

Validation of an Immunoassay for Detection and Quantitation of a Genetically Modified Soybean in Food and Food Fractions Using Reference Materials: Interlaboratory Study

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An immunoassay for detection of a specific genetically modified soybean (Roundup-Ready®) was validated on dried soybean powder in an interlaboratory study. Different percentages of genetically modified soybeans in nonmodified soybean matrix were evaluated in a blind study. Thirty-eight laboratories from 13 countries participated. The immunoassay was evaluated for 2 endpoints: (1) To give a semiquantitative result, i.e., determination of a given sample above or below a given threshold, or (2) to compute a quantitative result, i.e., percentage of genetically modified soybeans in the sample. Semiquantitative results showed that a given sample which contained <2% genetically modified soybeans was identified as below 2% with a 99% confidence level. Quantitative use of the assay resulted in a repeatability (r) and reproducibility (R) that were computed to be $RSD_r = 7\%$ and $RSD_R = 10\%$, respectively, for a sample containing 2% genetically modified soybeans. Application of this method depends on availability of appropriate reference materials for a specific food matrix. Only matrix-matched reference materials can be used for analysis of food or food fractions.

The requirements for labeling of food and food products derived from genetically modified organisms (GMO) within the European Union is set out by the Regulation 258/97/EEC on Novel Foods and Novel Food Ingredients (1).

Because 2 GMO products (Roundup Ready® soybean and BT176 corn) were on the market before the Novel Foods Regulation came into force, their labeling requirements are dealt with separately by Regulation 1139/98/EEC (2).

To ensure that consumers are fully informed of the content of the food they purchase, adequate information is provided on product labels. Labeling is mandatory if GMO products are no longer substantially equivalent to their conventional counterpart, e.g., if a new protein and/or the genetic modification of DNA can be detected. Analytical methods for detection of both newly expressed protein or inserted DNA for GMOs are necessary to determine compliance with labeling requirements. Such methods should be validated at the European Union level to encourage application of harmonized procedures. DNA-based methods for specific detection of the economically most important GMOs are already available, e.g., (3–7).

Recently, a DNA-based screening method based on detection of 2 genetic elements, the 35S promoter and the *nos* terminator, by means of polymerase chain reaction (PCR) was published (8) and validated on a European scale (9). These 2 genetic elements are important for expression of genes that are present in nearly all genetically modified plants to-date (10, 11). The method seems well suited to screening for the presence of GMOs. However, it does not identify specific GMOs in the sample because these elements, the 35S promoter and the *nos* terminator, are present in numerous GMOs.

No internationally validated method based on analysis of proteins is currently available. This study describes a validation study of a specific protein-based method for detecting RoundupReady soybeans in food-related matrixes.

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Experimental

Immunoassay

The immunoassay kit was provided by Strategic Diagnostics, Inc. (Newark, DE). The method was designed as a sandwich enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies against the protein CP4 EPSPS, which induces Roundup (Monsanto Co., Peapack, NJ) tolerance in modified plants, immobilized in the wells of the ELISA plate, and a polyclonal antibody conjugated with horseradish-peroxidase (HRP) as detection system. As a substrate for the HRP, 3-3',5,5'-tetramethylbenzidine (TMB) was used. Analysis was performed according to instructions in the kit.

Monoclonal Antibody Development

CP4 EPSPS protein was produced by fermentation (100 L) of *Escherichia coli* strain GB100, transformed with plasmid pMON21104. The protein was purified to >90% by a combination of cell extraction, ammonium sulfate precipitation, and hydrophobic and anion exchange chromatography. The *E. coli*-expressed CP4 EPSPS protein has been characterized. This CP4 EPSPS standard (lot No. 5192245) was stored in a buffer solution containing 50mM Tris-HCl, pH 7.5, 150mM KCl, 2mM DTT, and 50% (v/v) glycerol at ca 3.96 mg/mL total protein. A comprehensive safety assessment of the CP4 EPSPS protein has been published (12). The monoclonal antibody 39B10.1 was obtained from a Swiss Webster mouse immunized with this purified recombinant CP4 EPSPS protein. The mouse received the first injection of antigen in complete Freund's adjuvant intraperitoneally and subsequent injections in incomplete Freund's adjuvant intraperitoneally. All injections were between 14 and 21 days apart. The primary immunization injection and all subsequent booster injections contained 100 µg CP4 EPSPS protein.

