulative historical data) are unbalanced in structure due to an unequal allocation of genotypes to environments. Our linear mixed model, which is a random effects model, considers and accommodates the correlated and unbalanced data structure resulting from multisite field studies conducted over multiple seasons. The linear mixed model incorporated into this parametric tolerance interval approach has been widely used to statistically analyze data from agricultural experiments involving multisite field studies.²⁰

The conventional formula for the classical parametric tolerance interval was developed assuming responses are *iid* following a normal distribution,¹⁵ and our model-based parametric tolerance interval approach degenerates into the classical parametric solution under those conditions. When the data values are *iid* normal with sample size n , then m equals $n - 1$ and R_0 equals n , and formula 1.0 is simplified into the conventional formula for the two-sided tolerance interval.²⁷

When employing random effects models for unbalanced data, construction of the tolerance interval becomes substantially more complicated, but our approach is computationally feasible and straightforward to execute via SAS (see Supplementary Method in the Supporting Information) or other appropriate statistical software. Compared to simple cases such as those with normally distributed *iid* data, analytical formulas for the tolerance limits are not available for unbalanced and correlated data, and numerical methods or approximations become necessary.²⁷ For a one-way random effect model with balanced data, the unknown R_0 is approximated by its lower confidence limit to achieve the desired content with the nominal confidence level.³⁰ However, "no guidelines are given regarding the choice of the confidence level" for unbalanced data.²⁷ Alternatively, with a one-way random effect model, the tolerance interval has been successfully developed using generalized pivotal quantity machinery.^{31,32} This approach involves statistical resampling and heavy computation, which is difficult for most practitioners to implement. To our knowledge, an extension from the one-way random effect model to a two-way random effects model is not available with a generalized pivotal quantity approach.²⁷ Our random effects model is multiway, as it involves more than two random effects and our data sets are unbalanced; therefore, instead of solving for an unbiased solution using heavy computation, the unknown R_0 is simply replaced with its point estimate. As a result, our tolerance interval is expected to be narrower than a true 99%-content, 95%-confidence tolerance interval, and as such is conservative in the context of a GM crop safety assessment.

A simulation study was conducted to demonstrate that the actual coverage of our tolerance interval is indeed narrower than the nominal coverage level. The tolerance interval coverage was evaluated according to the approach of Liao.³³ In this exercise, 5000 data sets were simulated following an experimental design similar to the cumulative historical data set

and a given set of variance component values. For each of the simulated data sets (data not shown), the actual content of the computed tolerance interval was recorded and compared to the nominal content level (i.e., 99%). Of the 5000 sets of tolerance intervals, 94.24% of the sets had an actual content of at least 99%. With the size of the simulation ($n = 5000$), the standard error associated with 94.24% is 0.3295%. This result, which is less than the nominal confidence level of 95%, demonstrates that our tolerance interval is indeed narrower than a true 99%-content, 95%-confidence tolerance interval.

Sample Size. In the context of a cumulative historical data set with a correlated data structure, a recommended minimum sample size in terms of total number of data points is impractical because our tolerance interval solution is a complex function of various factors. Using the tolerance interval formula 1.0, the width of a tolerance interval (represented by the minimum and maximum calculated limits) is a function of three factors: $\hat{\sigma}_{\text{total}}^2$ (the estimated total variance), m (the Satterthwaite degrees of freedom), and \hat{R}_0 (the estimated ratio of $\hat{\sigma}_{\text{total}}^2$ over the variance of the estimated grand mean $\hat{\mu}$). As the natural variation of the population ($\hat{\sigma}_{\text{total}}^2$) increases, so does the width of the tolerance interval. Parameter R_0 can be considered the effective sample size of the population, as it is the ratio of the variance of the population ($\hat{\sigma}_{\text{total}}^2$) over the variance of the grand mean estimator μ (formula 1.0). As R_0 is dependent on each of the individual variance factors that make up the total variance of the population, R_0 would be different for each analyte of an individual data set. Therefore, it is not straightforward to determine a universal minimum sample size n that could be applied to all analytes. In contrast, we recommend minimum values for the two parameters m and R_0 associated with our tolerance interval approach.

