SUMMARY

(In accordance with 40 CFR part 152, this summary is available for public release after registration)

STUDY TITLE

Method Validation for the Determination of Aryloxyalkanoate Dioxygenase (AAD-12) in Soybean Tissues Using an Enzyme-linked Immunosorbent Assay (ELISA)

DATA REQUIREMENTS

Not Applicable

AUTHOR(S)

J. K. Smith-Drake, M. J. Sosa, G. Shan

STUDY COMPLETED ON

8-Dec-2009

PERFORMING LABORATORY

Regulatory Sciences and Government Affairs—Indianapolis Lab
Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, Indiana 46268-1054

LABORATORY STUDY ID

081008

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SUMMARY

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- The sensitivity of the method limit of detection (LOD) was 0.5 ng/mg dry weight (DW) and a limit of quantitation (LOQ) was 1.0 ng/mg DW or equivalent to for all tissues. The validated standard-curve quantitative range was 0.25 ng/mL to 10 ng/mL.

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- The efficiency of the tissue extraction process was determined by comparison of five sequential extractions. The apparent extraction efficiency was based on the amount of AAD-12 protein in the first extract relative to the total AAD-12 in all five extracts. The mean extraction efficiency for soybean tissues ranged from 85.8% to 97.2%.

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points. An acceptable recovery (67-100%) was achieved for each spiked concentration at or above the LOQ. The fortification recovery results verified the quantitative range for all tissues.

- Precision/ruggedness of the ELISA method was demonstrated using fortified control forage (whole plant) tissue and positive samples. Ruggedness results were compared within and across days. Data were interpolated from standard curves prepared by two analysts on two separate days. The intra-day percent coefficient of variation (%CV) ranged from 1.1% to 19.7%. The inter-assay %CV across days and analysts ranged from 4.6% to 14.1%.

- Equivalence of standard and test substance response in the AAD-12 ELISA was evaluated using serial dilutions of extracts of forage (whole plant), root, V5 leaf, seed, and V10 leaf positive tissues. For each tissue extract, five to seven of the eight dilutions fell within the quantitative range of the standard curve, and the %CV of the quantified results was less than 20%.

- False-positive and false-negative rates of the ELISA method were tested using unfortified control samples (matrix blanks) and samples fortified at 0.25 ng/mg. There were no false positives from the unfortified control samples and no false negatives from the 0.25 ng/mg fortified samples analyzed during this study.

- Specificity results indicated that there is no cross reactivity from Cry1F, Cry1Ac, Cry34Ab1, Cry35Ab1, PAT, and AAD-1 proteins tested.

Based on the results of this study, it is concluded that the AAD-12 ELISA method is suitable for quantitative measurement of the AAD-12 protein in soybean tissue.

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DATA REQUIREMENTS

Not Applicable

AUTHOR(S)

J. K. Smith-Drake 317-337-3459
[jksmith.drake@dow.com]
M. J. Sosa, G. Shan

STUDY COMPLETED ON

8-Dec-2009

PERFORMING LABORATORY

Regulatory Sciences and Government Affairs—Indianapolis Lab
Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, Indiana 46268-1054

LABORATORY STUDY ID

081008

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: AAD-12

Title: Method Validation for the Determination of Aryloxyalkanoate Dioxygenase (AAD-12) in Soybean Tissues Using an Enzyme-linked Immunosorbent Assay (ELISA)

- STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS:

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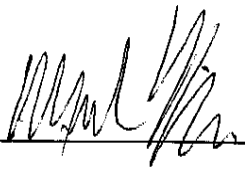
Company: Dow AgroSciences LLC

Company Agent: M. S. Krieger

Title: Regulatory Manager

Signature: 

Date: 8 December 2009

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STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

Title: Method Validation for the Determination of Aryloxyalkanoate Dioxygenase (AAD-12) in Soybean Tissues Using an Enzyme-linked Immunosorbent Assay (ELISA)

Study Initiation Date: 30-Jan-2008

This report represents data generated after the effective date of the EPA FIFRA Good Laboratory Practice Standards.

United States Environmental Protection Agency
Title 40 Code of Federal Regulations Part 160
FEDERAL REGISTER, August 17, 1989

Organisation for Economic Co-Operation and Development
ENV/MC/CHEM(98)17, Paris January 26, 1998

All aspects of this study were conducted in accordance with the requirements for Good Laboratory Practice Standards, 40 CFR 160.

M. S. Krieger
Sponsor
Dow AgroSciences LLC

8 December 2009
Date

M. S. Krieger
Submitter
Dow AgroSciences LLC

8 December 2009
Date

J.K. Smith-Drake
Study Director/Author
Dow AgroSciences LLC

08 DECEMBER 2009
Study Completion Date

Dow AgroSciences Quality Assurance Unit
Good Laboratory Practice Statement Page

Study ID: 081008

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GLP Quality Assurance Inspections

Date of GLP Inspection(s)	Date Reported to the Study Director and to Management	Phases of the Study which received a GLP Inspection by the Quality Assurance Unit
29, 30-Jan-2008	30-Jan-2008	Protocol Review
16-Jul-2009	16-Jul-2009	Precision Day 1: Analysis by ELISA
17-Jul-2009	17-Jul-2009	Repeat of Precision Day 1: Analysis by ELISA
15, 16, 20, 21, 22-Oct-2009	22-Oct-2009	Report and Raw Data Review: Test Substance Container and Sample Verification

QUALITY ASSURANCE STATEMENT:

The Quality Assurance Unit has reviewed the final study report and has determined that the report reflects the raw data generated during the conduct of this study.

TFroggatt 8-Dec-2009

Tracey Froggatt
Dow AgroSciences, Quality Assurance

Date

SIGNATURE PAGE

J. K. Smith-Drake
Author
Dow AgroSciences LLC

8 Oct 09
Date

M. J. Sosa
Co-Author
Dow AgroSciences LLC

8 Oct. 2009
Date

G. Shan
Co-Author
Dow AgroSciences LLC

8 Oct 2009
Date

E. Ma
Peer Reviewer
Dow AgroSciences LLC

8-Oct-2009
Date

K. A. Clayton
Group Leader, Biotechnology Regulatory Science
Dow AgroSciences LLC

08 Oct 2009
Date

STUDY PERSONNEL

Title: Method Validation for the Determination of Aryloxyalkanoate Dioxygenase (AAD-12) in Soybean Tissues Using an Enzyme-linked Immunosorbent Assay (ELISA)

Principal Analyst: (Principle Investigator) M. J. Sosa, Dow AgroSciences, LLC

Author/Co-authors: J. K. Smith-Drake, Dow AgroSciences, LLC
M. J. Sosa, Dow AgroSciences, LLC
G. Shan, Dow AgroSciences, LLC
G. L. Heady, Dow AgroSciences, LLC

TABLE OF CONTENTS

TABLE OF CONTENTS (CONT.)

Page

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ABSTRACT

The objective of this study was to validate the Dow AgroSciences Method GRM 08.04; a direct, double-antibody sandwich enzyme linked immunosorbent assay (ELISA) for measurement of the AAD-12 protein as expressed in the tissues of transgenic soybean plants. The validation included the evaluation of assay sensitivity, specificity, extraction efficiency, accuracy, precision and ruggedness. Soybean tissues included in the validation were as follows: V5 leaf, V10 leaf, forage (whole plant) at R3 growth stage, root at R3 growth stage, and seed.

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points. An acceptable recovery (67-100%) was achieved for each spiked concentration at or above the LOQ. The fortification recovery results verified the quantitative range for all tissues.

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- False-positive and false-negative rates of the ELISA method were tested using unfortified control samples (matrix blanks) and samples fortified at 0.25 ng/mg. There were no false positives from the unfortified control samples and no false negatives from the 0.25 ng/mg fortified samples analyzed during this study.

- Specificity results indicated that there is no cross reactivity from Cry1F, Cry1Ac, Cry34Ab1, Cry35Ab1, PAT, and AAD-1 proteins tested.

Based on the results of this study, it is concluded that the AAD-12 ELISA method is suitable for quantitative measurement of the AAD-12 protein in soybean tissue.

SCIENTIFIC TERMS AND ABBREVIATIONS

AAD-12	aryloxyalkanoate dioxygenase-12
°C	degrees Celsius
µg	microgram (10^{-6} g)
µL	microliter (10^{-6} L)
CV	coefficient of variation
DAS	Dow AgroSciences LLC
DW	dry weight
EE	extraction efficiency
ELISA	enzyme linked immunosorbent assay
g	gram
LOD	limit of detection
LOQ	limit of quantitation
mg	milligram (10^{-3} g)
mL	milliliter (10^{-3} L)
M	molar
mM	millimolar
ng	nanogram (10^{-9} g)
OD	optical density
PBST	phosphate buffered saline with 0.05% Tween 20
QC	quality control
R3 Stage	soybean growth stage- reproductive growth stage where pod is 5 mm long at one of the four uppermost nodes on the main stem with a full developed leaf.
RSD	relative standard deviation (equivalent to CV)
SGN	sample group number
STD	standard
STDEV	standard deviation

TSN	test substance number
V5	soybean growth stages – vegetative growth stage where plant has six nodes with unfolded leaflets
V10	soybean growth stages – vegetative growth stage where plant has ten nodes with unfolded leaflets
WP	whole plant; stem and leaf together at the R3 stage; also called forage

INTRODUCTION

Soybean plants have been modified by the insertion of the *aad-12 gene*, which encodes for the aryloxyalkanoate dioxygenase-12 (AAD-12) protein. When expressed in soybean plants this protein provides tolerance to 2,4-dichlorophenoxyacetic acid and pyridyloxyacetate herbicides.

A rapid, selective, and sensitive method for monitoring protein levels in plant and related products is of significant importance for registration, quality control, and other relevant studies. Immunoassay has been shown to be a useful tool for such a need (1).

An enzyme-linked immunosorbent assay (ELISA) has been developed for the determination of AAD-12 protein expressed in various soybean tissues (2). A validation study is needed to define important method parameters such as accuracy, precision, specificity, quantitative range, and limit of detection were assessed to validate the method in this study. The method will be used to analyze soybean tissue samples from Dow AgroSciences (DAS) Regulatory Laboratories and will support registrations.

MATERIALS AND METHODS

Materials

Test substances

The test substances were representative soybean tissue samples which were genetically modified to express the *aad-12* gene and AAD-12 proteins, and non-transgenic control soybean of the variety Maverick. The tissues, listed below, were collected from the greenhouse. The presence or absence of the AAD-12 protein in the transgenic or control plants was confirmed with specific immunoassay or bioassay.