Somatic cell fusions were performed using immunized mouse splenocytes and the myeloma cell line P3x63Ag8.653. The fusion was performed with polyethylene glycol using standard techniques (Kearney). Supernatants from microtiter plates containing growing hybridomas were screened by ELISA using CP4 EPSPS coated plates. The hybridoma 39B10 was cloned by limiting dilution, and a clone was selected by ELISA and designated 39B10.1. The hybridoma and the clones were cultured in 10% fetal bovine serum, 2 mM L-glutamine, and 3.4×10^{-5} M 2-mercaptoethanol in Isove's modified Dulbecco's medium in a 7% CO₂ incubator. The hybridoma and clones were derived from an animal housed in a murine pathogen-free vivarium. The P3x63Ag8.653 myeloma cell line is mycoplasma-negative.

Antibody from 39B10.1 was produced in ascites, purified, and characterized. Male imperial cancer research severe combined immunodeficiency (ICR SCID) mice were primed with 0.5 mL pristane (2,6,10,14-tetra- methylpentadecane). Fourteen days later, 1.2×10^6 cells were injected intraperitoneally. Ascites fluid was collected starting 21 days after cell injection and was completed 17 days later. Monoclonal antibody was purified by protein A axial flow chromatography.

Polyclonal Antibody Development

New Zealand White rabbits were immunized with purified recombinant CP4 EPSPS protein. The rabbits received the first injection of antigen in complete Freund's adjuvant intramuscularly and subcutaneously. All subsequent injections were in incomplete Freund's adjuvant. All injections were between 21 and 28 days apart. The primary immunization injection and all subsequent booster injections contained either 500 or 250 µg CP4 EPSPS, depending on the rabbit treatment group. The rabbits were bled on days 35, 56, 84, and 112 (14 days after incomplete adjuvant injection). The sera from these rabbits were analyzed in a direct binding ELISA. To obtain large volumes of sera, animals were production bled. Here, 250 µg CP4 EPSPS was injected intravenously. The rabbits were bled 14 days after this boost. Polyclonal antibody was purified by protein A axial flow chromatography. The protein A purified antibody was conjugated to HRP using the method initially reported by Nakane and Kawaoi and described in detail elsewhere (13).

Test Material

The test material used in this study consisted of the Roundup-Ready Soya reference material as produced by the Institute of Reference Material and Measurement of the European Commission's Joint Research Center (JRC Geel, IRMM, Retieseweg, Geel, Belgium). Details on the production of this material are available directly from this Institute (14) or on the Internet (<http://www.irmm.jrc.be/rm/cert-reports.html>).

Each laboratory received 3 known and 16 unknown test samples. The 16 unknown matrixes consisted of 0, 0.5, 1, and 2% GMO (weight % Roundup-Ready in conventional soybean powder). Each participant received 16 blind labeled samples, i.e., 3–5 replicates (randomly distributed) for each concentration level. Randomization ensured that, on average, 4 specimens of each concentration were distributed.

Design of the Validation Study

In total, 38 laboratories were contacted and results were received from 37 laboratories from 13 European Member States and Switzerland. The list of participants included official control, university, private, and food processing laboratories. The laboratories received calibration samples, blinded samples, and the ELISA kit. The test was designed to determine whether the GMO content of a sample was above or below a threshold of 2%. (The threshold was arbitrarily set, and did not anticipate any future decision of the European Commission.) Participants received 4 samples of known concentration with a GMO content of 0, 0.5, 1, and 2%.

Preliminary data created by the kit manufacturer stated that a 95% confidence interval in detecting a sample with a true GMO content of 2% could be achieved if the optical density (OD) was compared to that of a sample containing 1.25% GMO. Subsequently, laboratories were required to produce a reference sample of 1.25% by weighing the appropriate amounts of a 1 and a 2% sample.

The following quality criteria were applied to evaluate the validity of a result: Absorbance reading of the blank (only assay buffer, no sample extract), <0.2 OD; absorbance reading of the extract of the non-GMO sample (0%), <0.3 OD; coefficient of variation (CV) <15%. A slightly higher CV was allowed only for determining the blank value and the 0% sample, as OD values were rather low for these samples and small variations can easily lead to a CV >15%.