Minimum m and R_0 values were determined by plotting the functional relationship for critical components of the tolerance interval formula versus m and R_0 , with recommended minimum values corresponding to when the respective plot converges. For data collected under multisite field studies over multiple seasons with unbalanced allocations of genotypes to environments, both m and R_0 are increasing functions with respect to the number of unique environments, number of genotypes at each environment, and number of blocks within environments. Due to the unbalanced nature of cumulative historical data, it is cumbersome to provide an explicit formula for m and R_0 as functions of the number of unique environments and genotypes. As a result, it is impractical to provide a recommendation for the minimally required number of genotypes or environments. Additionally, m and R_0 are determined by the estimated variance components as well as the variance–covariance matrix of the estimated variance. Instead, it is more feasible to investigate the empirical behavior of the tolerance limits using functional curves that examine the relationship between $m/\chi_{m,\alpha}^2(0)$ and m and the relationship between $\chi_{1,1-\gamma}^2(1/R_0)$ and R_0 (Figure 4). As both m and R_0 approach infinity, the calculated tolerance interval stabilizes and the tolerance limits approach the true percentiles of the population.

As illustrated in Figure 4, $m/\chi_{m,\alpha}^2(0)$ starts to stabilize when m approaches 60, and $\chi_{1,1-\gamma}^2(1/R_0)$ starts to stabilize when R_0 approaches 50; therefore, we recommend a minimum m of 60 for the Satterthwaite degrees of freedom and a minimum R_0 of 50 for the effective sample size when tolerance intervals are constructed from data derived from multisite field studies. In the context of composition data, m and R_0 are estimated