List of non-transgenic soybean samples:

SGN	Tissue	Sample Description
081008-001-0001	Forage (Whole plant; leaf and stem; R3)	Maverick
081008-004-0001	Root (R3)	Maverick
081008-009-0001	Seed	Maverick
081008-010-0001	Leaves (V5)	Maverick
081008-011-0001	Leaves (V10)	Maverick

List of transgenic soybean samples:

SGN	Tissue	Description
081008-003-0001	Forage (Whole Plant; leaf and stem; R3)	AAD-12
081008-006-0001	Root	AAD-12
081008-012-0001	Seed	AAD-12
081008-013-0001	Leaves (V5)	AAD-12
081008-014-0001	Leaves (V10)	AAD-12 (QC)

Reference Substances

The reference substances employed in this study were a purified AAD-12 protein used as a calibration standard and as fortification material in the ELISA analysis. AAD-1, AAD-12, Cry1F, Cry1Ac, PAT, Cry35Ab1 and Cry34Ab1 proteins were used to test for cross reactivity.

Characterization of the reference standards and documentation of the source is located on file at Dow AgroSciences, LLC archives.

Protein	TSN	Purity or Concentration	Reference
Cry1F	104301	0.164 mg/mL	BIOT033236
AAD-1	105930	0.1805 mg/mL	BIOT09-203007
Cry1Ac	102337	0.26 mg/mL	BIOT08-162946
AAD-12	030732	0.2 mg/mL	BIOT09-203009
PAT	105742	0.3 mg/mL	BIOT063302
Cry35Ab1	104066	0.128 mg/mL	BIOT08-162948
Cry34Ab1	104874	0.248 mg/mL	BIOT09-203014

All test and reference substances were stored in temperature monitored freezers, and removed only for sample preparation and analysis.

Methods

Test System

The test system for this study was the AAD-12 protein ELISA. A specific sandwich ELISA kit (Catalog Number 20-0161 purchased from Beacon Analytical System, Inc. Saco, ME) was used to quantify levels of AAD-12 protein in genetically modified soybean tissues. A method, GRM 08.04, was developed specifically for the quantitation of the AAD-12 protein in the tissues of transgenic soybean plants (see Appendix A). Briefly, the AAD-12 protein is extracted from soybean samples (V5, V10, forage, and root) with phosphate buffered saline solution containing 0.05% Tween 20 (PBST) buffer with 0.75% ovalbumin (OVA) (PBST/OVA). The AAD-12 protein is extracted from soybean seeds with a phosphate buffered saline solution containing 0.05% Tween 20 (PBST) and 0.1% Triton X-100 (TRITON) (PBST/TRITON). The extract is centrifuged; the aqueous supernatant is collected, diluted and assayed using a specific AAD-12 ELISA kit. An aliquot of the diluted sample is incubated with enzyme-conjugated anti-AAD-12 protein monoclonal antibody in the wells of an anti-AAD-12 polyclonal antibody coated plate in a sandwich ELISA format. Both antibodies in the sandwich pair capture the AAD-12 protein in the sample. At the end of the incubation period, the unbound reagents are removed from the plate by washing with PBST. The presence of AAD-12 is detected by incubating the antibody-bound enzyme conjugate with an enzyme substrate, generating a colored product. Since the AAD-12 is bound in the antibody sandwich, the level of color development is proportional to the concentration of AAD-12 in the sample (i.e., lower protein concentrations result in lower color development). The color reaction is stopped by adding an acidic solution and the absorbance at 450 nm minus absorbance at 650 nm is measured using a plate reader. A calibration curve is estimated from the 7 standard concentrations using a quadratic regression equation with a coefficient of determination of >0.990.

Sensitivity and Quantitative Range

The limit of detection (LOD) and limit of quantitation (LOQ) for the determination of AAD-12 in each tissue were empirically defined on the basis of assay parameters (absorbance, background, and linear range), matrix interferences and/or doses constituting the standard curve. They were also supported by statistical approaches following the method of Keith et al. (3) and by testing each control sample fortified with 5 ng/mL (0.5 ng/mg) of AAD-12 protein.

Specificity

Cross-reactivity. The cross-reactivity of this AAD-12 ELISA to non-target proteins Cry1F, Cry1Ac, Cry34Ab1, Cry35Ab1, PAT and AAD-1 was tested in this study. These proteins were prepared at a concentrations of 1 µg/mL and 10 µg/mL in PBST/OVA. On the same plate, an AAD-12 standard curve was generated as a reference. The OD response for the non-target proteins was interpolated from the AAD-12 standard curve and percent cross-reactivity was calculated using the following formula:

% cross-reactivity = 100 x (measured conc. by AAD-12 std curve/theoretical conc. of target protein).

Matrix Testing

Sample extracts (matrix) for each soybean tissue (1X, 5X and 10X dilutions) of negative control were spiked with different concentrations to create standard curves. The matrix-spiked standard curves were interpolated from a non-spiked standard curve run on the same plate. A difference of greater than 15% between the observed (a non-spiked standard curve used to interpolate the matrix-spiked standard concentrations) and theoretical (concentration of the matrix-spiked standard curve) means for each standard concentration level was considered indicative of a potential matrix effect.

Extraction Efficiency

A series of five extractions were performed on transgenic soybean tissues known to express AAD-12. Briefly, 1.5 mL of buffer was added to the tissue sample (15 mg) and extracted as

described in the method (Appendix A). Following extraction and centrifugation, the extracted solution was removed by pipette. After the first extract, an aliquot of 200 µL of buffer was added and mixed with the sample, centrifuged and the supernatant removed and added to the first extraction solution. Another 1.5 mL of buffer was added to the tissue, and the extraction process was repeated. This procedure was repeated three more times to obtain 5 consecutive extractions. The concentration of AAD-12 in each extraction was determined using the AAD-12 ELISA test kit. At least five replicates were studied for each tissue sample. The apparent efficiency of the tissue extraction process was determined by comparison of the AAD-12 protein in the first extract relative to the total AAD-12 protein in all five extracts.

Accuracy

The accuracy of the method was determined by measuring the recovery of the AAD-12 protein from negative control matrices spiked with low (0.5 ng/mg DW), midpoint (1, and 4 ng/mg DW) and high (8 ng/mg DW) levels of AAD-12 protein. A minimum of five replications for each concentration was analyzed. The accuracy of the assay was indicated as percent of recovery.

Precision

The precision of the method was determined using the results of fortified soybean control samples analyzed by two analysts on multiple days. The control sample extracts were fortified with four levels of AAD-12 standard (0.25 ng/mg, 0.5 ng/mg, 4 ng/mg and 8 ng/mg). Each level of fortified extract was run in triplicate on each ELISA plate. The mean recovery concentration, standard deviation (stdev), and percent coefficient of variation (%CV) were calculated for each of the samples.

Positive samples (V5 leaf and forage (whole plant)) were tested for precision as well. The mean predicted concentration, standard deviation (stdev), and percent coefficient of variation (%CV) were calculated for each sample. Within and across day precision were calculated.

Equivalence of Standard and Test Substance Response in the ELISA

The purpose of this experiment was to verify that the AAD-12 protein standard and the AAD-12 protein in plant extracts exhibited a similar overall response in the ELISA. This was done for all transgenic tissues by assessing the agreement of the results from the dilution of a single extract interpolated from the quantitative range of the standard curve. The coefficient of variation for the interpolated results from all quantifiable dilutions was calculated for each tissue type.

False Positive and False Negative

Seed, leaf, forage (whole plant) and root tissues were tested for false-positive and false-negative occurrences. Fifteen unfortified control samples and fifteen samples fortified at 0.25 ng/mg were analyzed for each tissue to determine false-positive and false-negative rates. A false-positive result occurs when residue at or above 0.25 ng/mg is found in a sample known to be free of analytes. A false negative occurs when no residue is detected in a sample fortified at 0.25 ng/mg.

Data Analysis

ELISA readings were recorded from a Molecular Dynamics Microplate Reader using SOFTmax PRO software program. Concentration data were transferred to SAS, JMP or Microsoft Excel for calculations of mean, percent error, statistical mean, standard deviation, and %CV. Example calculations are provided in Appendix A.

RESULTS AND DISCUSSION

Calculated Limits of Quantitation and Detection

The limit of detection (LOD) of an immunoassay is defined as the analyte concentration that gives a response which has a statistically significant difference from the response of a zero analyte sample. The limit of quantitation (LOQ), or the working range of an assay, is generally defined as the highest and lowest concentrations which can be determined with an acceptable

degree of precision. In this study, the targeted LOD and LOQ for the determination of AAD-12 in each tissue were empirically defined on the basis of assay parameters (such as absorbance, background, signal-to-noise ratio, and linear range), matrix interferences, and the standard curve concentrations. The LOD and LOQ were also determined by statistical approaches (3). Following established guidelines, the LOD and LOQ were calculated using the standard deviation from the 0.5 ng/mg recovery results. The LOQ was calculated as ten times the standard deviation (10*s*), and the LOD was calculated as three times the standard deviation (3*s*) of the results of the analysis of a minimum of 5 samples per matrix. The calculated results and target LODs and LOQs for each tissue are summarized in Appendix A-Table 1. The target LOD is 0.5 ng/mg for all soybean matrices. The target LOQ is 1.0 ng/mg for all soybean matrices.

Specificity

Cross-reactivity

Several relevant proteins such as Cry1F, Cry1Ac, Cry34Ab1, Cry35Ab1, PAT, and AAD-1 were tested for cross reactivity. No cross reactivity was observed at the concentrations tested for these proteins (1 µg/mL and 10,000 ng/mL).

Matrix Effect

The results of the matrix tests are summarized in Table 1. A difference of greater than 15% between the observed and theoretical means for any of the seven standard concentration levels was considered indicative of a matrix effect. No matrix effect was observed in root at the 1X spiked-matrix level. No matrix effects were found at the 5X spiked-matrix level for V5 leaf, V10 leaf, forage (whole plant). However, matrix effects were found in seed at the 5X level. For AAD-12 quantification in soybean tissues, a 10X dilution is recommended for all tissue matricies.

Extraction Efficiency

Determining total AAD-12 protein levels in a sample is critical for examining extraction efficiency. Positive samples were extracted with extraction buffer five consecutive times and the AAD-12 protein concentration in each extract was determined by ELISA. The apparent extraction efficiency was based on the amount of AAD-12 protein in the first extraction relative to the total amount of AAD-12 in all five extractions. The extraction efficiencies of the AAD-12 protein from soybean tissues are shown in Appendix A-Table 2. The mean extraction efficiencies for forage (whole plant), root, seed, V5 leaf and V10 leaf ranged from 85.8-97.2%.