Laboratories were requested to repeat the analysis if the first series of results did not fulfill these requirements. All blinded samples, blank values, and the 0% test material were determined in duplicates; the reference sample of 1.25% GMO was analyzed in 3 replicates to get a reliable result.

The OD obtained by analyzing this reference sample was used as a decision criterion. If the OD of the unknown sample exceeded that of the reference sample (1.25% Roundup-Ready soybeans), the GMO content of the unknown sample was considered to be above the given threshold.

All participants were asked to send in the following results: all raw data (OD measurements) and an assessment of all unknown samples, i.e., whether they were above or below the threshold value of 2% (the arbitrary threshold).

Data Pretreatment

Several laboratories submitted results from more than one run; therefore, only one run was taken for the final data evaluation. For most laboratories, this was the first run performed, except for 3 cases. One laboratory mistakenly used a filter at 405 nm instead of 450 nm in the first run, and 2 laboratories had exceptionally large blank OD values in their first run. As corrective measures the data from the second run were taken, showing none of the irregularities given above, and meeting acceptable assay performance criteria discussed previously.

Five laboratories expressed erroneous OD values as differences from the mean blank value. For consistency in statistical analysis, the raw data were taken if available or computed from the reported OD values by adding the blank mean OD.

Several obvious mistakes in reporting results were detected from 6 laboratories and were excluded. The errors consisted of sample switching, exceptionally large differences between duplicates and saturation in absorbance readings, values typical for an empty well, and air bubbles in the wells. The individual samples affected were excluded from statistical analysis.

Results

Performance of Test Kit in Assessing 2% GMO Samples

The assay tested was designed to generate only positive or negative endpoints at a hypothetical threshold of 2%. Each sample was compared with the mean of a threshold reference sample. If the sample's average OD value exceeded that for the threshold, the sample was considered positive. Clearly, the chance of a positive response would be 50% if an unknown sample contained the same GMO level as the threshold. The threshold reference sample in this study was set at 1.25% GMO so that the chance of a positive response would be

Table 1. Summary of results

GMO in sample, %	No. of negative samples	No. of positive samples	Samples positive, %
0	149	0	0
0.5	148	0	0
1	140	7	4.8
2	1	142	99.3

nearly 100% for GMO levels of 2% or more. A negative response from this assay would then establish with >99% confidence that the sample did not contain GMO at levels of $\geq 2\%$. A positive response, however, would be interpreted as possibly containing $\geq 2\%$ GMO. False positives were allowed to ensure that no samples actually containing 2% GMO were falsely declared as negative.

All study samples in the accepted runs were compared to the reference sample containing 1.25% and classified as either positive or negative (Table 1). The sensitivity was computed to be 99.3% at 2% GMO levels; therefore, GMO levels of $\leq 1\%$ rarely gave a positive assay response.

A complete sensitivity curve for the assay was fit to these data using logistic regression. The sensitivity curve quantitated the chance of a positive result for every concentration of GMO in soybean flour. The logistic regression model used was:

Percent chance of positive

$$= 100 \times \left[1 + \left(\frac{\% \text{GMO}}{1.25} \right)^B \right]^{-1} \quad (1)$$

The coefficient B was determined by fitting (1) to the data using the LOGISTIC procedure in the statistic package SAS (SAS Institute, Inc., Cary, NC). This form of the logistic function fixes the expected probability of a positive response to 50% when the GMO concentration is 1.25%. (The application of a more general form of logistic regression did not reveal a significant deviation from this theoretical value.) The fitted coefficient B was computed to be 12.62 with a 95% profile confidence interval ranging from 10.07 to 15.83 (i.e., the true value of B ranges between 10.07 and 15.83 with 95% confidence). The complete sensitivity curve from (1) along with its 95% confidence bounds is shown in Figure 1.

