TITLE

Analytical Method for the Determination of Dicamba and Its Major Metabolites in Soy Matrices by LC/MS/MS

GUIDELINE

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PERFORMING LABORATORY

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AG-ME-1321-01

AMENDED REPORT NUMBER

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(original report number MSL0022390)

(1st amended report number MSL0022582)

Amendment 2

STATEMENT OF NO DATA CONFIDENTIALITY CLAIM

The text below applies only to use of the data by the United States Environmental Protection Agency (U.S. EPA) in connection with the provisions of the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA).

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COMPLIANCE STATEMENT

This project meets the U.S. EPA Good Laboratory Practice requirements as specified in 40 CFR Part 160.

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QUALITY ASSURANCE STATEMENT

Project Title: Analytical Method for the Determination of Dicamba and Its Major Metabolites in Soy Matrices by LC/MS/MS

Report Number: MSL0022390
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Reviews conducted by the Quality Assurance Unit confirm that the validation summary report accurately describes the methods and standard operating procedures followed and accurately reflects the raw data of the project.

Following is a list of reviews conducted by the Monsanto Regulatory Quality Assurance Unit on the project reported herein.

Dates of Inspection / Audit	Phase	Date Reported To: Lead Scientist	Management
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PROJECT INFORMATION

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Records Retention: The supporting raw data, the final report and the amended reports are retained in the Monsanto Regulatory Archives, Monsanto Company, St. Louis, MO, USA.

AMENDMENTS TO REPORT

The report for project AG-ME-1321-01 is amended as follows to correct errors in the original report, naming of chemical structures in Figures 1 and 2 and to add representative calibration standard curves to the report. The changes had no impact on the study data or results.

Title Page (Pg 1)

Added: 1) "Amended Report Date" with new date in place of report date
2) "Amended Report Number" with new number and reference to the original report

Changed total number of pages from 105 to 108

Quality Assurance Statement (Pg 5)

Changed "Study" title to "Project" title

Added: 1) "Report" instead of "Project" for original report number
2) "Amended Report Number" with new number

Project Information (Pg 6)

Added: 1) "Amended Report Number" with new number
2) "Mary Mierkowski and Michael J. Miller" as authors
3) "Amended Report Date" with new date

Table of Contents (Pg 8)

Added: Missing entry for Report Approvals

Altered pagination due to amendment

List of Figures (Pg 10)

Added Figures 3-6 to the list of figures

Results and Discussion / Dynamic Range, Accuracy of Standards (Pg 14)

Changed "The correlation coefficient was greater than 0.9992 for all analytes" to "The correlation coefficient was 0.9992 or greater for all analytes"

Added "Representative calibration standard curves are shown in Figures 3-6."

Figures 1 and 2 (Pg 31)

Corrected the names written for structures 3,6-dichlorosalicylic acid and $^{13}C_6$-3,6-dichlorosalicylic acid

Changed "Dicamba" to "dicamba" and "Dichlorogentisic" to "dichlorogentisic" for consistency in capitalization

Figures 3 through 6 (Pg 32-33)

Added representative calibration standard curve plots for each analyte

AMENDMENTS TO REPORT (AMENDMENT 2)

The report for project AG-ME-1321-01 is amended as follows to modify the presentation of the LOQs, to correct statements that incorrectly referred to the LOQ values as "dicamba equivalents", to modify references to the residue study report due to a change in the study report title, to add the residue study report number, to modify references to the metabolism study due to a change in the report number, to add an amendment to the analytical method SOP AG-ME-1321-01, to remove an obsolete copy of the validation plan, to modify the discussion of precision and accuracy results which were referenced to incorrect criteria in the obsolete validation plan, to remove references to "LLMV" and to correct minor typographical or grammatical errors. The changes increased the accuracy of the report and had no impact on the study data or results.