Accuracy

The mean recovery levels of AAD-12 from all tissues when fortified at levels equating to the LOQ, mid- and high-points of the standard curve are shown in Appendix A-Table 3. Spiked at the LOQ level or above, V5 leaf, V10 leaf and seed were between 67-100% for the mean recovery with percent coefficient of variances (%CVs) at or below 15%. (Appendix A-Tables 4-8).

Ruggedness/Precision

The precision data from tissue extract fortified at four levels are shown in Appendix A-Tables 9-10. The assay precision and ruggedness were examined using V5 leaf and forage (whole plant) extracts containing four levels of AAD-12 protein. The levels were 8 ng/mg, 4 ng/mg, 0.5 ng/mg and 0.25 ng/mg. The intra-day precision of the assay was less than or equal to 6.3%, 10.8%, 9.6% and 15.0% for the V5 leaf extract fortified at 8, 4, 0.5 and 0.25 ng/mg, respectively. The intra-day precision of the assay was less than or equal to 3.5 %, 13.1%, 10.1% and 10.7% for the forage (whole plant) extract fortified at 8, 4, 0.5 and 0.25 ng/mg, respectively. Positive V5 leaf and forage samples were also tested for assay ruggedness. The intra-day precision of the assay was less than or equal to 9.7% and 19.7% for the V5 leaf and whole plant, respectively.

The inter-assay precision across all days and analysts was 4.6%, 10.1%, 6.4% and 12.9% for the V5 leaf extracts fortified at 8, 4, 0.5 and 0.25 ng/mg, respectively. The inter-assay precision across all days and analysts was 6.0%, 10.5 %, 6.4% and 10.1% for the forage extracts fortified at 8, 4, 0.5 and 0.25 ng/mg, respectively. The inter-assay ruggedness across days and analysts was 11.3% and 14.1% for positive V5 leaf and forage, respectively.

Equivalency

Equivalence of standard and test substance response in the AAD-12 ELISA was demonstrated using up to eight serial dilutions of extracts from AAD-12 positive tissues. For each tissue extract, five or more of the dilutions fell with in the quantitative range of the standard curve, and the %CV of the quantified results was less than 20% (Appendix A Table 11).

False-Positive/False-Negative Rate

Unfortified control samples (matrix blanks) and samples fortified at 0.25 ng/mg (LOD=0.5 ng/mg) were analyzed to determine the false-positive and false-negative rate. There were no false positives from the unfortified control samples and no false negatives reported from samples analyzed in this study.

CONCULSIONS

Dow AgroSciences LLC analytical method GRM 08.04, “Method Validation for the Determination of Aryloxyalkanoate Dioxygenase (AAD-12) in Soybean Tissues Using an Enzyme-linked Immunosorbent Assay (ELISA)”, has been demonstrated to be suitable for its intended purpose. The method was validated over the concentration range of 0.025 to 1.0 ng/mg dry weight (DW) and has a validated limit of quantitation (LOQ) in all soybean tissues of 1.0 ng/mg DW and a limit of detection (LOD) in all soybean tissue of 0.5 ng/mg DW. The AAD-12 protein was recovered at acceptable levels from all tissues. The validated assay is

specific for AAD-12 protein when compared to the non-target proteins tested in previous studies. For AAD-12 protein quantification in soybean tissues a 10X dilution is recommended for all matrices. In addition, AAD-12 protein was efficiently extracted from all soybean tissues. The assay was shown to have acceptable accuracy and precision, and no false-positive or false-negative results were seen below the target LOD. This AAD-12 ELISA method has been demonstrated to be suitable for quantitative measurements of the AAD-12 protein in soybean tissue.

ARCHIVING

The protocol, raw data, and the original version of the final report are all filed in the Dow AgroSciences LLC archives at 9330 Zionsville Road in Indianapolis, Indiana 46268-1054.

REFERENCES

1. Lipton, C. R., Dautlick, J. X., Grothaus, G. D., Hunst, P. L., Magin, K. M., Mihaliak, C. A., Rubio, F. M., and Stave, J. W. 2000. Guidelines for the Validation and Use of Immunoassays for Determination of Introduced Proteins in Biotechnology Enhanced Crops and Derived Food Ingredients, *Food and Agricultural Immunology*, 12:156-164.

2. Smith-Drake, J. K., Sosa, M. J., and Shan, G. 2009. Determination of Aryloxyalkanoate Dioxygenase (AAD-12) Protein in Soybean Tissues by Enzyme-Linked Immunosorbent Assay – GRM 08.04. Unpublished report of Dow AgroSciences, LLC.

3. Keith, L. H., Crummett, W., Deegan, J., Jr., Libby, R. A., Taylor, J. K., Wentler, G. 1983. Principles of Environmental Analysis, *Anal. Chem.*, 55, 2210-2218.

Table 1. Summary of Matrix Effects

Tissue	SGN#	Matrix Dilution[a]			Lowest dilution w/o matrix effect
		1X	5X	10X	
V5 Leaf	081008-010-0001	Yes	No	No	1:5
V10-12 Leaf	081008-011-0001	Yes	No	No	1:5
R3 Forage (whole plant)	081008-001-0001	Yes	No	No	1:5
R3 Root	081008-004-0001	No	No	No	1:2
R8 Seed	081008-009-0001	Yes	Yes	No	1:10

[a] "Yes" represents a standard curve is affected by matrix when the mean percent error between the observed and theoretical values for all the seven standard concentration levels is greater than 15%. "No" represents no matrix effects or the mean percent error between the observed and theoretical values for all the seven standard concentration levels is less than 15%.

APPENDIX A – PROTOCOL AMENDMENTS AND DEVIATIONS

Appendix A Table 1. Study Amendments and Deviations

Protocol Amendments	**Description**
	NONE

Protocol Deviations	
1	Removal of pollen from the list of matricies and addition of information on AAD-12 seed
2	Document a change in the dilution used for the matrix effect.
3	Value of 0.25 for false + and false - experiments

APPENDIX B – GRM 08.04

Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, Indiana 46268-1054

GRM: 08.04
EFFECTIVE: 8-Dec-2009
SUPERSEDES: NEW

Determination of Aryloxyalkanoate Dioxygenase (AAD-12) Protein in Soybean Tissues by Enzyme-Linked Immunosorbent Assay

J. K. Smith-Drake, M. Sosa and G. Shan

1. SCOPE

This method is applicable for the quantitative determination of Aryloxyalkanoate Dioxygenase (AAD-12) protein expressed in soybean tissues using an enzyme-linked immunosorbent assay (ELISA) kit. The calibration standard curve quantitative range is from 0.25 ng/mL to 10 ng/mL in buffer. The AAD-12 protein level in soybean seed, leaf (V5 and V10), root, and forage at R3 stages can be determined with a limit of quantitation (LOQ) of 1.0 ng/mg and a limit of detection (LOD) of 0.5 ng/mg.

2. PRINCIPLE

An analytical method has been developed for the determination of AAD-12 protein expressed in soybean plants. The AAD-12 protein is extracted from soybean samples (leaf V5, leaf V10, forage, and root) with phosphate buffered saline solution containing 0.05% Tween 20 (PBST) buffer with 0.75% ovalbumin (OVA) (PBST/OVA). The AAD-12 protein is extracted from soybean seeds with a phosphate buffered saline solution containing 0.05% Tween 20 (PBST) and 0.1% Triton X-100 (TRITON) (PBST/TRITON). The extract is centrifuged; the aqueous supernatant is collected, diluted and assayed using a specific AAD-12 ELISA kit. An aliquot of the diluted sample is incubated with enzyme-conjugated anti-AAD-12 protein monoclonal antibody in the wells of an anti-AAD-12 polyclonal antibody coated plate in a sandwich ELISA format. Both antibodies in the sandwich pair capture the AAD-12 protein in the sample. At the end of the incubation period, the unbound reagents are removed from the plate by washing with PBST. The presence of AAD-12 is detected by incubating the enzyme conjugate with an enzyme substrate, generating a colored product. Since the AAD-12 is bound in the antibody sandwich, the level of color development is proportional to the concentration of AAD-12 in the sample (i.e., lower protein concentrations result in lower color development). The absorbance at 450 nm minus absorbance at 650 nm is measured using a plate reader. A calibration curve is estimated from the 7 standard concentrations using a quadratic regression equation with a coefficient of determination of >0.990. This AAD-12 ELISA is highly specific for the quantitation of AAD-12 protein. The confirmation of protein identity may be verified using a western blotting standard operating procedure.

3. SAFETY PRECAUTIONS

3.1. Each analyst must be acquainted with the potential hazards of the reagents, products, and solvents used in this method before commencing laboratory work. SOURCES OF INFORMATION INCLUDE: MATERIAL SAFETY DATA SHEETS, LITERATURE, AND OTHER RELATED DATA. Safety information on non Dow AgroSciences LLC products should be obtained from the container label or from the supplier. Disposal of reagents, reactants, and solvents must be in compliance with local, state, and federal laws and regulations.

3.2. Avoid contact of Stopping Solution (0.5% sulfuric acid or 1N hydrochloride acid) with skin and mucous membranes. Wear protective clothing and proper eye protection when working with this material. If this reagent comes in contact with skin, flush the affected area with water.

3.3. It is imperative that proper eye and personal protective equipment be worn when handling these reagents.

4. EQUIPMENT AND MATERIALS (Note 12.1)

4.1. Equipment

4.1.1. Balance, analytical, Model AE50, Mettler Instrument Corporation, Hightstown, NJ 08520.

4.1.2. Balance, analytical, Model AB54-S, Mettler Instrument Corporation.

4.1.3. Centrifuge, capable of holding 96-well plates, Model GR422, catalog number 11176916, Jouan, Inc., Winchester, VA 22602.

4.1.4. Centrifuge, rotor, RTR M4 Hrz 4 Place, catalog number 11175338, Jouan, Inc.

4.1.5. Centrifuge, capable of holding 2 mL Eppendorf tubes, Eppendorf–5417C, Brinkmann Instruments. Inc., Westbury, NY 11590.

4.1.6. Freezer, capable of maintaining –20 °C, Model 75F, U-Line Corporation, Milwaukee, WI 53223.

4.1.7. Freezer, capable of maintaining –80 °C, Model ULT2586, catalog number 13-989-233, Fisher Scientific, Pittsburgh, PA 15205.