The sensitivity curve and the data in Table 1 indicate that samples with $\geq 2\%$ GMO have more than a 99% chance of giving a positive assay response. This implies then that any negative sample, identified as below the hypothetical threshold of 2%, can be claimed to have levels <2% with at least 99% confidence. Because the assay is targeted to detect 2% GMO samples, it will naturally misclassify as positives some samples containing levels slightly <2%. The sensitivity curve shows, however, that this misclassification is unlikely to extend to levels <1%. To summarize, this binary assay is suitable to classify soybean flour samples containing <2% GMO. Al-

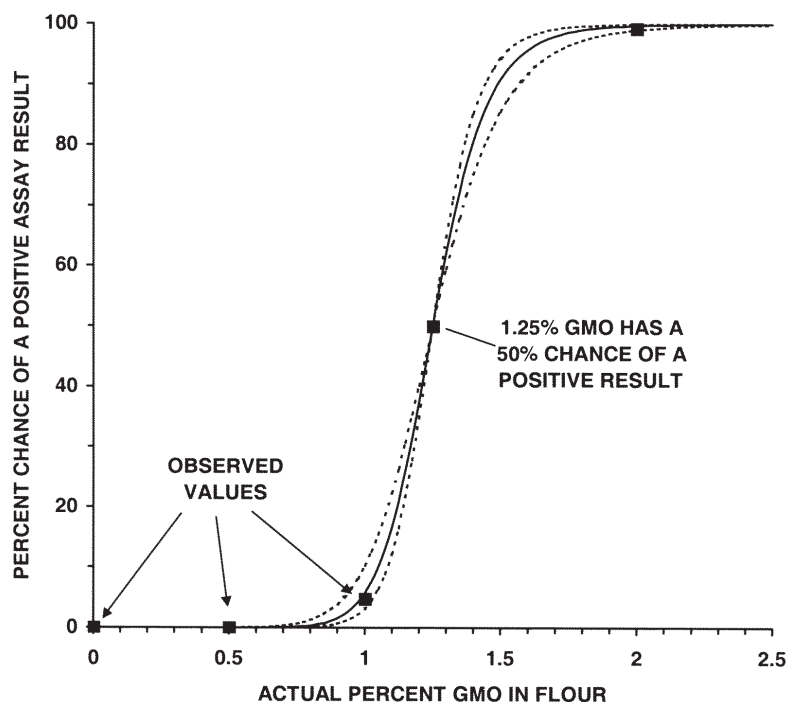


Figure 1. Predicted assay sensitivity to actual levels of GMO in soybean flour. Dashed lines indicate 95% confidence limits on the sensitivity curve.

though assay-positive samples could contain GMO at levels <2% Roundup-Ready Soya, they would be rarely at levels <1% GMO.

Quantitation Procedures

Although the GMO Soya test kit used in this study was designed specifically to test for the presence or absence of a GMO threshold, it can be used to measure GMO concentration. Information collected in this study allows assessment of accuracy and precision when the assay is used in a quantitative manner. The methods and results of the statistical assessment are described here. For statistical reasons, the data were transformed to give an approximately linear response at all laboratories before precision and accuracy of this test were assessed.

Establishing a Calibration Function

Immunoassays typically have a sigmoidal concentration-response curve, although in suitably chosen concentration ranges a simpler function may be adequate. For this assay, examination of all data indicated the presence of a similar slight curvature (concave) for all laboratories. On a logarithmic concentration scale, the curvature was in the opposite direction (convex). In more traditional assays, a nonlinear dose-response could be fit to a large number of reference levels. However, because the test kit was not primarily designed for a quantitative evaluation, only 3 levels of calibration samples were used: 2 GMO samples at 2% and 1.25% GMO; a single 0% GMO sample; and a blank. With only 3 reference levels, it would not be prudent to use a calibration function with more than 2 fitted parameters. Such an approach would be overly sensitive to errors in the reference sample and in-

crease the variation among predicted GMO values. Consequently, the approach taken here was to find a transformation of the GMO concentration that resulted in an approximately linear response at all laboratories.

Because the curvature was slight, we used a simple power transformation of the GMO concentration, g , to determine a transformed value, $t = g^p$. Given the appropriate value of p , OD and t should be approximately linear. That is, for each laboratory i ,

$$OD_i = A_i + B_i t + \text{error} = A_i + B_i g^p + \text{error} \quad (2)$$

approximately. To find the optimal value of p , both reference and test samples at each laboratory were used. At each laboratory, i , the median of sample OD values, \tilde{Y}_{ij} , was computed over all unique GMO levels, j . Medians were used instead of means to avoid possible distortion from outliers. For each value of p considered, the following quadratic model was fit to all the data simultaneously:

$$\begin{aligned} \tilde{Y}_{ij} &= A_i + B_i t_j + C t_j^2 + \text{error} \\ &= A_i + B_i g_j^p + C g_j^{2p} + \text{error} \end{aligned} \quad (3)$$

Although each laboratory could have a different value of A and B , there is only a single curvature coefficient, C , for the entire set of laboratories. Values of p between 0.7 and 1 were examined in steps of 0.01. The power $p = 0.83$ gave the smallest significance level for the coefficient C and was, therefore, selected for use in the quantitation of study samples.

