Title

Internal validation of protein based tests, FG72 ELISA

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STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA 10(d) (1) (A). (B). (C).

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

The work to which this report refers was performed according to the procedures herein described and provides an accurate record of the results obtained. The study was not conducted under the Good Laboratory Practice Standards as specified in 40 CFR 160.

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APPROVALS PAGE

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SUMMARY

Internal validation of protein based tests, FG72 ELISA

This report is issued as the validation document for the use and performance of the Strategic Diagnostic Incorporated (SDI) EPSPS ELISA plates and Agdia HPPD ELISA plates for the detection of 2mEPSPS and HPPD W336 proteins in double-herbicide tolerant soybean transformation event, FG72 soybean grain.

SDI EPSPS ELISA plates and Agdia HPPD ELISA plates were validated for soybean grain by spiking non-transgenic Jack soybean with various concentrations of 2mEPSPS and HPPD W336 protein standards. The 2mEPSPS and HPPD W336 proteins were spiked together into each non-transgenic sample. The standards were added to the extraction buffer at the prescribed concentrations prior to extraction in 5 replicates. Each replicate was analyzed using duplicate wells.

The SDI EPSPS ELISA plate has a limit of detection (LOD) for 2mEPSPS of 10.3 ng/g in grain. The limit of quantitation (LOQ) for 2mEPSPS is 40.0 ng/g in grain.

The Agdia HPPD ELISA plate has a LOD for HPPD W336 of 20.8 ng/g in grain. The LOQ for HPPD is 80.0 ng/g in grain.

The results of the validation of SDI EPSPS ELISA plate and Agdia HPPD ELISA plate for the grain matrix indicate that the plates can detect the protein of interest in soybean FG72 grain.

STUDY IDENTIFICATION

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Study Initiation Date: July 17, 2009
Experimental Termination Date: July 31, 2009

ACRONYMS and SCIENTIFIC TERMS

2mEPSPS – Modified 5-enolpyruvyl-shikimate-3-phosphate synthase
BTID – Identification number given to samples
COA – Certificate of Analysis
CV - Coefficient of Variation (relative standard deviation)
ELISA - Enzyme Linked Immunosorbent Assay
FG72 - OECD ID: MST-FGØ72-3
g - Gram
GLP - Good Laboratory Practice
HPPD W336 – modified 4-hydroxyphenylpyruvate dioxygenase
kDa – kiloDaltons
LOD – Limit of Detection
LOQ – Limit of Quantitation
mL – milliLiter
mM – milliMolar
NT- Non-transgenic
PI – Principal Investigator
SD - Standard Deviation
SOP – Standard Operating Procedure
USA - United States of America

1. VALIDATION SCOPE

The validation of SDI EPSPS ELISA plates and Agdia HPPD ELISA plates specific to FG72 grain.

2. BACKGROUND INFORMATION

FG72 Soybean

The transgenic soybean event FG72 contains the *hppdpfw336*[1] and *2mepsps*[2] genes which, when expressed in the plant, produce the HPPD W336 protein (hydroxyl phenyl-pyruvate-dioxygenase) and the 2mEPSPS protein (5-enolpyruvylshikimate-3-phosphate synthase), respectively. The expression of the HPPDW336 protein confers tolerance to isoxaflutole herbicide and the expression of the 2mEPSPS protein confers tolerance to glyphosate herbicide. Planting double-herbicide-tolerant soybean varieties containing transformation event FG72 provides growers with new options for weed control using IFT herbicide (registered in North America as Balance Pro®) in combination with glyphosate herbicide.

ELISA

The ELISA analysis is used to quantify the amount of proteins in Raw Agricultural Commodities and Processed Fractions. The assay, which uses a double antibody sandwich format, has antibodies specific to the protein of interest coated onto the ELISA plate. The sample is incubated in the wells of the plate with the enzyme conjugate of a second antibody to the protein of interest. The two antibodies form an enzyme-labeled sandwich of the protein of interest. After the incubation, the plate is washed to remove unbound material. A second incubation is done with a color or substrate solution. The optical density readings of the samples compared to the standards are used to determine the amount of protein in the samples.

3. EXPERIMENTAL

3.1 Standards

The reference substances used in this study are described in Table 1. 2mEPSPS and HPPD W336 purified protein references (produced in *E. coli)* were used as standards and to fortify non-transgenic samples (grain). 2mEPSPS and HPPD W336 were supplied by Bayer CropScience NV (Gent, Belgium). These reference substances were used for validation and recovery studies.