4.1.8. Incubator, Precision, Economy, catalog number 51221087, Jouan, Inc.

4.1.9. Pipettor, Eppendorf Maxipetter, catalog number 21-278-43C, Fisher Scientific, or equivalent.

4.1.10. Pipettor, Eppendorf 0.5-10 µL, catalog number 05-402-45, Fisher Scientific or equivalent.

4.1.11. Pipettor, Eppendorf 10-100 µL, catalog number 05-402-48, Fisher Scientific or equivalent.

4.1.12. Pipettor, Eppendorf 100-1000 µL, catalog number 05-402-50, Fisher Scientific or equivalent.

4.1.13. Pipettor, Eppendorf 500-5000 µL, catalog number 05-402-91, Fisher Scientific or equivalent.

4.1.14. Pipettor, Eppendorf 8 channel, 30-300 µL, catalog number 13-688-502, Fisher Scientific or equivalent.

4.1.15. Pipettor, Eppendorf 12 channel, 10-100 µL, catalog number 13-688-504, Fisher Scientific or equivalent.

4.1.16. Pipettor, Eppendorf 12 channel, 30-300 µL, catalog number 13-388-505, Fisher Scientific or equivalent.

4.1.17. Pipet Aid, portable, catalog number 13-681-19, Fisher Scientific.

4.1.18. Plate reader, Spectra Max 190 or M2 microplate reader with SOFTmax PRO software, capable of reading 450 and 650 nm, Molecular Devices, Sunnyvale, CA 94089

4.1.19. Refrigerator, capable of maintaining 4 °C, catalog number 13-991-86, Fisher Scientific.

4.1.20. Shaker/Grinder, Model Geno/Grinder, catalog number 2000-115, Certiprep, Metuchen, New Jersey 08840.

4.1.21. Stir plate, Model 220T, catalog number 14-493-220T, Fisher Scientific.

4.1.22. Vortex, Genie-2 Model, catalog number 12-812, Fisher Scientific.

4.1.23. Washer, 96-well microplate, Model Elx 405, Bio-Tek Instruments, Inc., Winooski, VT 05404.

4.1.24. Water purification system, Model Milli-Q UV Plus, Millipore Corporation, Milford, MA 01757.

4.1.25. Wiley Mill, Intermediate Thomas Wiley Cutting Mill, catalog number 08-338, Fisher Scientific.

4.2. Materials

4.2.1. Basin, Reagent, non-sterile, catalog number 13-681-100, Fisher Scientific.

4.2.2. Bead, 1/8" chrome steel, catalog number 039347, Small Parts Inc., Miami Lakes, FL 33014-0650.

4.2.3. Cap, for 2.0-mL conical tube, catalog number 02-681-361, Fisher Scientific.

4.2.4. Pipet, 10-mL disposable serological, catalog number 13-678-11E, Fisher Scientific.

4.2.5. Pipet tip, 10-µL, Continental Lab Products, catalog number 21-102-8, Fisher Scientific.

4.2.6. Pipet tip, 200-µL, Costar, catalog number 07-200-300, Fisher Scientific.

4.2.7. Pipet tip, 200-µL, Eppendorf, catalog number 21-371-3, Fisher Scientific.

4.2.8. Pipet tip, 1000-µL, Eppendorf, catalog number 21-372-4, Fisher Scientific.

4.2.9. Pipet tip, 1000-µL, Fisher Scientific, catalog number 21-197-8A, Fisher Scientific.

4.2.10. Pipet tip, 5000-µL, Maxitip, Eppendorf, catalog number 21-379-50, Fisher Scientific.

4.2.11. Plate, 96-well, non-binding for sample dilution, catalog number 14-245-145, Fisher Scientific.

4.2.12. Plate cover, 96-well, catalog number 07-200-375, Fisher Scientific.

4.2.13. Plate stand, 96-well, catalog number Z36, 335-9, Sigma, St. Louis, MO 63178.

4.2.14. Tube, 2.0-mL polypropylene conical micro-centrifuge, catalog number 02-681-344, Fisher Scientific.

4.2.15. Tube, 5-mL polypropylene centrifuge with cap, catalog number 14-959-11A, Fisher Scientific.

4.2.16. Tube, 15-mL polypropylene centrifuge with cap, catalog number 05-538-59A, Fisher Scientific.

4.2.17. Weigh dish, small, catalog number 02-204A, Fisher Scientific.

5. REAGENTS, STANDARDS AND PREPARATION

5.1. Reagents (Note 12.1)

5.1.1. AAD-12 Microtiter Plate ELISA Test Kit, catalog number 20-0161, Beacon Analytical Systems, Inc., Saco, Maine 04072 (Note 12.2). Store at 2-8 °C. Contents:

a. Antibody coated 96-well microtiter plates
b. AAD-12 Antibody Conjugate (1x solution)
c. Substrate Solution (120 mL)
d. Stop Solution (120 mL)
e. AAD-12 Microtiter Plate ELISA Assay User's Guide (Note Reference 13.1)

5.1.2. Phosphate buffered saline solution containing 0.05% Tween 20 (PBST), pH 7.4, and catalog number P-3563, Sigma. Store at 25 °C.

5.1.3. Triton X-100 (Triton), catalog number X100-100ML, Sigma-Aldrich. Store at 25 ºC.

5.1.4. Albumin chicken egg (OVA), grade V, Sigma-Aldrich, A5503. Store refrigerated.

5.2. Standards

5.2.1. Obtain AAD-12 microbial protein from Test Substance Coordinator, Dow AgroSciences LLC, 9330 Zionsville Road, Building 304, Indianapolis, IN 46268.

5.2.2. If needed, quality control samples (positive and negative) may be obtained from Dow AgroSciences LLC, 9330 Zionsville Road, Building 304, Indianapolis, IN 46268.

5.3. Reagent and Standard Preparations

5.3.1. Phosphate Buffered Saline, pH 7.4, with 0.05% Tween 20 (PBST) (Note 12.3)

a. Add one packet to 1.0 liter of de-ionized water.
b. Add a stir bar and mix to dissolve on the stir plate.
c. Store at 20-25 ºC up to 3 months or at 2-8 ºC for a maximum of 6 months. Discard if any visible contamination is observed.

5.3.2. Phosphate Buffered Saline, pH 7.4, with 0.05% Tween 20 plus 0.1% Triton (w/v) (PBST/TRITON) to be used with grain:

a. Add 1 g Triton to 1.0 liter PBST in the container (Section 5.3.1).
b. Add a stir bar and mix on stir plate to dissolve.
c. Store at 2-8 ºC up to 3 months. Discard if any visible contamination is observed.

5.3.3. Phosphate Buffered Saline, pH 7.4 with 0.05% Tween 20 plus 0.75% OVA (w/v) (PBST/OVA) to be used with leaf V5, leaf V10, forage and root.

a. Add 0.75 g OVA to 100 mL PBST (Section 5.3.1).
b. Add a stir bar and mix on stir plate to dissolve.
c. Filter through a 0.22 µ sterile filter.
d. Store at 2-8 ºC for 24 hours.

5.3.4. AAD-12 Stock Solution, 2000 ng/mL and 100 ng/mL

a. When working with seed the appropriate buffer is PBST/TRITON. When working with leaf V5, leaf V10, forage and root the appropriate buffer is PBST/OVA
b. The starting AAD-12 standard may be lyophilized powder or aliquoted liquid stock solutions. For example, one stock solution used in DAS Regulatory Laboratories is a 0.2-mg/mL solution.
c. Vortex the stock solution and then add 30 µL of the 0.2-mg/mL AAD-12 stock solution into 2.970 µL of appropriate buffer and mix well to make the 2000-ng/mL stock solution. Similarly, add 200 µL of the 2000-ng/mL stock solution into 3800 µL of appropriate buffer and mix well to make the 100-ng/mL stock solution. Keep them on ice and use within 2 hours. Discard if any visible contamination is observed. For other concentrations dilutions may be performed as needed.

5.3.5. Fortification Solutions

Dilute appropriate aliquots of the stock solution to volume with the appropriate buffer in 15-mL tubes to obtain the desired concentrations for the fortification of recovery samples, as shown in the table below[a]:

Initial Stock Soln. Conc.	Aliquot of Stock Soln.	Buffer Vol. Added	Final Soln. Volume	Spiking Soln. Final Conc.	Equivalent Sample Conc[b]
ng/mL	mL	mL	mL	ng/mL	ng/mg
2000	0.400	9.600	10.0	80	8.0
2000	0.200	9.800	10.0	40	4.0
2000	0.050	9.950	10.0	10	1.0
2000	0.025	9.975	10.0	5	0.5
0	0	10.000	10.0	0.000	0

[a] Spiking solutions can be stored on ice for up to 1 hour after preparation.
[b] The equivalent sample concentrations are based on fortifying the 15-mg samples with 1.5 mL of spiking solutions.

6. INSTRUMENT SETTINGS

To obtain results from the AAD-12 ELISA kit, use the following parameter settings on Microplate Reader:

Parameter	Reader Abbreviation	Setting
Read Mode	Read Mode	Endpoint
Data Reduction	Data Reduction	quadratic
Number of Standard Replicate		3
Number of Standards		7
Control	Ctl	0
Standard #1 Concentration	Std01	10
Standard #2 Concentration	Std02	8
Standard #3 Concentration	Std03	4
Standard #4 Concentration	Std04	2
Standard #5 Concentration	Std05	1
Standard #6 Concentration	Std06	0.5
Standard #7 Concentration	Std07	0.25
Minimum Correlation	Correlation Flag	0.990
Wavelength	Dual Wavelength	450 nm-650 nm
Data Mode	Data Mode	Absorbance
Units	Units	ng/mL
Precision of Standards	Rep %CV Flag	15

7. DETERMINATION OF RECOVERY OF AAD-12 PROTEIN IN SOYBEAN TISSUES

7.1. Preparation of Recovery Samples

7.1.1. Extract the recovery samples at the same time and manner as the unknowns. Store the recovery extracts in the same manner as the unknown samples.

7.1.2. Soybean tissues are stored frozen at -80 ºC until lyophilized. After lyophilization, samples are ground and then stored at -80 ºC until weighed for analysis.

7.1.3. For all sample types, weigh 15-mg portions of the prepared control soybean tissue samples and dispense into 2-mL polypropylene tubes. Add two or three metal beads to each tube. For laboratory recovery samples, add 1.5 mL of the appropriate spiking/extraction solution from Step 5.3.5. A reagent blank and a control should be carried through the method with each sample set. The reagent blank contains 1.5 mL of the appropriate buffer solution. For seed the appropriate buffer is PBST/TRITON. For leaf V5, leaf V10, forage and root the appropriate buffer is PBST/OVA.