Title Page (Pg 1)

Changed: 1) The amended report date to the latest amended report date
2) The amended report number to the new number and added a reference to the 1st amended report

Changed total number of pages from 108 to 112

Compliance Statement (Pg 3)

Changed the sponsor representative from "Monte A. Marshall" to "Sharon J. Moran"

Quality Assurance Statement (Pg 5)

Changed : 1) "Amended Report Number" to "1st Amended Report Number"

Added: 1) "2nd Amended Report Number" with the new number
2) The second amended report audit information

Project Information (Pg 6)

Changed: 1) "Amended Report Number" to "1st Amended Report Number"
2) "Amended Report Date" to "1st Amended Report Date"
3) "amended report" to "amended reports" in "Records Retention"

Added: 1) "2nd Amended Report Number" with the new number
3) "2nd Amended Report Date" with new date

Corrected the copyright statement

Table of Contents (Pg 10)

Added: "Amendments to Report (Amendment 2)"

Altered pagination due to addition of a second report amendment and an analytical method amendment

Introduction and Summary (Pg. 14), Limit of Quantitation (Pg. 17), Conclusion (Pg. 22)

Changed the soybean residue study REG-08-096 title to "Magnitude of Residues of Dicamba in Soybean Raw Agricultural and Processed Commodities after Application to MON 87708". Added the residue study report number ("MSL0022660").

AMENDMENTS TO REPORT (AMENDMENT 2 CONTINUED)

Introduction and Summary (Pg. 14), Precision and Accuracy (Pg. 17), Sample Extracts 72 Hour Reinjection Stability (Pg. 19), Conclusion (Pg. 22)

Modified and clarified the discussion regarding comparisons to the acceptance criteria to reflect the precison criterion in the revised validation (RSD $\leq$ 20%) rather than the incorrect criterion (RSD $\leq$ 10%) in the original (obsolete) plan.

Introduction and Summary (Pg. 14), Radiovalidation (Pg. 19), Conclusion (Pg. 22), Purpose and Scope of Radiovalidation (Pg. 88)

Changed references to the metabolism study report number from "MSL-20277" to "MSL0022659" to reflect the amended report number.

Introduction and Summary (Pg. 14), Limit of Quantitation (Pg. 17), Conclusion (Pg. 22)

Removed references to "dicamba equivalents" from statements regarding the LOQs.

Calculation of Residues (Pg. 16)

Changed "Injected Concentration (ng/mL analyte)" to "Injected Concentration (µg/mL analyte)"

Limit of Quantitation (Pg. 17)

Changed "0.0050" to "0.005".

Sample Extracts 72 Hour Reinjection Stability (Pg. 19)

Discussion modified to remove references to "LLMV", to correct typographical or grammatical errors, and to clarify the discussion and achieved results.

Table 8 (Pg. 32)

Replaced LOD/LOQ table with a revised table displaying the LOQs as 3 decimal places rather than 4 and removed statement regarding dicamba equivalents.

Added: "[b] The LOD and LOQ are expressed as the concentration of the individual analyte."

Appendix I (Pgs. 37 and 82)

Changed "Appendix I. Analytical Method (With Amendment)" to "Appendix I. Analytical Method (With Amendments)"

Added SOP amendment 2

Appendix III (Pg. 104)

Deleted obsolete validation plan and associated amendment

Added explanatory note

Pgs. 14, 15, 18, 20, 22, 88, 93, 94

Minor errors in grammar or typographical errors were corrected

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ABBREVIATIONS AND ACRONYMS

ACN	acetonitrile
amu	atomic mass unit
API	atmospheric pressure ionization
aq	aqueous
conc.	concentration
DCGA	3,6-dichlorogentisic acid
DCSA	3,6-dichlorosalicylic acid
DI	deionized
ESTC	Environmental Sciences Technology Center
g	gram
HPLC	high-performance liquid chromatography
IS	internal standard
L	liter
LC	liquid chromatography
LC/MS/MS	liquid chromatography tandem mass spectrometry
LLMV	lower limit of method validation
LOQ	limit of quantitation
M	molar
mL	milliliter
mm	millimeter
mM	millimolar
MRM	multiple reaction monitoring
MS	mass spectrometry
NA	not applicable
ND	not detected
Q	quadrupole
QC	quality control
RSD	relative standard deviation
µg	microgram
µg/L	microgram per liter
µm	micrometer
UPLC	ultra performance liquid chromatography
V	volt