Table 1 Reference Substances

Reference Substance (protein)	Certificate of analysis / batch number	Chemical name	Molecular Weight
2mEPSPS	BB506-009	modified 5-enolpyruvyl-shikimate-3-phosphate synthase	47 kDa
HPPD W336	BB509-001	modified hydroxyl phenyl-pyruvate-dioxygenase	40 kDa

3.2 Samples

The non-transgenic soybean (variety: Jack) were grown under Study Number HT08SOY002. Samples were received under Study Number DQ09B003[3]. Samples were maintained in a freezer at approximately -20°C until sample preparation. The amounts of 2mEPSPS and HPPD W336 were determined at Bayer CropScience, NC.

3.3 Sample Preparation for ELISA

Grain samples were ground in a blender pre-chilled with dry ice according to SOP 98034. Small amounts of dry ice were added to the blender periodically to ensure the samples remained frozen during preparation. A separate blender was used for each sample. The ground samples were stored in a freezer at approximately -20°C for overnight or longer to allow the dry ice to sublimate before extraction.

3.4 Extraction for ELISA

Total protein was extracted from the raw agricultural product of soybean grain according to SOP 98033. PBST Extraction/Dilution Buffer, Agdia, Inc. Catalog Number ACC 00501A, was used. A representative fraction (approximately 0.1 g) of ground sample was mixed with 4 mL extraction buffer in a 50 mL low-protein binding polypropylene centrifuge tube, and then shaken for 30 minutes at ≈ 4°C on a shaker (IKA-SCHÜTTLER MTS 4) at 250 rpm. The liquid extract was transferred to a clean centrifuge tube and separated by centrifugation at approximately 18000 x g for 10 minutes at ≈ 4°C. The clear supernatant was then used for 2mEPSPS and HPPD W 336 analyses. Duplicate extracts were prepared for each sample.

3.5 ELISA

A commercial ELISA kit (Strategic Diagnostics, Inc. (SDI, Newark, DE), catalogue number 7020100) is available for EPSPS-type proteins. This ELISA was validated in the soybean grain matrix for the 2mEPSPS protein as described in SOP 98052.01. SOP 98052.01 was modified by changing the extraction/dilution buffer to PBST Extraction/Dilution Buffer, (Agdia, Inc. Elkhart, IN, Catalog Number ACC 00501), and the sample to buffer ratio was 0.1g:4mL.

A commercial ELISA kit (Agdia, Inc., catalogue number R54900) is available for the HPPD protein. This ELISA was validated in the soybean grain matrix for the HPPD W336 protein as described in SOP 98060.

3.6 ELISA Plate Calibration and Spiking Concentrations

Protein standards used to generate a calibration curve were included in duplicate on each 2mEPSPS ELISA plate at the following concentrations: 32, 16, 8, 4, 2, 1 and 0.5 ng/mL. Protein standards used to generate a calibration curve were included in duplicate on each HPPD ELISA plate at the following concentrations: 32, 16, 8, 4, 2, and 1 ng/mL.

For 2mEPSPS validation, non-transgenic soybean grain samples were fortified at concentrations of 100, 32, 16, 8, 4, 2, 1 and 0.5 ng/mL and analyzed using the 2mEPSPS calibration curve generated on the plate analyzed. For HPPD validation, non-transgenic soybean grain samples were fortified at concentrations of 100, 32, 16, 8, 4, 2, and 1 ng/mL and analyzed using the HPPD calibration curve generated on the plate analyzed. The fortification standards were added to the extraction buffer at the indicated concentrations prior to extraction in five replicates. .

3.7 Determination of Protein Concentration

SoftMax Pro™ software (Molecular Devices, Version 4.0) was used to derive the concentration of 2mEPSPS and HPPD W336 proteins. Absorbance units were adjusted for the buffer blank and then any background due to the matrix was subtracted using values from wells containing non-transgenic extracts assayed on the same plate. The absorbance readings corrected for both buffer blank and non-transgenic background were converted to the 2mEPSPS and HPPD W336 protein concentration using the standard curve.

3.8 Statistical Analysis

Descriptive statistics (mean, standard deviation, and % coefficient of variance) were calculated in Excel for each sample matrix and analyte[4]. Bayer CropScience conducted all statistical analyses. All statistical analyses were done on data with full precision. Results may be rounded to two or three significant figures.