7.1.4. Cap all of the tubes. Extract the samples using the Geno/Grinder automatic shaker/grinder at a dial setting of 500 and the toggle switch at the 1X setting (approximately 1500 strokes per minute) for 3 minutes as one cycle.

7.1.5. Centrifuge the samples at 3,000 (or greater) rpm for 5 minutes or until separated (no visible particles in the supernatant). The supernatant can be transferred to a separate tube or aliquoted for analysis as described in Section 7.2. Keep the extract on ice and assay it within 2 hours.

7.1.6. Recommended dilution of 10X for all samples due to matrix effects observed in the validation.

7.1.7. Assay each sample according to the procedure described in Section 7.2.

7.2. Assay Procedure

7.2.1. AAD-12 ELISA Kit Preparation (See Note 12.2 and Reference 13.1)

Bring ELISA plate to 20-25 °C by removing it from the refrigerator at least 30 minutes prior to performing the assay. Store all ELISA kit reagents on ice or refrigerated until used.

7.2.2. Standard Calibration

Prepare standard calibration solutions in 5-mL polypropylene tubes by diluting the 100 ng/mL stock solution from Section 5.3.4 with the appropriate buffer as follows. The following example preparation provides enough standard for 1 plate. Adjust volumes as necessary for additional plates. Store tubes on the benchtop on ice and use within 2 hours of preparation.

Conc. of Stock Soln. (ng/mL)	Aliquot of Stock Soln (µL)	Starting Buffer Volume (µL)	Final Soln. Volume (µL)	Final Standard Conc. (ng/mL)	Remaining Volume after Aliquot (µL)
2000	200	3800	4000	100	3850
100	150	1350	1500	10	700
10	800	200	1000	8	500
8	500	500	1000	4	500
4	500	500	1000	2	500
2	500	500	1000	1	500
1	500	500	1000	0.5	500
0.5	500	500	1000	0.25	1000

7.2.3. ELISA Analysis

7.2.3.1. Conduct each test in an individual microtiter plate. The average of replicate analyses of a sample or standard constitutes a single result. A calibration curve and the appropriate control must be included in each plate.

7.2.3.2. Prepare sample dilutions as needed and transfer diluted samples to the non-binding 96-well microtiter plate (130-150 µL/well) containing the standard calibration solutions and record the location on the 96-well assay template sheet (Figure 1).

7.2.3.3. Transfer the ELISA standard calibration solutions from Step 7.2.2 to a non-binding 96-well microtiter plate (130-150 µL/well) and record the location on the 96-well assay template sheet (Figure 1).

7.2.3.4. Dispense approximate 12 mL of the AAD-12 antibody conjugate per plate into a reagent basin.

7.2.3.5. Pipet 100 µL of the AAD-12 antibody conjugate from the reagent basin to each well of the antibody coated 96-well microtiter plate. **Discard any unused AAD-12 antibody conjugate solution.**

7.2.3.6. Add 100 µL of the ELISA standard solutions and diluted samples from the non-binding 96-well microtiter plate from Steps 7.2.3.2 and 7.2.3.3 to the antibody coated 96-well microtiter plate, keeping the same orientation as the 96-well assay template. Change pipet tips with each sample.

7.2.3.7. Cover the plate with an adhesive plate sealer. Gently swirl the ELISA plate on the benchtop or on a plate shaker for approximately ten seconds to mix the reference standards and diluted samples with the AAD-12 antibody conjugate.

7.2.3.8. Allow the microtiter plate to incubate at room temperature (20-30 °C) for 60 minutes either on the benchtop or in a room temperature incubator.

7.2.3.9. Wash the plate five times with 350 µL/well PBST using an automatic plate washer. Tap out excess liquid on a paper towel.

7.2.3.10. Dispense approximate 12 mL of the color reagent (Substrate Solution) per plate into a reagent basin.

7.2.3.11. Pipet 100 µL of the color reagent from the reagent basin to each well of the antibody coated 96-well microtiter plate. Cover the plate and gently mix. **Discard any unused color reagent solution.**

7.2.3.12. Allow the microtiter plate to incubate at room temperature (20-30 °C) for 15-30 minutes either on the benchtop or in a room temperature incubator (Note 12.4).

7.2.3.13. Dispense approximate 12 mL per plate of the Stop Solution into a reagent basin.

7.2.3.14. Add 100 µL of Stop Solution to each well to stop the reaction. Mix the plate gently. The addition of stop solution should be completed without interruption. Protect the microtiter plate from sunlight; otherwise, color intensity is influenced.

7.2.3.15. Read the absorbance at 450 nm minus 650 nm using a 96-well microtiter plate reader. All readings should be completed within 30 minutes of adding the stop solution.

8. DETERMINATION OF AAD-12 PROTEIN IN SOYBEAN TISSUES

8.1. Prepare the samples as described in Step 7.1.3.

8.1.1. For all sample types, weigh 15-mg portions of the prepared unknown soybean samples and dispense into 2-mL polypropylene tube. Add two or three metal beads to each tube. Add 1.5 mL of the appropriate buffer solution. For seed the appropriate buffer is PBST/TRITON. For leaf V5, leaf V10, forage and root the appropriate buffer is PBST/OVA.

8.2. Extract the samples as described in Steps 7.1.4 and 7.1.5.

8.3. Assay each sample according to the procedure described in Section 7.2. If the sample contains more than 10 ng/mL of AAD-12 protein, perform an additional dilution of the sample from Step 7.2.3.2 prior to assay (e.g., for a 1:10 dilution, pipet 225 µL of appropriate buffer onto a non-binding dilution plate, add 25 µL of sample from Step 8.3 plate, and mix with the pipettor). Assay the diluted aliquot as described in Section 7.2.3.

9. DATA ANALYSIS AND CALCULATIONS

9.1. Calibration Curve

9.1.1. SOFTmax PRO software is available for use with the Spectramax plate reader. SOFTmax PRO allows the creation of computer generated data files containing all of the parameters required for acquiring and analyzing data from any Spectramax instrument. The calibration curve for the AAD-12 ELISA kit is constructed using a quadratic curve regression of the known concentration of the standard calibration solutions and their subsequent absorbance (optical density).

9.1.2. The equation fits the best parabola to the standard curve based on the equation:

$$y = A + Bx + Cx^2$$

Where:

y = mean absorbance value (OD)
x = reference standard concentration

An example of a calibration curve is presented in Figure 2.

9.2. Calculation of AAD-12 in Unknown Samples

9.2.1. The SOFTmax PRO software will calculate the concentration of AAD-12 in each sample as noted in Section 9.1 above. The absorbance value and calculated concentration as well as individual well results, mean sample result, standard deviation and the percent coefficient of variation are reported on the SOFTmax PRO data report.

9.3. Example Calculations

$$\text{Method Factor (MF)} = \frac{\text{weight of tissue (mg)}}{\text{extraction volume (mL)}}$$

Final concentration calculation:

$$\underset{\text{(ng/mg)}}{\text{AAD-12 Concentration}} = \underset{\text{(ng/mL)}}{\text{Mean Result}} / \text{MF}$$

Then:

AAD-12 Concentration (ng/mg)/1000 = AAD-12 Concentration (μg/mg)

Example: For example a 15.0-mg whole plant/forage sample extracted with 1.5 mL of buffer, the mean results of two dilutions of the sample were 76.80 and 70.27 ng/mL.

$$\text{AAD-12 concentration} = \frac{[(76.80+ 70.27)/2] \text{ ng/mL}}{15.0 \text{ mg}/1.5\text{mL}}$$

$$= 7.354 \text{ ng/mg}$$

9.4. Calculation of Percent Recovery

The percent recovery is calculated as the average of all replicate (well) concentrations divided by the fortification concentration.

$$\text{Mean \% recovery} = \frac{\text{the average of protein concentration found}}{\text{fortification concentration}} \times 100$$

$$\text{Recovery} = \frac{7.354 \text{ ng/mg}}{8.0 \text{ ng/mg}} \times 100\%$$

$$\text{Recovery} = 91.9 \%$$

9.5. Predicted Concentration (predconc)

9.5.1. The predicted concentration is the basis for the mean percent error calculation. The predicted concentration is determined using the coefficients of the curve and optical density (OD) readings in the quadratic formula. The regression equation was applied as follows:

$$y = C_1x^2 + C_2x + C_3$$

(where x = predicted concentration and y = OD)

$$\text{predicted concentration} = \frac{-C_2 + \sqrt{C_2^{\,2} - 4C_1 * (C_3 - OD)}}{2C_1}$$

For example, given equation parameters of $C_1 = -0.007$, $C_2 = 0.307$, $C_3 = 0.087$, and OD = 0.388

$$\text{Predconc} = \frac{-0.307 + \sqrt{0.307^2 - 4\ (-0.007)\ (0.087 - 0.388)}}{2\ (-0.007)} = 1.005$$

9.6. Mean Percent Errors

9.6.1. Mean percent errors are determined for each standard concentration of the database curves. The percent error is calculated from the predicted concentration and the theoretical concentration (tconc).

$$\text{Mean percent error} = \left|\frac{\text{predconc} - \text{tconc}}{\text{tconc}}\right| \text{x } 100$$

For example, given the predicted concentration from one of standard curve in method validation for the 1.00-ng/mL standard

Predconc = 1.005 ng/mL

$$\text{mean percent error} = \left|\frac{1.005 - 1.00}{1.00}\right| \text{x } 100 = 0.5\% \text{ error}$$

9.7. Standard Deviation

$$\text{Standard deviation} = \sqrt{\frac{\sum_{i=1}^{N}(y - \bar{y})^2}{N - 1}}$$

Where: y = individual data values,
N = number of data values

9.8. Coefficient of Variation

$$\%\text{ coefficient of variation (CV)} = \frac{\text{standard deviation}}{\text{mean}} \text{ x } 100$$

10. QUALITY CONTROL

10.1. Analytical Batch Definition

An analytical batch of samples is defined as a group of 96 wells. The size of the batch is based on the capacity of the unit (1 solid microplate) of the Beacon AAD-12 ELISA test kit. An analytical batch of less than 96 wells can be analyzed. The first 24 wells (well positions in columns 1, 2, and 3: A1-H1, A2-H2, A3-H3) are used for triplicate analysis of the seven concentrations of the standard. The Quality Control (QC) Sample may be included in each batch if available. Following the Quality Control Sample, up to 35 samples may be analyzed in duplicate (two wells). If more samples are to be analyzed than can be accommodated in one plate, the remaining samples should be analyzed as a different analytical batch with a new standard curve.