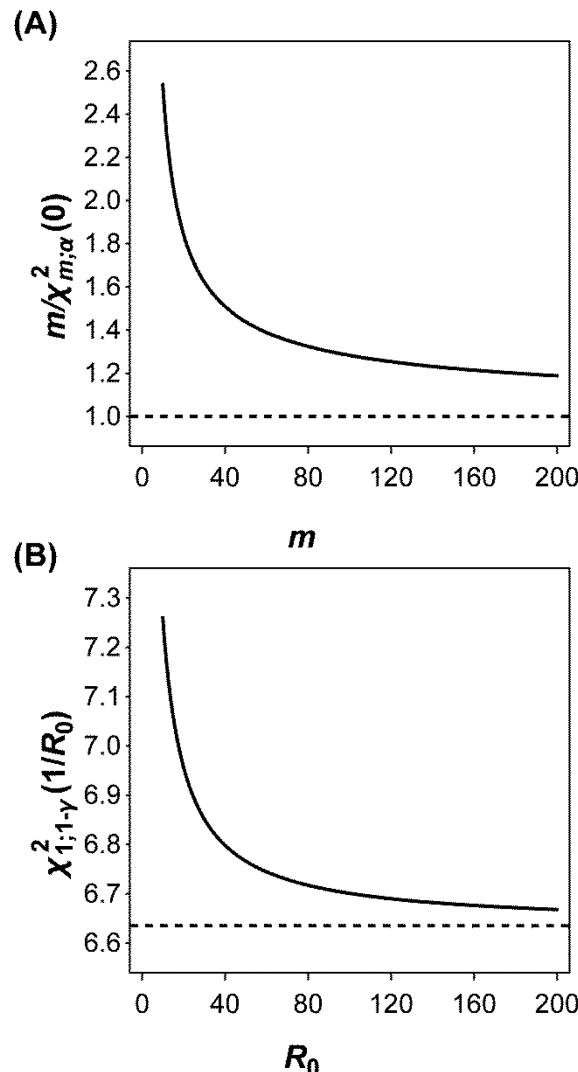


Figure 4. (A) Plot of $m/\chi_{m,\alpha}^2(0)$ versus m . Solid line corresponds to $m/\chi_{m,\alpha}^2(0)$. Dashed line corresponds to the limit of $m/\chi_{m,\alpha}^2(0)$ as m approaches infinity. (B) Plot of $\chi_{1,1-\gamma}^2(1/R_0)$ versus R_0 . Solid line corresponds to $\chi_{1,1-\gamma}^2(1/R_0)$. Dashed line corresponds to the limit of $\chi_{1,1-\gamma}^2(1/R_0)$ as R_0 approaches infinity. m is the Satterthwaite degrees of freedom, and R_0 is the estimated ratio of the total variance over the variance of the grand mean. $m/\chi_{m,\alpha}^2(0)$ and $\chi_{1,1-\gamma}^2(1/R_0)$ are two critical components of the tolerance interval equation that determine the width of a tolerance interval (represented by the minimum and maximum calculated limits).

individually for each analyte, and then estimated m and R_0 values are examined across analytes to evaluate the adequacy of the data set for the purpose of generating tolerance intervals. For example, m and R_0 were estimated for each individual analyte in a data set composed of cumulative maize grain data from 2003 to 2011 (referred to here as the 2003–2011 maize grain cumulative historical data set; data not shown). The minimum estimated m value for a given analyte in this cumulative historical data set was 76, which exceeded the recommended minimum of 60. The estimated R_0 values for 2 of 55 analytes in the 2003–2011 maize grain cumulative historical data set were below the recommended minimum of 50, and the lower of the two R_0 values was 47. Therefore, this cumulative historical data set was deemed adequate for generating tolerance intervals for maize grain analytes.

Tolerance Interval Construction and Results. Tolerance intervals are constructed by estimating parameters (grand mean and variance components) using linear mixed model analysis and subsequently using these parameter values in formula 1.0. Within the 2003–2011 maize grain cumulative historical data set, two analytes (tryptophan and oleic acid) are used as examples to illustrate how tolerance intervals are constructed. For each individual variance component that contributed to the total variance of tryptophan and oleic acid, the estimated values are provided in Table 2, along with the corresponding

Table 2. Variance Components

source of variation	tryptophan		oleic acid	
	variation	% total variation	variation ^a	% total variation
genotype	6.70×10^{-6}	6.7	0.0131	55.6
environment	7.11×10^{-5}	71.6	0.00387	16.4
genotype × environment	3.51×10^{-6}	3.5	0.00206	8.7
block (environment)	0	0.0	0	0.0
residual	1.80×10^{-5}	18.1	0.00456	19.3

^aVariance component estimates were obtained on the log-transformed scale.

percentages of the total variance. Environmental variance was the largest contributor to the total variation of tryptophan values (71.6% of the total variance), whereas genotypic variation was the largest contributor to the total variation of oleic acid values (55.6% of the total variance). Any given composition analyte has a natural variation that is influenced by various individual contributors (e.g., genotypic factors, environmental factors). However, it is important to recognize that the natural variation is the sum of these variation sources and the key objective here is to capture and estimate the total variation, not to characterize or quantify the individual factors that make up the total variation.

Tolerance intervals (99%-content, 95%-confidence) were constructed on the basis of the grand mean and the total variance estimated from the linear mixed model analysis. Tolerance interval results for tryptophan and oleic acid are presented in Table 3 along with the corresponding range of

Table 3. Tolerance Interval Results

analyte	tolerance interval	data range
tryptophan ^a	0.0344–0.0940	0.0348–0.0927
oleic acid ^b	18.1–44.0	17.0–42.8

^aPercent dry weight. ^bPercent total fatty acids.

individual data values for each analyte. To illustrate the reasonableness of the tolerance interval coverage, the tryptophan and oleic acid tolerance interval results were compared to the individual data points for each multisite field study included in the cumulative historical data set using dot plots (Figure 5). Of the 1038 samples included in the cumulative historical data set, all of the tryptophan sample values were within the respective tolerance interval, and all but three of the oleic acid sample values were within the respective tolerance interval. It is expected that 1% of all data values from a population would fall outside a 99%-content tolerance interval. However, a sample set such as the 2003–2011 maize grain cumulative historical data set is unlikely to cover all