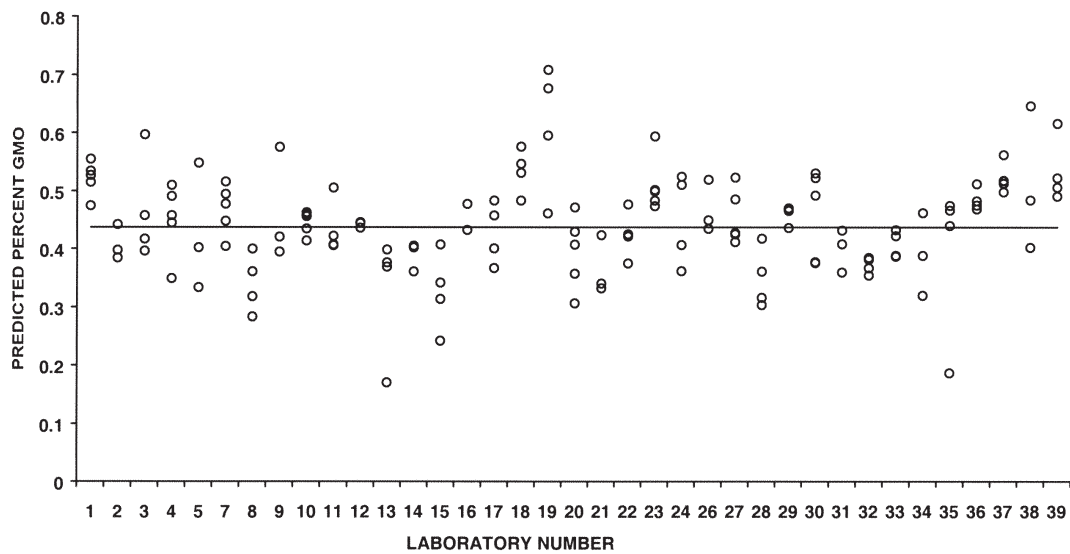


Figure 2. Summary of 0.5% GMO sample results for all laboratories. Horizontal line is median computed for all data.

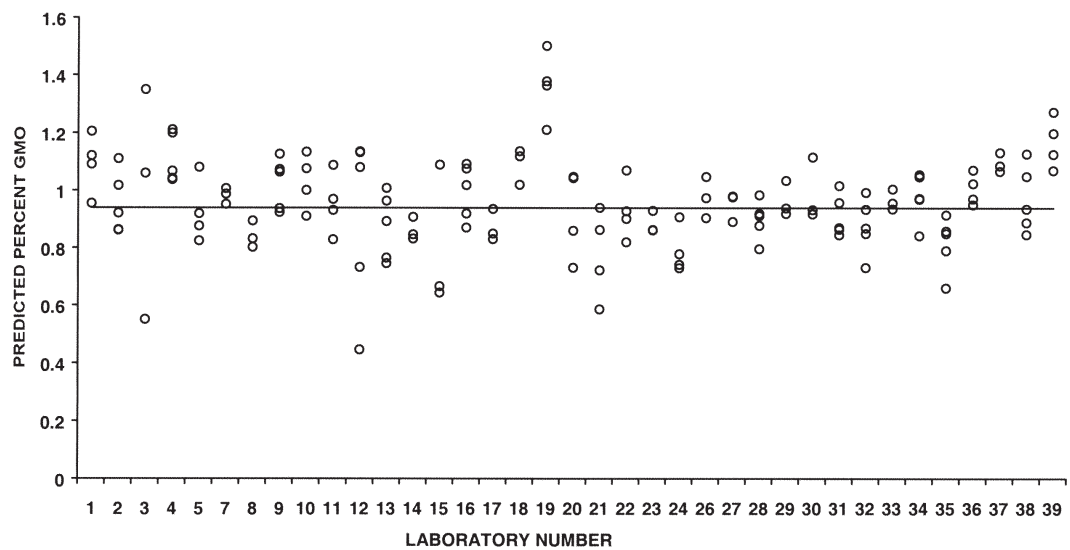


Figure 3. Summary of 1% GMO sample results for all laboratories. Horizontal line is median computed for all data.