10.2. Study Samples

All study samples should be assayed in duplicate. If the concentration of AAD-12 in the sample exceeds the range of the assay, dilute the sample with the appropriate assay buffer (typically a 5- or 10-fold dilution is performed) and then assay the diluted sample aliquot. Multiply the result by the appropriate method factor and dilution factor to obtain the final result.

10.3. Criteria for Acceptance of an Analytical Batch

Each run shall meet the accept criteria in the procedure to be valid as listed below. If the data fail to meet these performance criteria, the analyst should evaluate the results; determine the potential source of the variation, and repeat the analysis if necessary.

Assay Buffer Blank	Absorbance (450 nm-650 nm) < 0.150
10 ng/mL standard	Absorbance (450 nm-650 nm) ≥ 0.900
Calibration curve	r^2 (Correlation of determination) > 0.990
All positive reference standard, OD	CV (OD) of triplicates ≤ 15%
Unknown or QC samples, solution	CV (OD) of replicates ≤ 20%
Quality control samples, solution or positive tissue (if applicable)	Measured value ≤ ±20% expected value or within the range provided

11.0 RESULTS AND DISCUSSION

11.1 Method Validation

11.1.1 Calculated Limits of Detection and Quantitation

The targeted LOD and lower LOQ for the determination of AAD-12 in each tissue were empirically defined on the basis of individual assay, matrix properties and/or concentrations of the standard curve. These LODs and LOQs were further determined

by statistical approaches. Following established guidelines (13.2), the theoretical LOQ and LOD for the determination of AAD-12 protein were calculated using the standard deviation from the results of the recovery samples fortified at 0.5 ng/mg. The theoretical LOQ was calculated as ten times the standard deviation (10s), and the theoretical LOD was calculated as three times the standard deviation (3s), of the results of a minimum of 5 samples. The results are listed in Table 1. The target LOD is 0.5 ng/mg dry weight (DW) for all soybean matrices. The target LOQ is 1.0 ng/mg DW for all soybean matrices. In all cases the target LOD's and LOQ's were higher than the values calculated using the standard deviation. In actual plant samples, numerical results should be reported and noted as less than the LOQ for samples with the AAD-12 protein levels that are above the LOD, but less than the validated LOQ. For results less than the LOD, it should be reported as not detected.

11.1.2 Extraction Efficiency

The efficiency of the sample extraction process was determined by comparison of five serial extractions. The apparent extraction efficiency was based on the amount of AAD-12 protein in the first extraction relative to the total amount of AAD-12 in all five extractions. The extraction efficiencies of the AAD-12 protein from the soybean tissues are shown in Table 2. The average extraction efficiency for soybean tissue ranged from 85.8-97.2%.

11.1.3 Standard Curve

The coefficient of determination (r^2) values for the quadratic regression equations describing the absorbance as a function of standard concentration ranged from 0.994 to 1.000 for analytical batches or plates analyzed during the method validation.

11.1.4 Accuracy/Recovery

The mean recovery levels of AAD-12 protein from sample extracts when spiked at levels equal to the LOQ (1.0 ng/mg DW), LOD (0.5 ng/mg DW), midpoint (4.0 ng/mg DW) and an upper (8.0 ng/mg DW) are summarized in Table 3. Individual accuracy results for each matrix are listed from Table 4 thru Table 8. For results spiked at the LOQ (1.0 ng/mg DW) or above, the mean recovery ranged from 67% to 100% with a %CV ranging from 1.6% to 10.3%.

11.1.5 Ruggedness/Precision

The assay precision and ruggedness were examined using V5 leaf and forage (whole plant) extracts containing four levels of AAD-12 protein. The levels were 8.0 ng/mg, 4.0 ng/mg, 0.5 ng/mg and 0.25 ng/mg.

The intra-day precision of the assay was less than or equal to 6.3%, 10.8%, 9.6% and 15.0% for the V5 leaf extract fortified at 8.0, 4.0, 0.5 and 0.25 ng/mg, respectively (Table 9). The intra-day precision of the assay was less than or equal to 3.5 %, 13.1%,

10.1% and 10.7% for the forage (whole plant) extract fortified at 8.0, 4.0, 0.5 and 0.25 ng/mg, respectively (Table 9).

Positive V5 leaf and forage samples were also tested for assay ruggedness. The intra-day precision of the assay was less than or equal to 9.7% and 19.7% for the V5 leaf and whole plant, respectively (Table 9).

The inter-assay precision across all days and analysts was 4.6%, 10.1%, 6.4% and 12.9% for the V5 leaf extracts fortified at 8.0, 4.0, 0.5 and 0.25 ng/mg, respectively (Table 10). The inter-assay precision across all days and analysts was 6.0%, 10.5 %, 6.4% and 10.1% for the forage extracts fortified at 8.0, 4.0, 0.5 and 0.25 ng/mg, respectively (Table 10). The inter-assay ruggedness across days and analysts was 11.3% and 14.1% for positive V5 leaf and forage, respectively (Table 10).

11.1.6 Equivalency

Equivalence of standard and test substance response in the AAD-12 ELISA was demonstrated using up to eight serial dilutions of extracts from AAD-12 positive tissues. For each tissue extract, five to seven of the dilutions fell within the quantitative range of the standard curve, and the CV of the quantified results was less than 20% (Table 11).

11.1.7 False-Positive/False-Negative

Non-fortified control samples (15 samples per matrix) and fifteen samples (per matrix) fortified at 0.25 ng/mg (LOD=0.5 ng/mg) were analyzed during the study to verify the false-positive and false-negative levels. A false-positive result occurs when a residue (OD value) at or above the established LOD is found in a control sample known to be free of analyte. A false-negative occurs when no residue is detected in a fortified sample. There were no false positives from the non-fortified control samples and no false negative observed from the samples fortified below the LOD.

11.2 Confirmatory Method

If needed, the detection of the AAD-12 protein in soybean tissue samples may be confirmed by western blotting, using Dow AgroSciences, LLC, SOP ECL-27 “SDS-Page and Western Blotting”.

11.3 Assay Time

The time required to analyze a typical batch (35 samples or recoveries in duplicate, 8 standards in triplicate), including the sample extraction, is 4-5 hours.

11.4 Limitation of the Method

This ELISA method is limited to samples where the amount of AAD-12 protein can be

correlated with the level of AAD-12 present in the microbial standard or reference material used.

12 NOTES

12.1 Equipment, glassware, materials, reagents, and chemicals considered to be equivalent to those specified may be substituted with the understanding that their performance must be confirmed by appropriate tests. Common laboratory supplies are assumed to be readily available and are, therefore, not listed.

12.2 The AAD-12 Microtiter Plate ELISA Test Kit, catalog number 20-0161, Beacon Analytical Systems, Inc., Saco, Maine 04072 was used for performing this method validation. An equivalent kit such as AAD-12 kit from Acadia BioSciences, LLC may be used as well.

12.3 PBST solution may be made from individual ingredients to achieve the same concentrations.

12.4 Substrate color development is dependent on environmental parameters and can vary from day to day and lab to lab. The AAD-12 ELISA test kit is designed to give optimum performance at ambient temperatures of 20 °C to 30 °C. The absorbance of the highest reference material should be greater than 0.90 optical density (OD) and should not fall outside the linear range of the spectrometer. At temperatures greater than 30 °C, OD values will increase more rapidly, a reduced substrate incubation time may be necessary. At low temperatures (less than 20 °C) the substrate incubation time should be increased.

12.5 The use of a zero standard is not required for the standard curve. It is at the discretion of the scientist if a zero standard will be used in the standard curve

13 REFERENCES

13.1 Beacon Analytical Systems, Inc., AAD-12 Plat Kit Instructional Insert, Catalog Number 20-0161.

13.2 Keith, L. H.; Crummett, W.; Deegan, J., Jr.; Libby, R. A.; Taylor, J. K.; Wentler, G. 1983, Principals of Environmental Analysis, *Anal. Chem., 55*, 2210-2218.

13.3 Shan, G. and Skoczenski, B. A. 2009. Development and characterization of enzyme linked-immunosorbent assay (ELSIA) for the detection of AAD-12 protein. Unpublished DAS internal report 091127.

Table 1. Summary of LOD and LOQ Calculation of AAD-12 ELISA in Soybean Tissue

Tissue	Spiked Level ng/mg	Average Recovery ng/mg	Standard Deviation *s*	3 x *s*	Target LOD ng/mg	10 x *s*	Target LOQ ng/mg
Forage (Whole Plant)	0.5	0.3	0.06	0.18	0.5	0.60	1.0
Root	0.5	0.3	0.05	0.15	0.5	0.50	1.0
V5 Leaf	0.5	0.3	0.05	0.15	0.5	0.50	1.0
Seed	0.5	0.4	0.01	0.03	0.5	0.10	1.0
V10 Leaf	0.5	0.5	0.01	0.03	0.5	0.10	1.0

Table 2. Summary of Extraction Efficiency of AAD-12 from Soybean Tissue

Sample	SGN#	Mean Extraction Efficiency (%)	Standard Deviation	CV%	%EE Range
Forage (Whole Plant)	081008-003-0001	93.7	1.1	1.1	92.3-95.2
Root	081008-006-0001	90.0	0.4	0.5	89.6-90.6
V5 Leaf	081008-013-0001	97.2	0.2	0.3	96.9-97.6
Seed	081008-012-0001	85.8	5.6	6.6	79.1-91.1
V10 Leaf	081008-014-0001	93.3	1.4	1.5	91.1-94.7

Table 3. Summary of Accuracy Results

Matrix	Fortification Level		Recovery Rate (%)		CV%	n
	ng/mg	ng/mL[a]	Mean	Range		
Forage (Whole Plant)	8	80	71	59-77	9.4	5
	4	40	70	60-79	9.5	5
	1	10	67	57-76	10.3	5
	0.5	5	58	46-77	15.8	5
	1-8	10-80	69	60-79	13.8	20
Root	8	80	72	66-77	6.0	5
	4	40	71	64-76	6.2	5
	1	10	69	62-76	7.9	5
	0.5	5	61	51-76	13.0	5
	1-8	10-80	71	62-77	10.7	20
Leaf V5	8	80	75	66-80	7.4	5
	4	40	76	67-83	8.6	5
	1	10	73	66-82	7.8	5
	0.5	5	65	53-78	12.9	5
	1-8	10-80	75	66-83	10.8	20
Seed	8	80	75	72-77	2.4	5
	4	40	75	74-77	1.6	5
	1	10	74	72-76	2.4	5
	0.5	5	73	71-75	2.6	5
	1-8	10-80	74	72-77	2.4	20
Leaf V10	8	80	99	97-101	1.8	5
	4	40	100	92-105	5.2	5
	1	10	96	94-99	2.8	5
	0.5	5	93	91-94	1.4	5
	1-8	10-80	98	92-105	4.2	20

[a] Samples were diluted 10X prior to analysis.