4. RESULTS AND DISCUSSION

4.1 Validation of 2mEPSPs and HPPD W336 in the Grain Matrix

A summary of the validation data is shown for grain in Table 2 for 2mEPSPS and HPPD W336 proteins. The complete data sets are given in Appendix 1, Table A1-2 to A1-3 for 2mEPSPS and HPPD W336 proteins. The Critical dates of analyses of 2mEPSPS and HPPD are given in Appendix 1, Table A1-1.

Table 2 Validation of Sample Extraction for the 2mEPSPS and HPPD ELISA with Fortified Non-transgenic Controls of Grain

Validation of Soybean Grain for the 2mEPSPS ELISA BTID 1754A				Validation of Soybean Grain for the HPPD ELISA BTID 1754A			
2mEPSPS Fortified (ng/mL)	2mEPSPS Detected [a] (ng/mL)	% 2mEPSPS Recovery	2mEPSPS Recovery	HPPD Fortified (ng/mL)	HPPD Detected [a] (ng/mL)	% HPPD Recovery	HPPD Recovery
	Mean ± SD	Mean ± SD	% CV		Mean ± SD	Mean ± SD	% CV
100	82.5 ± 2.7	82.5 ± 2.7	3.32	100	123 ± 4	123 ± 4	3.01
32	23.0 ± 0.5	71.7 ± 1.6	2.21	32	31.4 ± 1.3	98.2 ± 3.9	4.01
16	10.4 ± 0.3	64.8 ± 1.6	2.45	16	18.0 ± 0.9	112 ± 6	5.26
8	5.14 ± 0.20	64.2 ± 2.6	3.98	8	8.34 ± 0.54	104 ± 7	6.47
4	2.59 ± 0.19	64.8 ± 4.7	7.23	4	3.51 ± 0.24	87.8 ± 5.9	6.78
2	1.21 ± 0.07	60.4 ± 3.5	5.74	2	1.43 ± 0.26	71.6 ± 13.1	18.2
1	0.62 ± 0.10	61.5 ± 9.7	15.7	1 [b]	0.43 ± 0.19	43.0 ± 19.2	44.6
0.5 [b]	0.45 ± 0.12	89.5 ± 23.2	26.0				

[a] The 2mEPSPS and HPPD proteins detected and their recovery are expressed as the average of 20 data points: 5 samples, 2 replicate extracts per sample and 2 replicate assays per extract.

[b] Validity criteria were not met at indicated concentration.

4.2 Limit of Detection and Limit of Quantitation

The limit of detection (LOD) is determined for each matrix using the standard curve and the concentration derived from the background absorbance of the negative control samples. The LOD is the concentration corresponding to an absorbance value three standard deviations above the mean background absorbance value.

The limit of quantitation (LOQ) is defined as the lowest concentration of the standard that meets the criteria for the LOQ. Validation criteria are: a) analyte recoveries from fortified matrix samples are ≥ 60% and ≤ 130% and: b) the coefficient of variance (relative standard deviation) is less than 25%. When a lower recovery is caused by the nature of a specific matrix or the effect of the matrix, the lowest concentration of the standard that gives a smaller coefficient of variance than 25% is used as the LOQ.

An analytical reading giving rise to an analyte concentration below the LOD is reported as ND (Non-detectable). The value of ND in a calculation is zero. Values below the LOQ but about the LOD can be identified as '<LOQ' and the value of the LOQ is used in calculations. A summary of the results is presented below in Table 3. The complete data tables are shown in Appendix 1, Tables A1-4 to A1-5.

Table 3 Limits of Detection and Quantitation of 2mEPSPS and HPPD W336 Protein in Grain as Detected by ELISA

Protein Analyte	Matrix	LOD	LOQ
		ng/g Sample	ng/g Sample
2mEPSPS	Grain	10.3 [a]	40.0 [a]
HPPD W336	Grain	20.8 [a]	80.0 [a]

[a] Calculated based on the extraction of 0.1 g matrix per 4 mL extraction buffer and expressed in ng/g in the sample.

5. CONCLUSIONS

The SDI EPSPS ELISA plate validation indicated that the plates had an LOD of 10.3 ng/g and an LOQ of 40.0 ng/g in soybean grain.

The Agdia HPPD ELISA plate validation indicated that the plates had an LOD of 20.8 ng/g and an LOQ of 80.0 ng/g in soybean grain.