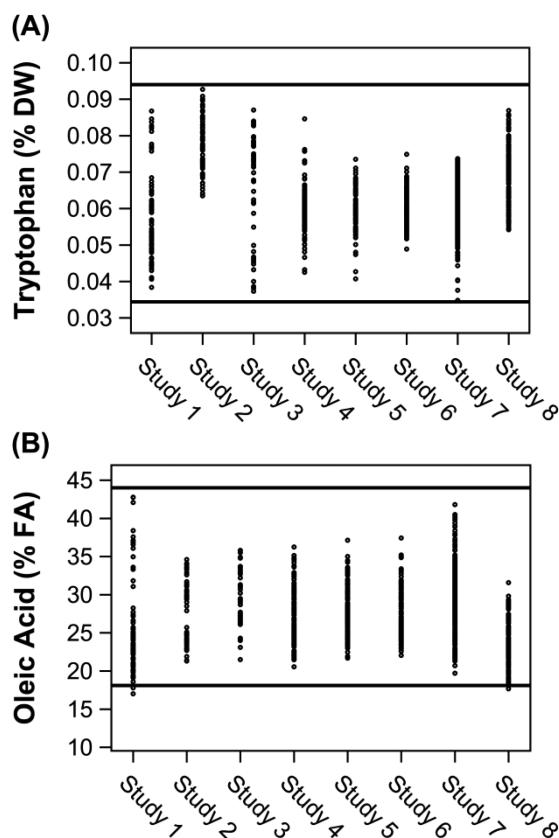


Figure 5. Dot plots for (A) tryptophan (% dry weight) and (B) oleic acid (% total fatty acids). The horizontal lines are tolerance interval limits constructed from the 2003–2011 maize grain cumulative historical data set for (A) tryptophan (0.0344, 0.0940) and (B) oleic acid (18.1, 44.0).

extremes of the population and may have <1% of data values outside the calculated tolerance interval for a given analyte. The same sample set may also have >1% of data values outside the calculated tolerance interval for a different analyte due to the random nature of sampling. As shown in Figure 5, this tolerance interval approach appropriately reflects the natural variation observed in this cumulative historical data set without over-extrapolation.

Tolerance Interval Application. During a substantial equivalence assessment, tolerance intervals can be used to interpret statistical differences within the biological context of natural variation. A statistically significant difference between the GM crop and the non-GM comparator in the compositional assessment may not be biologically relevant. Like any statistical test, the statistical power (i.e., the power of detecting a difference) is driven by the size of the study and the variation in the study data,³⁴ and detection of a statistical difference does not address whether the difference is biologically relevant. Composition data may vary more or less for some analytes compared to other analytes. Therefore, individual analytes end up having different statistical powers, and some analytes may be statistically overpowered due to smaller data variation. For any statistical comparison, there is a possibility of identifying a statistically significant difference by chance (known as type I error or false-positive error). In other words, across all composition analytes for which statistical comparisons are conducted between a GM crop and a non-GM comparator at the significance level of 0.05, statistically significant differences

are expected to be identified for 5% of the analytes due to false-positive error. Additionally, even if a statistically significant difference could be attributed to the genetic modification, the magnitude of the difference is usually negligible when compared to the influence from genetics and environments as demonstrated previously in compositional equivalence evaluations of GM crops.^{35–37} Consequently, it is critical to place any statistical difference within biological context. Tolerance intervals derived from cumulative historical data are a powerful tool to examine biological relevance of significant differences because the data are collected from the population with a history of safe use. Our tolerance interval approach makes statistical inference to the natural range of population values for a given analyte. This inferred natural range then becomes the basis of biological context for compositional equivalence evaluation.