Table 4. Recovery of AAD-12 Protein from Forage (Whole Plant) (SGN 081008-001-0001)

Sample Number	Date of Analysis	AAD-12 (ng/mg) Spike ng/mg	AAD-12 (ng/mg) Recovery ng/mg	Percent Recovery	Statistical Calculations[a]
Control A	29 Jun 09	0.00	ND[b]		
Control B	29 Jun 09	0.00	ND		
Control C	26 Jun 09	0.00	ND		
Control D	26 Jun 09	0.00	ND		
Control E	30 Jun 09	0.00	ND		
LOD A	29 Jun 09	0.5	0.297[c]	59	$\bar{x}$ = 0.3
LOD B	29 Jun 09	0.5	0.229[c]	46	s = 0.06
LOD C	26 Jun 09	0.5	0.288[c]	58	CV= 15.8 %
LOD D	26 Jun 09	0.5	0.244[c]	49	LOD(3s)[d] = 0.18
LOD E	30 Jun 09	0.5	0.384[c]	77	LOQ(10s)[e] = 0.6
1.0 A (LOQ)	29 Jun 09	1.0	0.757	76	
1.0 B	29 Jun 09	1.0	0.650	65	
1.0 C	26 Jun 09	1.0	0.660	66	$\bar{x}$ = 0.7
1.0 D	26 Jun 09	1.0	0.568	57	s = 0.08
1.0 E	30 Jun 09	1.0	0.738	74	CV= 10.3 %
4.0 A	29 Jun 09	4.0	3.156	79	
4.0 B	29 Jun 09	4.0	2.884	72	
4.0 C	26 Jun 09	4.0	2.774	69	$\bar{x}$ = 2.8
4.0 D	26 Jun 09	4.0	2.406	60	s = 0.27
4.0 E	30 Jun 09	4.0	2.822	71	CV = 9.5 %
8.0 A	29 Jun 09	8.0	6.175	77	
8.0 B	29 Jun 09	8.0	5.860	73	
8.0 C	26 Jun 09	8.0	5.505	69	$\bar{x}$ = 5.6
8.0 D	26 Jun 09	8.0	4.743	59	s = 0.56
8.0 E	30 Jun 09	8.0	5.930	74	CV = 9.4 %
			$\bar{x}$ =	69[f]	
			s =	9.6	
			CV =	13.8	
			n =	20	

[a] Calculated with more digits than are displayed. All calculations were done using Microsoft Excel, Version 2003, using full precision.

[b] ND = None detected at a detection limit of 0.5 ng/mg.

[c] Region of less certainty (positive above limit of detection (0.5 ng/mg), but below limit of quantification (1.0 ng/mg)).

[d] Calculated limit of detection.

[e] Calculated limit of quantitation.

[f] The overall mean recovery is calculated from samples fortified with AAD-12 protein at LOQ or above

Table 5. Recovery of AAD-12 Protein from Root (SGN 081008-004-0001)

Sample Number	Date of Analysis	AAD-12 ng/mg		Percent Recovery	Statistical Calculations[a]
		Spike	Recovery		
Control A	29 Jun 09	0.00	ND[b]		
Control B	29 Jun 09	0.00	ND		
Control C	26 Jun 09	0.00	ND		
Control D	26 Jun 09	0.00	ND		
Control E	30 Jun 09	0.00	ND		
LOD A	29 Jun 09	0.5	0.311[c]	62	$\bar{x}$ = 0.3
LOD B	29 Jun 09	0.5	0.254[c]	51	*s* = 0.05
LOD C	26 Jun 09	0.5	0.302[c]	60	(3s)[d] = 0.15
LOD D	26 Jun 09	0.5	0.268[c]	54	(10s)[e] = 0.5
LOD E	30 Jun 09	0.5	0.381[c]	76	CV = 13.0 %
1.0 A (LOQ)	29 Jun 09	1.0	0.748	75	
1.0 B	29 Jun 09	1.0	0.664	66	
1.0 C	26 Jun 09	1.0	0.660	66	$\bar{x}$ = 0.7
1.0 D	26 Jun 09	1.0	0.624	62	*s* = 0.06
1.0 E	30 Jun 09	1.0	0.762	76	CV = 7.9 %
4.0 A	29 Jun 09	4.0	3.058	76	
4.0 B	29 Jun 09	4.0	2.904	73	
4.0 C	26 Jun 09	4.0	2.797	70	$\bar{x}$ = 2.8
4.0 D	26 Jun 09	4.0	2.567	64	*s* = 0.18
4.0 E	30 Jun 09	4.0	2.897	72	CV = 6.2 %
8.0 A	29 Jun 09	8.0	6.199	77	
8.0 B	29 Jun 09	8.0	5.850	73	
8.0 C	26 Jun 09	8.0	5.721	72	$\bar{x}$ = 5.7
8.0 D	26 Jun 09	8.0	5.270	66	*s* = 0.34
8.0 E	30 Jun 09	8.0	5.618	70	CV = 6.0 %
			$\bar{x}$ =	71[f]	
			s =	7.6	
			CV =	10.7	
			n =	20	

[a] Calculated with more digits than are displayed. All calculations were done using Microsoft Excel, Version 2003, using full precision.

[b] ND = None detected at a detection limit of 0.5 ng/mg.

[c] Region of less certainty (positive above limit of detection (0.5 ng/mg), but below limit of quantification (1.0 ng/mg)).

[d] Calculated limit of detection.

[e] Calculated limit of quantitation.

[f] The overall mean recovery is calculated from samples fortified with AAD-12 protein at LOQ or above

Table 6. Recovery of AAD-12 Protein from V5 Leaf (SGN 081008-010-0001)

Sample Number	Date of Analysis	AAD-12 ng/mg Spike	AAD-12 ng/mg Recovery	Percent Recovery	Statistical Calculations
Control A	29 Jun 09	0.00	ND[b]		
Control B	29 Jun 09	0.00	ND		
Control C	26 Jun 09	0.00	ND		
Control D	26 Jun 09	0.00	ND		
Control E	30 Jun 09	0.00	ND		
LOD A	29 Jun 09	0.5	0.343[c]	69	$\bar{x}$ = 0.3
LOD B	29 Jun 09	0.5	0.263[c]	53	*s* = 0.05
LOD C	26 Jun 09	0.5	0.337[c]	68	(3s)[d] = 0.15
LOD D	26 Jun 09	0.5	0.284[c]	57	(10s)[e] = 0.5
LOD E	30 Jun 09	0.5	0.389[c]	78	CV = 12.9%
1.0 A (LOQ)	29 Jun 09	1.0	0.816	82	
1.0 B	29 Jun 09	1.0	0.698	70	
1.0 C	26 Jun 09	1.0	0.715	71	$\bar{x}$ = 0.7
1.0 D	26 Jun 09	1.0	0.662	66	*s* = 0.06
1.0 E	30 Jun 09	1.0	0.761	76	CV = 7.8%
4.0 A	29 Jun 09	4.0	3.320	83	
4.0 B	29 Jun 09	4.0	3.165	79	
4.0 C	26 Jun 09	4.0	3.114	78	$\bar{x}$ = 3.0
4.0 D	26 Jun 09	4.0	2.690	67	*s* = 0.25
4.0 E	30 Jun 09	4.0	2.888	72	CV = 8.6%
8.0 A	29 Jun 09	8.0	6.207	78	
8.0 B	29 Jun 09	8.0	6.436	80	
8.0 C	26 Jun 09	8.0	6.008	75	$\bar{x}$ = 6.0
8.0 D	26 Jun 09	8.0	5.245	66	*s* = 0.46
8.0 E	30 Jun 09	8.0	6.200	78	CV = 7.4%
			$\bar{x}$ =	75[f]	
			s =	8.1	
			CV =	10.8	
			n =	20	

[a] Calculated with more digits than are displayed. All calculations were done using Microsoft Excel, Version 2003, using full precision.

[b] ND = None detected at a detection limit of 0.5 ng/mg.

[c] Region of less certainty (positive above limit of detection (0.5 ng/mg), but below limit of quantification (1.0 ng/mg)).

[d] Calculated limit of detection.

[e] Calculated limit of quantitation.

[f] The overall mean recovery is calculated from samples fortified with AAD-12 protein at LOQ or above

Table 7. Recovery of AAD-12 Protein from Seed (SGN 081008-009-0001)

Sample Number	Date of Analysis	AAD-12 ng/mg Spike	AAD-12 ng/mg Recovery	Percent Recovery	Statistical Calculations
Control A	06 Jul 09	0.00	ND[b]		
Control B	06 Jul 09	0.00	ND		
Control C	06 Jul 09	0.00	ND		
Control D	07 Jul 09	0.00	ND		
Control E	07 Jul 09	0.00	ND		
LOD A	06 Jul 09	0.5	0.360[c]	72	$\bar{x}$ = 0.4
LOD B	06 Jul 09	0.5	0.369[c]	74	*s* = 0.01
LOD C	06 Jul 09	0.5	0.357[c]	71	(3s)[d] = 0.03
LOD D	07 Jul 09	0.5	0.376[c]	75	(10s)[e] = 0.1
LOD E	07 Jul 09	0.5	0.354[c]	71	CV = 2.6%
1.0 A (LOQ)	06 Jul 09	1.0	0.717[c]	72	
1.0 B	06 Jul 09	1.0	0.764	76	
1.0 C	06 Jul 09	1.0	0.730	73	$\bar{x}$ = 0.7
1.0 D	07 Jul 09	1.0	0.740	74	*s* = 0.02
1.0 E	07 Jul 09	1.0	0.731	73	CV = 2.4%
4.0 A	06 Jul 09	4.0	2.974	74	
4.0 B	06 Jul 09	4.0	2.977	74	
4.0 C	06 Jul 09	4.0	2.976	74	$\bar{x}$ = 3.0
4.0 D	07 Jul 09	4.0	3.088	77	*s* = 0.05
4.0 E	07 Jul 09	4.0	2.996	75	CV = 1.6%
8.0 A	06 Jul 09	8.0	6.011	75	
8.0 B	06 Jul 09	8.0	5.879	73	
8.0 C	06 Jul 09	8.0	6.120	77	$\bar{x}$ = 5.9
8.0 D	07 Jul 09	8.0	5.830	73	*s* = 0.14
8.0 E	07 Jul 09	8.0	5.774	72	CV = 2.4%
			$\bar{x}$ =	74[f]	
			s =	1.8	
			CV =	2.4	
			n =	20	

[a] Calculated with more digits than are displayed. All calculations were done using Microsoft Excel, Version 2003, using full precision.