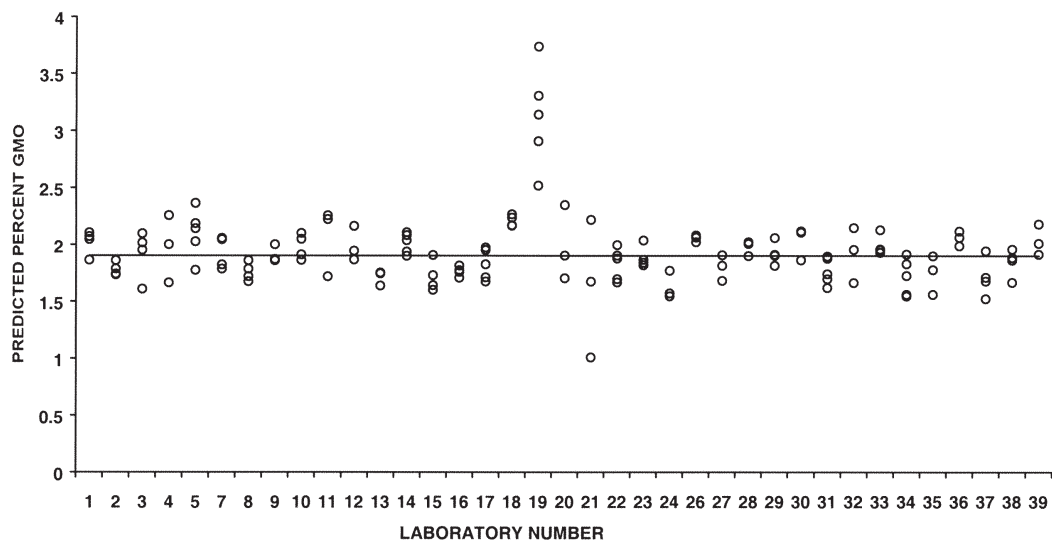


Figure 4. Summary of 2% GMO sample results for all laboratories. Horizontal line is median computed for all data.

Quantitation of Study Samples

A calibration curve was fit to the reference samples at each laboratory independently using the simple linear regression model:

$$\tilde{Y}_g = A + Bg^{0.83} + \text{error} \quad (4)$$

Here, \tilde{Y}_g is the median OD for all the reference values at %GMO level g . The median OD was again used to reduce the influence of any single aberrant OD values in reference samples. Once A and B were found, the predicted percent GMO in test samples was computed from the sample OD as:

$$\text{Predicted \% GMO} = \left(\frac{\text{OD} - A}{B} \right)^{\frac{1}{0.83}} \quad (5)$$

If the OD for any sample was less than A , then no quantitation was possible. For convenience, the predicted value in this case was set either equal to 0 or coded as N (i.e., nonquantifiable). Regardless of the number or code assigned, such samples obviously should be included in those considered not detected.

Removal of Outliers

Results obtained for the 0.5, 1, and 2% GMO levels are graphically illustrated in Figures 2–4, respectively. Aberrant laboratories and other outliers are commonplace in interlaboratory studies. The general philosophy in the ISO 5725:1994 standard for collaborative studies was used to remove outlier laboratories at each GMO level. Cochran's test was applied to remove laboratories with an extreme variation, followed by several Grubbs tests to remove laboratories with extreme average levels. Such cycles of Cochran and Grubbs tests are performed until no additional laboratories are identified for removal. The outlier procedures used here were slightly simplified to only the more stringent $p < 0.01$ level to reject laboratories. The Cochran test assumes equal number of replicate samples at each level across laboratories. In this study, the number of replicates ranged between 3 and 5. Simulation studies indicated that, with this data set, this range was too great to give reliable results. Consequently, a simulation-based approach was used to obtain the appropriate critical values for the Cochran test. The Cochran test statistic was simulated 5000 times with the specific array of replicates used at each level among the laboratories. The 99th percentile of this simulated distribution was used as the upper 0.01 critical value

Table 2. Outliers removed from data evaluation^a

GMO level, %	Extreme variation (Cochran)	Extreme average level (Grubbs)
0.5	None	None
1	3, 12, 15	19
2	21	19

^a Laboratories are identified by number.

for the test. Because laboratory averages are less sensitive to minor differences in number of replications, no modifications were made to the Grubbs tests. Using these standardized procedures, several laboratories were excluded (Table 2). The results of all remaining laboratories were used in the statistical analysis of accuracy and precision for each level.