1 INTRODUCTION AND SUMMARY

The purpose of this project was to develop and investigate the performance of a method for quantitative analysis of dicamba and its endogenous metabolites, analyzed as the chemophores, 5-hydroxydicamba, 3,6-dichlorosalicylic acid (DCSA) and 3,6-dichlorogentisic acid (DCGA), in soybean matrices (refer to Figure 1 for structures). Analyte-specific stable-labeled internal standards were used in the analytical method AG-ME-1321-01 (Appendix I) to compensate for matrix effects and procedural recovery (refer to Figure 2 for structures). The method was validated according to the validation plan (Appendix III). Radiovalidation, which demonstrated the extraction efficiency and recovery of the method, was conducted using soybean hay and seed samples from study 06-98-M-1, "Metabolism of Dicamba in Dicamba-Tolerant Soybeans", MSL0022659, in which [^{14}C]dicamba was utilized as test substance. The method also was evaluated in soybean processed fractions using spiked-matrix recovery experiments. Verification data for processed fractions were included in the analytical method AG-ME-1321-01 in Appendix I.

The calibration standard curves met the criteria [correlation coefficient (r) of ≥ 0.99 and back-calculated data points within the range of 70 – 120% for a minimum of five calibration levels] at all levels in all sets for each analyte.

The analytical method met acceptance criteria (average recovery must be between 70 and 120% with RSD of ≤ 20% at each fortification level) at the 0.010 µg/g fortification level and above for all analytes in soybean forage and hay. In soybean seed, the precision and accuracy for dicamba, DCSA and DCGA at the 0.010 µg/g fortification level and above met acceptance criteria. The acceptance criterion for accuracy was not met for 5-hydroxydicamba at the 0.010 µg/g level, which is below the statistically-determined LOQ, in soybean seed (66.9% recovery). In addition, it was noted in the soybean residue study REG-08-096 ("Magnitude of Residues of Dicamba in Soybean Raw Agricultural and Processed Commodities after Application to MON 87708", MSL0022660) that recoveries of 70-120% were difficult to achieve consistently for dicamba and 5-hydroxydicamba at the 0.010 µg/g fortification level. The limit of quantitation (LOQ) of the method was determined in study REG-08-096 by statistical analysis of recovery results from low-level fortifications. The LOQs for the four analytes in soybean forage, hay and seed ranged from 0.005 to 0.021 µg/g.

Radiovalidation showed that average extractabilities for pre- and postemergence hay and postemergence seed samples were 94.7%, 94.2% and 48.1%, respectively, and were quite comparable to the extractabilities obtained in the metabolism study. The average total recoveries of radioactivity through the method for pre- and postemergence ^{14}C-hay samples and postemergence ^{14}C-seed samples were 62.9%, 64.3% and 25.5%, respectively. There was generally good agreement between the LC/MS/MS quantitation results and actual residue levels except for the case of low-level DCGA residues in seed.

2 MATERIALS AND METHODS

The analytical method (AG-ME-1321-01) is presented in Appendix I.