To provide an example of how a tolerance interval is utilized to assist with the substantial equivalence assessment of GM crop composition data, one multisite field study is presented here. This study (identified as study 4 in Figure 5) was conducted in North America during the 2010 growing season at nine field sites. The study contained four GM maize hybrids and one non-GM near-isogenic control maize hybrid (referred to as control maize hybrid). All GM maize hybrids and the control maize hybrid were from the same genetic background. The study also contained six non-GM commercial maize hybrids (referred to as reference maize hybrids). At each site, three of the six reference maize hybrids were randomized together with all four GM maize hybrids and the control maize hybrid in a randomized complete block design according to European Food Safety Authority (EFSA) guidance.³⁸ The resulting grain composition data were statistically analyzed to compare the across-site means of each of the four GM maize hybrids to the across-site mean of the control maize hybrid.

Tryptophan and oleic acid concentrations are summarized in side-by-side box plots for each GM and control maize hybrid, as well as for the pooled data from the six reference maize hybrids (Figure 6). The tryptophan and oleic acid tolerance intervals constructed from the 2003–2011 maize grain cumulative historical data set are also provided in Figure 6. For tryptophan, a statistically significant difference (at the significance level of 0.05) was identified between one GM maize hybrid and the control maize hybrid (identified as GM2 and COMP91, respectively, in Figure 6A). When the mean values designated by the diamond within each box plot are compared, the statistical difference between the GM2 and control means for tryptophan is not visually obvious, with a magnitude of <4% (GM2 mean = 0.0610% dry weight, control mean = 0.0632% dry weight). For oleic acid, a statistically significant difference was identified for three of the GM maize hybrids (identified as GM2, GM3, and GM4 in Figure 6B) compared to the control maize hybrid (COMP91). The statistical difference between each GM mean compared to the control mean for oleic acid was <20% (GM2 mean = 31.0% total fatty acid, GM3 mean = 31.4% total fatty acid, GM4 mean = 32.7% total fatty acid, control mean = 27.3% total fatty acid); this magnitude of difference is more visually obvious when the mean values designated by the diamond within each box plot are compared.

The next step in the substantial equivalence assessment was to compare any relevant GM maize line data ranges to the corresponding tolerance interval for each identified statistically significant difference, to examine the statistical difference in the context of the crop population with an established history of