Accuracy and Precision

A simple analysis of variance was used to measure assay bias, repeatability, and reproducibility. The mixed model procedure in SAS (Proc MIXED) was used to compute the mean and between- and within-laboratory variance components (V_b and V_w , respectively) at each GMO level. The repeatability standard deviation, S_r , is the square root of the within-laboratory variance component, V_w . The reproducibility standard deviation, S_R , is the square root of the total variance $V_b + V_w$. The repeatability and reproducibility values (r and R) are 2.8 times the respective standard deviations (15). These 2 values are limits on the expected differences between identical samples in the same run (r) and at different laboratories (R ; Table 3).

Table 3. Summary of accuracy and precision statistics for quantitative assay

	GMO in soy bean flour, %		
	0.5	1	2
No. of laboratories			
Retained in analysis	37	33	35
Removed as outliers	0	4	2
Accuracy			
Mean			
GMO, %	0.440	0.952	1.902
True value, %	88.1	95.2	95.1
Bias (mean-true value)			
GMO, %	-0.060	-0.048	-0.098
% of True value	-11.9	-4.8	-4.9
Precision ^a			
Repeatability			
s_r	0.062	0.092	0.146
RSD_r	12.4	9.2	7.3
r ($2.8 \times s_r$)	0.176	0.260	0.414
Reproducibility			
s_R	0.083	0.123	0.186
RSD_R	16.6	12.3	9.3
R ($2.8 \times s_R$)	0.236	0.349	0.527

^a s_r , s_R , r , and R are expressed in units of % GMO; RSD_r and RSD_R are expressed as percent of true value.