6. ARCHIVING

The protocol and final report are archived under study number DQ08Q004 in the Archives of Bayer CropScience, 2 T. W. Alexander Drive, Research Triangle Park, NC 27709, USA. Raw data, computer generated listings of raw data and supporting documentation are archived under study number DQ09B003 in the Archives of Bayer CropScience, 2 T. W. Alexander Drive, Research Triangle Park, NC 27709, USA.

7. REFERENCES

No.	Author(s). Year. Title. Source. Edition. Pages.
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2	**Lebrun, M., Sailland, A., Freyssinet, G., and Degryse, E. 1997.** Mutated 5-enolpyruvylshikimate-3-phosphate synthase, gene encoding for said protein and transformed plants containing said gene. International patent publication WO 97/04103-A2. 06.02.97. 25 pages.
3	**Poe, Martha R. 2009.** Analyses of the Raw Agricultural Commodity of Soybean Event FG72 for HPPDW336 and 2mEPSPS Proteins. USA 2009. Study Identification Number DQ09B003.
4	**Devore, J; Peck, R. 1986.** Statistics. West Publishing Company, St. Paul

APPENDIX 1

Supporting Data

Table A1-1 Critical Dates for Validation of 2mEPSPS and HPPD Protein for Grain Samples

Biotech Sample ID:	Matrix	Sample received	Sample ground	Sample extracted	Assayed
1754A	Grain	11/13/2008	4/3/2009	7/21/2009	7/21/2009

Table A1-2 Validation of 2mEPSPS ELISA for Grain of Non-transgenic Soybean on BTID Sample 1754A

ng 2mEPSPS / mL spiked	Sample I 2mEPSPS 1 (ng/mL extract)	Sample I 2mEPSPS 2 (ng/mL extract)	Sample II 2mEPSPS 1 (ng/mL extract)	Sample II 2mEPSPS 2 (ng/mL extract)	Sample III 2mEPSPS 1 (ng/mL extract)	Sample III 2mEPSPS 2 (ng/mL extract)	Sample IV 2mEPSPS 1 (ng/mL extract)	Sample IV 2mEPSPS 2 (ng/mL extract)	Sample V 2mEPSPS 1 (ng/mL extract)	Sample V 2mEPSPS 2 (ng/mL extract)	2mEPSPS (ng/mL) Average [a]	2mEPSPS (ng/mL) SD [a]	2mEPSPS (ng/mL) %CV [a]
100	80.3	80.6	82.8	83.7	82.5	77.3	87.0	84.0	85.3	81.9	82.5	2.7	3.32
32	22.3	22.4	22.7	22.8	22.4	23.3	23.1	23.3	23.8	23.5	23.0	0.5	2.21
16	10.4	10.1	10.3	10.5	10.8	10.6	9.91	10.2	10.5	10.4	10.4	0.3	2.45
8	5.27	5.56	5.08	5.26	5.07	5.29	5.02	4.96	4.97	4.88	5.14	0.20	3.98
4	2.56	2.29	2.60	2.62	3.01	2.71	2.45	2.47	2.58	2.62	2.59	0.19	7.23
2	1.07	1.25	1.31	1.27	1.20	1.26	1.13	1.22	1.19	1.20	1.21	0.07	5.74
1	0.47	0.49	0.61	0.75	0.59	0.66	0.56	0.73	0.59	0.71	0.62	0.10	15.7
0.5	0.35	0.76	0.45	0.47	0.44	0.39	0.43	0.44	0.40	0.35	0.45	0.12	26.0