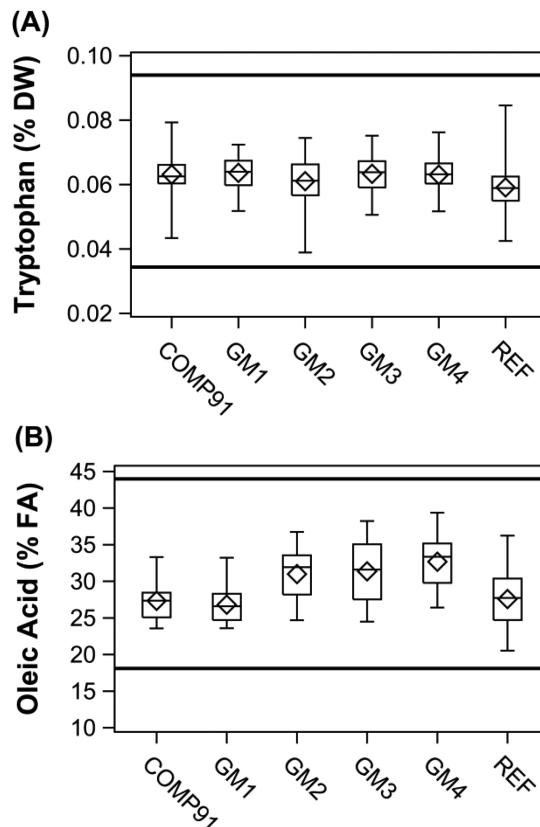


Figure 6. Box plots by genotype for (A) tryptophan (% dry weight) and (B) oleic acid (% total fatty acids) in a single study (study 4 in Figure 5). Data were plotted for the near-isogenic control maize hybrid (coded COMP91), the four genetically modified maize hybrids (coded GM1, GM2, GM3, and GM4), and combined data from the six reference maize hybrids (coded REF). The diamond within each box plot represents the mean value. The whiskers were drawn from the first quartile and third quartile to the minimum and maximum data points, respectively. The horizontal lines are tolerance interval limits constructed from the 2003–2011 maize grain cumulative historical data set for (A) tryptophan (0.0344, 0.0940) and (B) oleic acid (18.1, 44.0).

safe use. For both analytes, all box plots fell within the respective tolerance interval (constructed from the 2003–2011 maize grain cumulative historical data set). Because the tryptophan and oleic acid data ranges of the corresponding GM maize hybrids fell within the respective range of natural variation for the analyte, it can be concluded that the statistical differences are not biologically relevant.

Additionally, Figure 6 illustrates a limitation of sampling commercial reference data in a single study, in that the interquartile ranges for the pooled data from the reference maize hybrids are not noticeably larger than those of the individual GM or control maize hybrids. The limited number of environments and genotypes represented in one multisite field study conducted in a single year results in reference data that do not adequately represent the population. This reference data may even be a biased sample set due to extreme environmental conditions experienced in that particular year. As illustrated in Table 4, m and R_0 values derived from the single-study reference hybrids are well below the recommended minimums of 60 and 50, respectively. The best way to ensure that individual m and R_0 values for a given data set are above the recommended minimums is to include more extensive sampling

Table 4. Comparison of Key Parameters for Sample Size Adequacy

analyte	cumulative historical data ^a		single study data	
	<i>m</i> ^b	<i>R</i> ₀ ^c	<i>m</i>	<i>R</i> ₀
tryptophan	81	57	23	12
oleic acid	140	66	22	12

^aThe 2003–2011 maize grain cumulative historical data set. ^b*m* is the Satterthwaite degrees of freedom. ^c*R*₀ is the estimated ratio of the total variance over the variance of the grand mean.

from a large number of genotypes and representative environments, which may require multiple years of data accumulation.

Importance of Cumulative Historical Data. A cumulative historical data set provides a greater number of environments (i.e., field sites) and a greater diversity of environmental and other site-specific factors including soil characteristics (e.g., soil type, pH, organic matter), environmental conditions (e.g., temperatures, precipitation, solar radiation, humidity, wind), management practices (e.g., crop history, tillage practices, pesticide applications, fertilizer applications, irrigation), and biotic factors (e.g., insects, pathogens, weeds). For example, the environmental diversity of the North American sites used in the 2003–2011 maize grain cumulative historical data set is illustrated in Figure 7. An environmental classification system that defines long-term abiotic macroenvironment types for maize production³⁹ was used to construct Figure 7. Cumulative historical data also incorporate increased genotypic diversity compared to a single-year multisite field study because of the availability and inclusion of more genotypes over time. Tolerance intervals generated from cumulative historical data are more robust than

reference data from a single study due to sample size adequacy and increased environmental and genotypic diversity and are, therefore, more representative of the actual corresponding range of values for the population.

Over the past few years, some interest has been expressed in the application of an equivalence testing approach to the substantial equivalence assessment of GM crops. One approach is to grow non-GM commercial reference lines in the same study at the same field sites as the corresponding GM and non-GM near-isogenic control lines and to utilize the resulting non-GM commercial reference composition data to generate an equivalence interval for each analyte.³⁸ The purpose of the equivalence test is to assess whether the mean of the GM crop (i.e., 90% confidence interval of the mean) for each given analyte falls within the corresponding equivalence interval at a given level of statistical confidence (i.e., 95%). This approach is conceptually appealing but has a number of limitations.