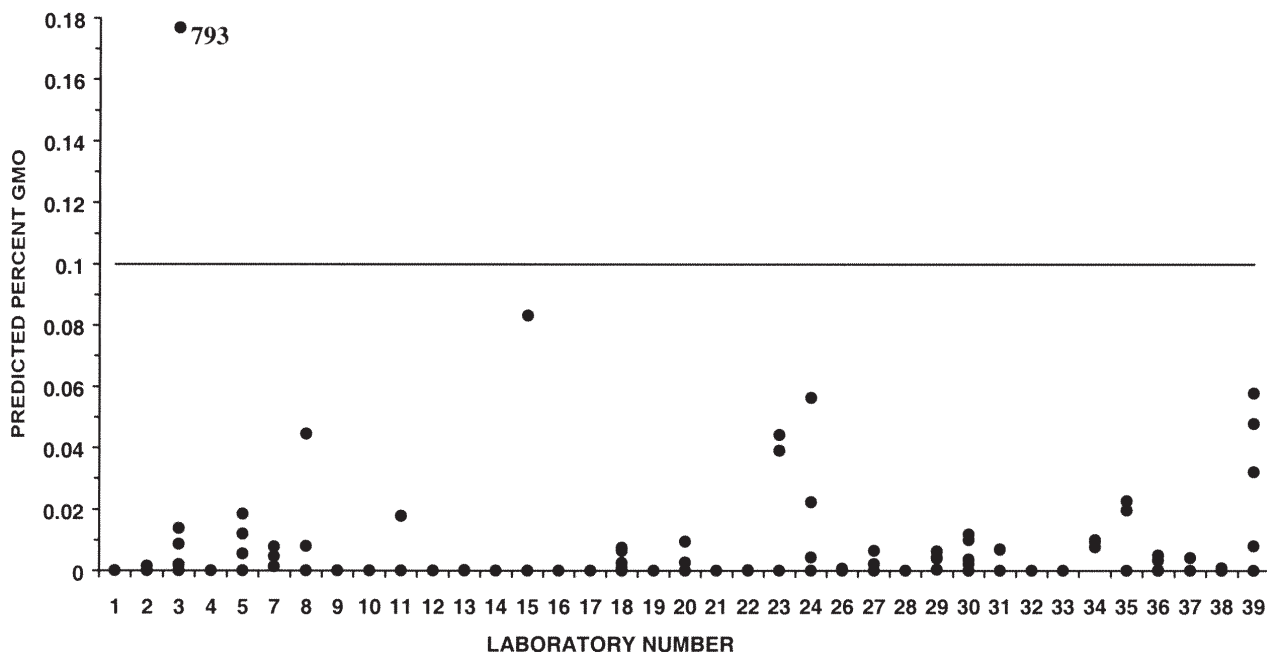


Figure 5. Summary of 0% GMO sample results for all laboratories. All samples yielding a noncomputable value were set equal to 0% GMO. Horizontal line is proposed detection criterion.

Detection Limits

Results obtained for samples free of GMO (0% GMO) are illustrated graphically in Figure 5. All apparent GMO concentration values except those for the single sample No. 793 were below 0.1%. Sample No. 793 (from laboratory 3) demonstrated some inconsistency in the individual well OD results (i.e., 0.139 and 0.386), suggesting some problem in the measurement. For the 1% GMO levels, this laboratory was rejected because of extreme variation among samples.

Approximately 40% of the 0% GMO samples were quantitated using the calibration method (5). After a square root transformation, these data appear reasonably consistent with the upper half of a normal distribution. A predicted %GMO value of about 0.1% would correspond to about the 99.9th percentile of this distribution. Thus, 0.1% seems a reasonably conservative criterion of detection for this assay. Samples with predicted GMO levels exceeding 0.1% would be considered detected, i.e., they would be labeled "GMO-containing."

The smallest actual GMO level that must be in a sample to yield consistent detection must obviously be much greater than the 0.1% detection criterion. (If a sample actually contained 0.1% GMO, such a sample would be expected to give a predicted value exceeding 0.1% at most 50% of the time.) The accuracy and precision results from the 0.5% GMO level above can be used to estimate this consistent detection level. If a sample contained a true GMO level of μ , then it would be consistently detected by this assay if:

$$0.1 < \mu\rho_{\mu} - 2\sigma_{\mu} \quad (6)$$

Here ρ and σ are the expected recovery and the reproducibility standard deviation, respectively, when the

GMO level is μ . Assuming that these values are approximately the same as those for the 0.5% GMO sample in Table 3, then (6) can be approximated as:

$$0.1 < \mu(0.880838) - 2 \times 0.082937 \quad (7)$$

or

$$\mu > 0.3018\% \text{ GMO} \quad (8)$$

Thus, samples containing between 0.3 and 0.35% GMO (or greater) would be consistently detected by this assay. Samples with <0.3% would be detected less reliably.

Conclusions

A test based on an immunoassay for the specific detection of Roundup-Ready soybeans was validated on reference material in the form of dried powdered soybeans. Thus, the interpretation given here is valid only for the reference material used in this study. The immunoassay was evaluated in 2 ways: (1) To detect whether a given sample was above or below a certain threshold, and (2) to quantitate the percentage of genetically modified soybean present in the sample.

The detection limit was approximately 0.35% GMO on a dry weight basis. For this study, the threshold was set at 2%, without anticipating any decisions of the European Commission. To check compliance with a threshold by the set-up used for this study, any sample scoring negative contains <2% GMO, and any sample scoring positive contains at least 0.85% GMO with a confidence level of 99%. The validation was set up to avoid false negative results; however, this increases the proportion of false positive results. The reference

sample used contained 1.25% GMO, but by altering this value the method can easily be adjusted to any threshold between 1 and 2.5%. Operating the immunoassay in a quantitative manner resulted in a repeatability (r) of $RSD_r = 7\%$ and a reproducibility (R) of $RSD_R = 10\%$ at a level of 2% GMO.

The validation study was performed on the only currently available reference material for GMOs, that simulates but does not reflect an actual food fraction. By modifying the extraction procedure accordingly, and using appropriate reference materials to control for cross-reactivity, sample matrix effects, and extraction efficiency, similar results may be obtained for food fractions, if the protein is present in a form that is recognizable by the antibodies.

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