ng 2mEPSPS / mL spiked	% 2mEPSPS Recovery 1	% 2mEPSPS Recovery 2	% 2mEPSPS Recovery 3	% 2mEPSPS Recovery 4	% 2mEPSPS Recovery 5	% 2mEPSPS Recovery 6	% 2mEPSPS Recovery 7	% 2mEPSPS Recovery 8	% 2mEPSPS Recovery 9	% 2mEPSPS Recovery 10	% 2mEPSPS Recovery Average [a]	% 2mEPSPS Recovery SD [a]	% 2mEPSPS Recovery %CV [a]
100	80.3	80.6	82.8	83.7	82.5	77.3	87.0	84.0	85.3	81.9	82.5	2.7	3.32
32	69.7	70.0	70.8	71.2	70.0	72.7	72.3	72.9	74.3	73.3	71.7	1.6	2.21
16	64.8	63.1	64.5	65.8	67.3	66.5	61.9	64.0	65.4	64.8	64.8	1.6	2.45
8	65.8	69.5	63.5	65.8	63.3	66.1	62.8	62.0	62.2	61.0	64.2	2.6	3.98
4	64.0	57.3	65.0	65.4	75.3	67.7	61.3	61.7	64.5	65.6	64.8	4.7	7.23
2	53.6	62.7	65.4	63.3	59.9	62.9	56.4	61.0	59.5	60.0	60.4	3.5	5.74
1	47.3	48.6	61.1	75.1	59.0	65.6	56.0	73.2	58.6	70.9	61.5	9.7	15.7
0.5	69.0	151.2	89.6	93.6	88.4	78.8	85.2	88.8	80.2	69.8	89.5	23.2	26.0

[a] Averages, standard deviations and %CV values are calculated with full precision and then rounded to 2 or 3 significant figures.

Table A1-3 Validation of HPPD ELISA for Grain of Non-transgenic Soybean on BTID Sample 1754A

ng HPPD/ mL spiked	Sample I HPPD 1 (ng/mL extract)	Sample I HPPD 2 (ng/mL extract)	Sample II HPPD 1 (ng/mL extract)	Sample II HPPD 2 (ng/mL extract)	Sample III HPPD 1 (ng/mL extract)	Sample III HPPD 2 (ng/mL extract)	Sample IV HPPD 1 (ng/mL extract)	Sample IV HPPD 2 (ng/mL extract)	Sample V HPPD 1 (ng/mL extract)	Sample V HPPD 2 (ng/mL extract)	HPPD (ng/mL) Average [a]	HPPD (ng/mL) SD [a]	HPPD (ng/mL) %CV [a]
100	116	124	120	127	124	130	122	122	124	123	123	4	3.01
32	32.8	30.6	32.6	30.9	32.4	32.1	30.8	28.6	31.8	31.6	31.4	1.3	4.01
16	18.9	19.5	18.1	18.9	17.4	17.1	16.6	17.0	18.5	17.8	18.0	0.9	5.26
8	8.09	7.23	9.17	8.83	8.65	8.23	8.77	8.13	8.22	8.11	8.34	0.54	6.47
4	3.15	3.14	3.55	3.71	3.65	3.53	3.75	3.28	3.78	3.58	3.51	0.24	6.78
2	0.94	0.97	1.42	1.51	1.53	1.70	1.55	1.56	1.53	1.61	1.43	0.26	18.2
1	0.09	0.13	0.38	0.48	0.44	0.69	0.50	0.61	0.42	0.56	0.43	0.19	44.6

ng HPPD/ mL spiked	% HPPD Recovery 1	% HPPD Recovery 2	% HPPD Recovery 3	% HPPD Recovery 4	% HPPD Recovery 5	% HPPD Recovery 6	% HPPD Recovery 7	% HPPD Recovery 8	% HPPD Recovery 9	% HPPD Recovery 10	% HPPD Recovery Average [a]	% HPPD Recovery SD [a]	% HPPD Recovery %CV [a]
100	116	124	120	127	124	130	122	122	124	123	123	4	3.01
32	102	95.6	102	96.6	101	100	96.3	89.3	99.5	98.9	98.2	3.9	4.01
16	118	122	113	118	109	107	104	106	116	111	112	6	5.26
8	101	90.4	115	110	108	103	110	102	103	101	104	7	6.47
4	78.7	78.6	88.9	92.7	91.2	88.3	93.7	82.1	94.5	89.6	87.8	5.9	6.78
2	47.2	48.4	71.0	75.3	76.6	85.2	77.3	77.8	76.7	80.6	71.6	13.1	18.2
1	9.40	12.5	38.3	48.4	44.2	68.6	49.5	61.0	41.7	56.1	43.0	19.2	44.6

[a] Averages, standard deviations and %CV values are calculated with full precision and then rounded to 2 or 3 significant figures.