As discussed previously, the limited number of environments and genotypes contained in a single multisite field study results in an inadequate representation of the population. This particular equivalence interval approach relies heavily on the estimated value of genotypic variance (i.e., parameter σ_G^2 is the key component of the equivalence interval), but without extensive testing of the genotypes under their relevant environments, it is impossible to obtain an accurate estimate of the genotypic variance. In the previously described single multisite field study, the non-GM near-isogenic control maize, although considered as safe as the non-GM commercial reference lines, has a tryptophan mean confidence interval (0.0607% dry weight, 0.0649% dry weight) that does not entirely fall within the equivalence interval (0.0530% dry weight, 0.0646% dry weight) established by the non-GM commercial reference lines within the study. It is therefore

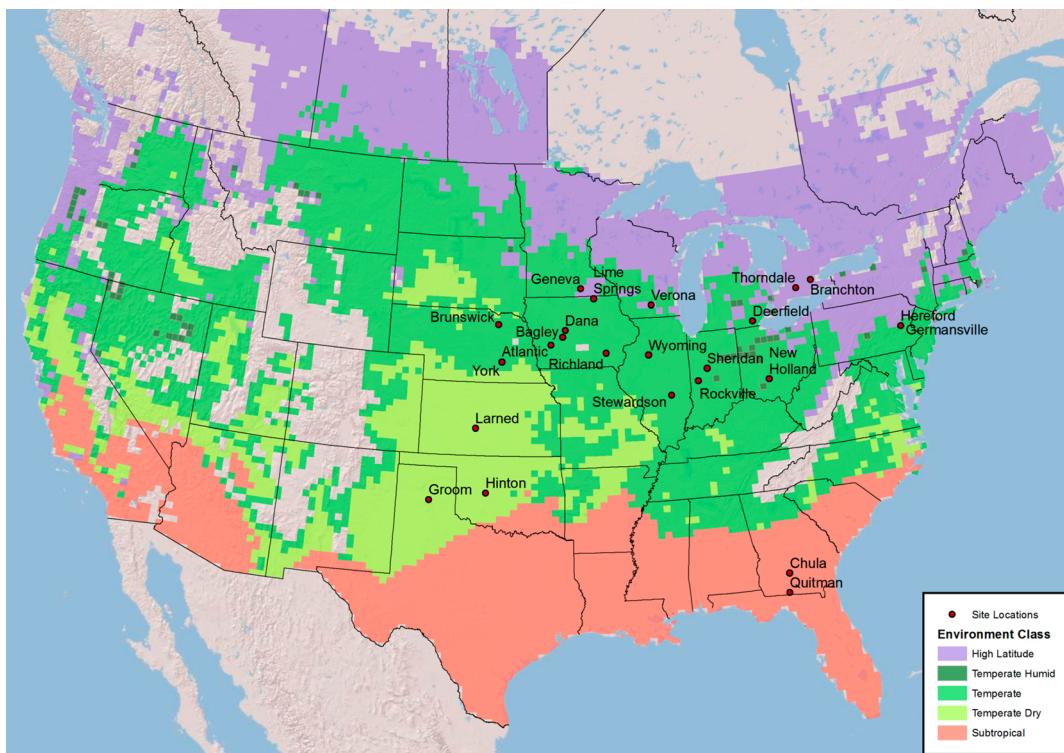


Figure 7. Environmental classification of long-term abiotic macroenvironment types for maize production in North America using the classification system described by Löffler et al.³⁹ and 30 year (1982–2012) weather data.

noted that the equivalence test, as well as any statistical test, cannot be relied on as the sole tool to evaluate the compositional equivalence of a GM crop. In fact, because the equivalence limits are estimated values that depend on which reference lines are included in a given study, this particular equivalence testing approach is not a legitimate statistical equivalence test. A classical equivalence test requires the equivalence limits to be fixed *a priori*.⁴⁰ Tolerance intervals generated from cumulative historical data are more robust and provide a more adequate inference to the natural ranges of the population values compared to equivalence intervals generated from a single multisite field study.