2.1 Test Procedures

2.1.1 Test Substances

Analytical Standards

- Dicamba: 3,6-dichloro-2-methoxybenzoic acid, CAS # 1918-00-9, purity, >95%
- 5-Hydroxydicamba: 2,5-dichloro-3-hydroxy-6-methoxybenzoic acid, CAS # 7600-50-2, purity >95%
- DCSA: 3,6-dichloro-2-hydroxybenzoic acid, CAS # 3401-80-7, purity >95%
- DCGA: 2,5-dichloro-3,6-dihydroxybenzoic acid, CAS # 18688-01-2, purity >95%

Internal Standards

- $^{13}C_6$-Dicamba, 3,6-dichloro-2-methoxybenzoic-1,2,3,4,5,6-$^{13}C_6$ acid, CAS # 1173023-06-7
- $^{13}C_6$-5-Hydroxydicamba, 2,5-dichloro-3-hydroxy-6-methoxybenzoic-1,2,3,4,5,6-$^{13}C_6$ acid
- $^{13}C_6$-DCSA, 3,6-dichloro-2-hydroxybenzoic-1,2,3,4,5,6-$^{13}C_6$ acid, CAS #1173019-34-5
- $^{13}C_6$-DCGA, 2,5-dichloro-3,6-dihydroxybenzoic-1,2,3,4,5,6-$^{13}C_6$ acid

2.1.2 Test Systems

The test systems for this method validation were ground soybean seed, hay and forage. Untreated soybean seed, hay and forage from study #06-63-R-4 (Magnitude of Glyphosate Residues in Soybean Raw Agricultural Commodities Following Application of a Glyphosate-based Formulation to MON 89788 and Roundup Ready Soybean) were used as control matrices for this study.

2.2 Analytical Procedure

2.2.1 Reference Substances

The analytical reference standards used in this study were the same as the test substances (section 2.1.1). The same stock solutions were used to prepare the chromatographic calibration standards and fortification solutions.

2.2.2 Sample Analysis

The analytical method for dicamba and its endogenous metabolites, analyzed as the chemophores, 5-hydroxydicamba, 3,6-dichlorosalicylic acid (DCSA) and 3,6-dichlorogentisic acid (DCGA), in soybean matrices is provided in Appendix I. The method incorporates an acid hydrolysis step to hydrolyze DCSA and DCGA glucose conjugates, the major metabolites of dicamba in dicamba-tolerant soybeans, to DCSA and DCGA.

Spiked recovery experiments conducted during method development revealed an issue for DCGA and $^{13}C_6$-DCGA. The recoveries were poor when the analytes were spiked directly onto hay and forage as well as when the analytes were spiked into a container containing matrix and extraction solution. The issue was circumvented by spiking only dicamba, 5-hydroxydicamba, DCSA, and the corresponding $^{13}C_6$ analogs, directly onto the matrix and extracting. DCGA and $^{13}C_6$-DCGA were spiked into the 10 mL extract aliquot following the extraction.

Soy matrices were extracted using 40:60 acetonitrile:water. An aliquot of the extract was hydrolyzed in 1N HCl at 95 ^{o}C in a water bath. The hydrolysate was partitioned with 40:60 ethyl acetate:isooctane, and the organic phase was partially concentrated. Water was then added to the organic phase, and the sample was concentrated until only the aqueous solution remained. Following evaporation of the organic layer, the samples were filtered, acidified and quantitated by LC/MS/MS with turboionspray ionization in negative ion mode.

2.2.3 Calculation of Residues

The LC/MS/MS Analyst® software automatically derives the calibration curve using the area ratio ($AR_{analyte}$) versus the concentration of the standards (µg/mL analyte) from all standards injected with the chromatographic set. A weighted quadratic curve (1/analyte conc.) is used. The resulting equation defining the standard curve is shown below:

$$A\,(\mu g/mL\ analyte)^2 + B\,(\mu g/mL\ analyte) + C = AR_{analyte} \quad where,$$

$AR_{analyte}$ is the detector response (area ratio)
A, B and C are curve constants

The results are calculated automatically by the Analyst® software. The calculation may be checked manually by applying the solution for quadratic equations as shown below. (**Note:** Subtract the response $AR_{analyte}$ from C first.)

$$\text{Injected Concentration (µg/mL analyte)} = \frac{-B \pm \sqrt{(B^2 - 4AC)}}{2A}$$

Further details are provided in the analytical method (Appendix I).