Table A1-4 Determination of the 2mEPSPS LOD for Grain of Non-Transgenic Soybean

Matrix	Biotech Sample ID	ng EPSPS/ mL spiked[c]	Sample I EPSPS 1 (OD value)	Sample I EPSPS 2 (OD value)	Sample II EPSPS 1 (OD value)	Sample II EPSPS 2 (OD value)	Sample III EPSPS 1 (OD value)	Sample III EPSPS 2 (OD value)	Sample IV EPSPS 1 (OD value)	Sample IV EPSPS 2 (OD value)	Sample V EPSPS 1 (OD value)	Sample V EPSPS 2 (OD value)	EPSPS (OD value) Average	SD (OD value)	3x SD	3x SD + avg[d]	LOD[d] ng/mL	LOD[d] ng/g
Grain[a]	1754A	0	0.010	0.003	0.022	0.043	0.024	0.041	0.020	0.028	0.019	0.024	0.023	0.012	0.037	0.060		
		neg. ctrl.	0.013	0.011	0.031	0.000	0.009	0.006	0.028	0.007	0.018	0.027	0.015	0.010	0.031	0.046		
		both											0.019	0.012	0.036	0.055		
Grain[b]	1754A	0	-0.009	-0.016	0.003	0.024	0.004	0.022	0.001	0.009	-0.001	0.005	0.004	0.012	0.037	0.041		
		neg. ctrl.	-0.006	-0.008	0.012	-0.019	-0.010	-0.013	0.009	-0.012	-0.001	0.008	-0.004	0.010	0.031	0.027		
		both											0.000	0.012	0.036	0.036	0.258	10.32

[a] OD values were subtracted with the plate background only.
[b] OD values were subtracted with both the plate background and the average 2mEPSPS OD value for the measurements calculated from both the 0 ng/mL spike and the negative control.
[c] "0" identifies the data for the 0 ng/mL spike. "Neg. ctrl." identifies the data for the negative control on the same plate as the 0 ng/mL spike. "Both" identifies the data obtained by averaging the data from the 0 ng/mL spike and the negative control.
[d] The value of 0 ng/g + 3 x SD was entered into the formula for the standard curve on the plate containing the 0 ng/mL spike and the negative control. The result is the LOD in ng/mL which is multiplied by the dilution during extraction (ratio of mL of extraction buffer / grams of matrix extracted) to convert to ng/g.

Table A1-5 Determination of the HPPD W336 LOD for Grain of Non-Transgenic Soybean

Matrix	Biotech Sample ID	ng HPPD/ mL spiked[c]	Sample I HPPD 1 (OD value)	Sample I HPPD 2 (OD value)	Sample II HPPD 1 (OD value)	Sample II HPPD 2 (OD value)	Sample III HPPD 1 (OD value)	Sample III HPPD 2 (OD value)	Sample IV HPPD 1 (OD value)	Sample IV HPPD 2 (OD value)	Sample V HPPD 1 (OD value)	Sample V HPPD 2 (OD value)	HPPD (OD value) Average	SD (OD value)	3x SD	3x SD + avg[d]	LOD[d] ng/mL	LOD[d] ng/g
Grain[a]	1754A	0	-0.035	-0.027	-0.002	-0.009	-0.015	-0.005	-0.018	-0.013	-0.014	0.012	-0.013	0.013	0.039	0.026		
		neg. ctrl.	-0.001	-0.005	-0.019	-0.017	-0.009	-0.008	-0.015	-0.005	0.000	0.000	-0.008	0.007	0.021	0.013		
		both											-0.010	0.010	0.031	0.021		
Grain[b]	1754A	0	-0.024	-0.017	0.008	0.001	-0.005	0.005	-0.008	-0.002	-0.004	0.022	-0.002	0.013	0.039	0.036		
		neg. ctrl.	0.009	0.005	-0.009	-0.006	0.001	0.002	-0.005	0.005	0.011	0.010	0.002	0.007	0.021	0.024		
		both											0.000	0.010	0.031	0.031	0.521	20.8

[a] OD values were subtracted with the plate background only.
[b] OD values were subtracted with both the plate background and the average HPPD OD value for the measurements calculated from both the 0 ng/mL spike and the negative control.
[c] "0" identifies the data for the 0 ng/mL spike. "Neg. ctrl." identifies the data for the negative control on the same plate as the 0 ng/mL spike. "Both" identifies the data obtained by averaging the data from the 0 ng/mL spike and the negative control.
[d] The value of 0 ng/g + 3 x SD was entered into the formula for the standard curve on the plate containing the 0 ng/mL spike and the negative control. The result is the LOD in ng/mL which is multiplied by the dilution during extraction (ratio of mL of extraction buffer / grams of matrix extracted) to convert to ng/g.