Advantages of Tolerance Interval Approach. Environmental and genotypic variances are generally large contributors to the overall total variation of composition data values.^{9–11,13} Appropriate and adequate sampling of both environments and genotypes is critical to accurately estimating the total variance of the population and the resulting tolerance intervals. Our model-based parametric tolerance interval approach has many advantages over other approaches for use in substantial equivalence assessment of a given GM crop. This approach considers the correlated and unbalanced data structure of multisite field studies over multiple seasons, as opposed to nonparametric and classical parametric approaches, which assume the data are *iid*. The approach can be executed using SAS or other appropriate statistical software and is more computationally feasible than some other alternatives that require statistical resampling and heavy computation. The approach is conservative in that it provides a tolerance interval narrower than a true 99%-content, 95%-confidence tolerance interval. The conservative nature of this approach is acceptable in the context of GM crop safety assessment because it is less likely to incorrectly conclude that a statistical difference is not biologically relevant. The two parameters associated with this tolerance interval approach, m and R_0 , can be used to evaluate the adequacy of a data set in the context of the correlated and unbalanced data structure. The use of m and R_0 for data set evaluation is of greater practical value compared to a universal minimum sample size n applied to all composition analytes regardless of data structure. Tolerance intervals generated from cumulative historical data are more robust than reference data ranges or equivalence intervals derived from a single study, because cumulative historical data provide a more adequate sample size as well as greater environmental and genotypic diversity. It is appropriate to use these tolerance intervals as reference ranges to evaluate biological relevance of statistical differences identified during substantial equivalence assessment of a GM crop.

The use of literature ranges in a substantial equivalence assessment is a complementary way to evaluate the biological context of statistical differences. In the case of the 2003–2011 maize grain cumulative historical data set, some tolerance intervals were narrower than the corresponding literature ranges, and some were wider (data not shown). The width of a given literature range may be influenced by the number of maize genotypes represented (which may be larger or smaller than the number of genotypes represented by Pioneer brand non-GM commercial maize lines in the corresponding tolerance interval derived from the 2003–2011 maize grain cumulative historical data set). The width of the literature range may also be restricted by the infrequent updates of existing data sources, such as the International Life Sciences Institute (ILSI) Crop Composition Database (which is a compilation of non-

GM crop composition data from a number of companies engaged in the agricultural biotechnology industry),^{41,42} or by the lack of recent publications to add to the literature ranges. Because access to a diverse range of non-GM commercial lines can be restricted due to proprietary concerns, and given the premise that all non-GM commercial lines have a history of safe use, it is therefore worthwhile to consider establishing a common set of tolerance intervals and/or equivalence intervals across the agricultural biotechnology industry. Currently, the necessary information for model-based tolerance interval construction is not available from the ILSI Crop Composition Database⁴² (e.g., distinguishing between different lines and different locations). There might be future opportunity to expand the existing ILSI Crop Composition Database with this additional information or to establish a different database that is suitable for this purpose.

In conclusion, under the substantial equivalence framework for crop composition assessment, it is important to acknowledge the value of cumulative historical data. One way to utilize cumulative historical data is to generate a set of tolerance intervals using an approach that appropriately considers the correlated data structure. Our tolerance interval approach has a solid theoretical basis, provides an ease of implementation, and offers a practical solution to the representation of natural variation derived from crop populations with an established history of safe use for practitioners in the field of GM crop safety assessment.

ASSOCIATED CONTENT

Supporting Information

Supplementary method, SAS code for construction of the tolerance interval, and weather data in Supplementary Tables 1–5. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

GM, genetically modified; *iid*, identically and independently distributed; CRM, comparative relative maturity; OECD, Organisation for Economic Co-operation and Development;

HPLC-UV, high-performance liquid chromatography with ultraviolet detection; FAME, fatty acid methyl esters; GC-FID, gas chromatography with flame ionization detection; LMM, linear mixed model; SAS, Statistical Analysis System; EFSA, European Food Safety Authority; ILSI, International Life Sciences Institute

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