[b] ND = None detected at a detection limit of 0.5 ng/mg.

[c] Region of less certainty (positive above limit of detection (0.5 ng/mg), but below limit of quantification (1.0 ng/mg)).

[d] Calculated limit of detection.

[e] Calculated limit of quantitation.

[f] The overall mean recovery is calculated from samples fortified with AAD-12 protein at LOQ or above

Table 8. Recovery of AAD-12 Protein from V10 Leaf (SGN 081008-011-0001)

Sample Number	Date of Analysis	AAD-12 ng/mg Spike	AAD-12 ng/mg Recovery	Percent Recovery	Statistical Calculations
Control A	29 Jun 09	0.00	ND[b]		
Control B	29 Jun 09	0.00	ND		
Control C	26 Jun 09	0.00	ND		
Control D	26 Jun 09	0.00	ND		
Control E	30 Jun 09	0.00	ND		
LOD A	29 Jun 09	0.5	0.468[c]	94	$\bar{x}$ = 0.5
LOD B	29 Jun 09	0.5	0.463[c]	93	*s* = 0.01
LOD C	26 Jun 09	0.5	0.472[c]	94	(3s)[d] = 0.03
LOD D	26 Jun 09	0.5	0.463[c]	93	(10s)[e] = 0.1
LOD E	30 Jun 09	0.5	0.455[c]	91	CV = 1.4%
1.0 A (LOQ)	29 Jun 09	1.0	0.940[c]	99	
1.0 B	29 Jun 09	1.0	0.990	99	
1.0 C	26 Jun 09	1.0	0.989	99	$\bar{x}$ = 1.0
1.0 D	26 Jun 09	1.0	0.947	95	*s* = 0.03
1.0 E	30 Jun 09	1.0	0.938	94	CV = 2.8%
4.0 A	29 Jun 09	4.0	4.179	105	
4.0 B	29 Jun 09	4.0	3.963	99	
4.0 C	26 Jun 09	4.0	3.699	92	$\bar{x}$ = 4.0
4.0 D	26 Jun 09	4.0	3.880	97	*s* = 0.22
4.0 E	30 Jun 09	4.0	4.214	105	CV = 5.2%
8.0 A	29 Jun 09	8.0	8.085	101	
8.0 B	29 Jun 09	8.0	7.844	98	
8.0 C	26 Jun 09	8.0	8.006	100	$\bar{x}$ = 8.0
8.0 D	26 Jun 09	8.0	7.755	97	*s* = 0.15
8.0 E	30 Jun 09	8.0	8.085	101	CV = 1.8%
			$\bar{x}$ =	98[f]	
			s =	4.1	
			CV =	4.2	
			n =	20	

[a] Calculated with more digits than are displayed. All calculations were done using Microsoft Excel, Version 2003, using full precision.

[b] ND = None detected at a detection limit of 0.5 ng/mg.

[c] Region of less certainty (positive above limit of detection (0.5 ng/mg), but below limit of quantification (1.0 ng/mg)).

[d] Calculated limit of detection.

[e] Calculated limit of quantitation.

[f] The overall mean recovery is calculated from samples fortified with AAD-12 protein at LOQ or above

Table 9. Summary of Precision Results (Intra-day, Inter-analyst)

Spiked Concentration or Sample		Day 1	Day 2
V5 Leaf Spike			
8 ng/mg	Mean	7.35	7.63
	Stdev (ng/mg)	0.46	0.26
	CV%	6.3	3.4
	Range (ng/mg)	7.03-7.68	7.45-7.82
4 ng/mg	Mean	3.48	3.81
	Stdev (ng/mg)	0.37	0.39
	CV%	10.8	10.4
	Range (ng/mg)	3.22-3.75	3.53-4.09
0.5 ng/mg	Mean	0.42	0.45
	Stdev (ng/mg)	0.04	0.01
	CV%	9.6	1.6
	Range (ng/mg)	0.39-0.45	0.44-0.45
0.25 ng/mg	Mean	0.20	0.23
	Stdev (ng/mg)	0.03	0.03
	CV%	15.0	12.5
	Range (ng/mg)	0.18-0.22	0.21-0.25
Forage Spike			
8 ng/mg	Mean	7.26	8.01
	Stdev (ng/mg)	0.08	0.28
	CV%	1.1	3.5
	Range (ng/mg)	7.21-7.32	7.81-8.21
4 ng/mg	Mean	3.53	3.80
	Stdev (ng/mg)	3.4	0.5
	CV%	9.6	13.1
	Range (ng/mg)	3.29-3.77	3.45-4.16
0.5 ng/mg	Mean	0.43	0.45
	Stdev (ng/mg)	0.04	0.01
	CV%	10.1	1.9
	Range (ng/mg)	0.40-0.46	0.45-0.46
0.25 ng/mg	Mean	0.21	0.23
	Stdev (ng/mg)	0.02	0.02
	CV%	7.6	10.7
	Range (ng/mg)	0.20-0.22	0.21-0.25
V5 Leaf (SGN 081008-013-0001)	Mean	314	265
	Stdev (ng/mg)	10.6	25.9
	CV%	3.4	9.7
	Range (ng/mg)	307-322	247-284
Forage (whole plant) (SGN 081008-003-0001)	Mean	71	61
	Stdev (ng/mg)	2.6	11.9
	CV%	3.7	19.7
	Range (ng/mg)	69-73	52-69

Table 10. Summary of Precision Results (Inter-day, Inter-analyst)

Spiked Concentration or Samples	Mean ng/mg	Standard Deviation ng/mg	CV%	Range ng/mg
		V5 Leaf		
8	7.49	0.35	4.6	7.03-7.82
4	3.64	0.37	10.1	3.22-4.09
0.5	0.43	0.03	6.4	0.39-0.45
0.25	0.21	0.03	12.9	0.18-0.25
Forage (Whole Plant)				
8	7.64	0.46	6.0	7.21-8.21
4	3.66	0.38	10.5	3.29-4.16
0.5	0.44	0.03	6.4	0.40-0.46
0.25	0.22	0.02	10.1	0.20-0.25
V5 Leaf	289.9	32.6	11.3	247.07-321.98
Forage (Whole Plant)	65.7	9.3	14.1	52.06-72.84

Table 11. Equivalence of Standard and Positive Tissue Sample response in the ELISA

Sample	SGN#	Number of Quantifiable Dilutions	Mean ng/mg	Standard Deviation ng/mg	CV%	Range ng/mg
Forage (Whole Plant)	081008-003-0001	7	58.0	2.2	3.73	54.748-61.816
Root	081008-006-0001	7	10.7	0.7	6.20	9.642-11.814
V5 Leaf	081008-013-0001	5	210.3	13.6	6.47	189.113-225.697
Seed	081008-012-0001	5	19.9	3.1	15.47	15.856-23.231
V10	081008-014-0001	6	12.9	2.6	19.86	10.947-17.293

Figure 1. Template for Plate Set-up

Template Sheet

Analyst: _____ Protocol Number: _____ **ELISA Kit Information**

Date: _____ Experimental Purpose: _____ Lot #

Protein Assay: _____ Kit: _____

Standard TSN: _____ Plate: _____

Standard Lot: _____ Conjugate: _____

Sample Matrix: _____ STDs Buffer: _____ Substrate: _____

Sample ID: _____ Sample Buffer/Extraction: _____ Stop Solution: _____

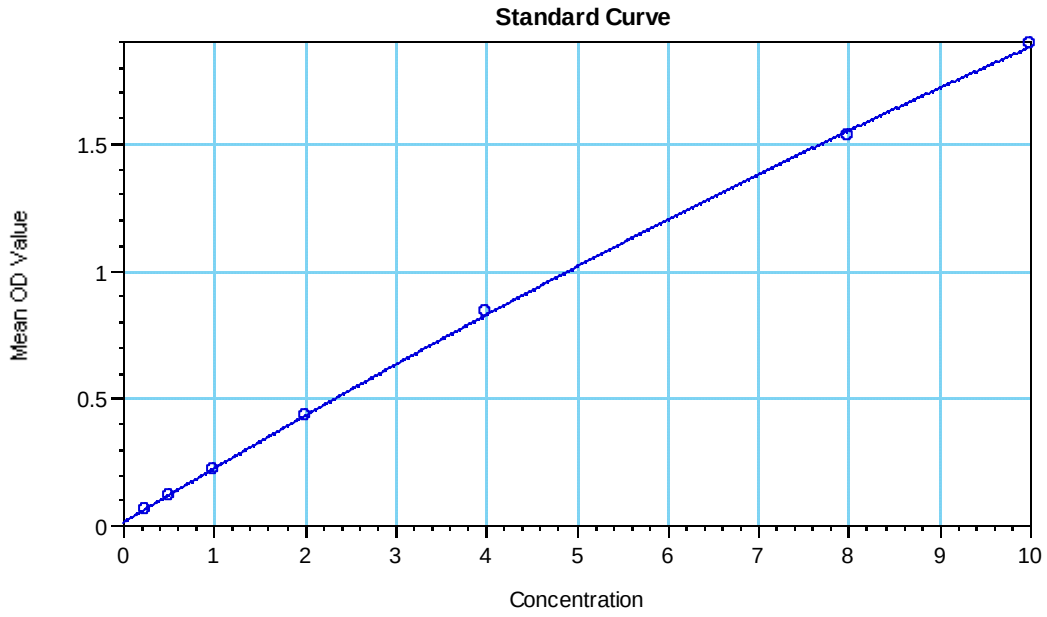
DSTAR (yes/no): _____ Sample Buffer/Dilution: _____ Buffer: _____

Plate Identification: _____

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Comments: _____

Figure 2. Standard Curve



Quadratic Fit: y = A + Bx + Cx^2:	A	B	C	R^2
STD#1 (Standards: Concentration vs Mean OD V...	0.0106	0.215	-0.00283	1