3 RESULTS AND DISCUSSION

3.1 Dynamic Range, Accuracy of Standards

Acceptance criteria: Separate standard curves for each analyte must each have a correlation coefficient (r) of ≥ 0.99 and yield back-calculated data points within the range of 70 – 120% for a minimum of five calibration levels.

Results: The calibration standard curves met the criteria at all levels in all sets for each analyte. The correlation coefficient was 0.9992 or greater for all analytes in all sets. The back-calculated percent of theoretical concentrations ranged from 74 to 119% for all analytes (refer to Tables 1-4). Representative calibration standard curves are shown in Figures 3-6.

3.2 Precision and Accuracy

Soybean seed, hay and forage were analyzed as control samples and fortified with analytes at 0.010, 0.020 and 0.100 µg/g (seven replicates at each level). Fortifications at the 0.005 µg/g level were conducted to attempt to define the LOQ of the method. Results from the 0.005 µg/g fortifications are not included in the following discussion.

Additional fortifications at 2.00 µg/g (two replicates for each matrix) were analyzed to determine accuracy for samples requiring dilution. Results of the analyses are shown in Tables 5-7.

Acceptance criteria: For fortified samples, average recovery must be between 70 and 120% with RSD of ≤ 20% at each fortification level (0.010 µg/g to 0.100 µg/g). The accuracy for fortifications at concentrations above the calibration standard range of the method (2.00 µg/g) must be between 70 and 120%.

Results (0.010, 0.020 and 0.100 µg/g fortifications)

The analytical method meets acceptance criteria for all analytes at 0.010 µg/g to 0.100 µg/g fortification levels in soybean forage. The maximum percent RSD for all analytes was 9.45%. The spiked matrix recoveries for all analytes ranged from 80.9 to 110%.

In soybean hay, the maximum percent RSD for dicamba, 5-hydroxydicamba, DCSA and DCGA, respectively, were 10.5, 9.13, 2.94 and 3.88%. The spiked matrix recoveries for all analytes ranged from 80.0 to 107%.

In soybean seed, the precision and accuracy for dicamba, DCSA and DCGA meet acceptance criteria at all evaluated fortification levels. The maximum percent RSD for these analytes at any fortification level was 5.85%. The spiked matrix recoveries for these analytes ranged from 95.9 to 106%.

The acceptance criterion for accuracy was not met for 5-hydroxydicamba at the 0.010 µg/g level in soybean seed. The percent RSD was 9.19%, but the spiked matrix recovery was only 66.9% at this level. The acceptance criteria for precision and accuracy were met for 5-hydroxydicamba at all higher fortification levels. At higher levels, the maximum percent RSD was 6.53% and the spiked matrix recoveries ranged from 71.3 to 102%.

Results (2.00 µg/g fortification)

The average recovery for fortifications at concentrations above the calibration standard range of the method (2.00 µg/g) demonstrated that the dilution scheme developed for this method was acceptable. The spiked matrix recoveries for all analytes ranged from 72.5 to 104%.

3.3 Limit of Quantitation (LOQ)

Matrix samples fortified at 0.005 µg/g (0.5X the expected LOQ of 0.010 µg/g) were added to each chromatographic set in an attempt to define the limit of quantitation. It was determined by a statistician that this fortification level was not low enough to determine the LOQ. Additional spiked matrix samples at concentration levels of 0.0005 and 0.001 µg/g were added to study REG-08-096 ("Magnitude of Residues of Dicamba in Soybean Raw Agricultural and Processed Commodities after Application to MON 87708", MSL0022660) to determine the LOQ. The limits of quantitation of the method

for the four analytes across forage, hay and seed ranged from 0.005 to 0.021 µg/g (ppm) (refer to Table 8).

3.4 Analyte Stability

The stability of the analytes was tested in stock solutions, working calibration standards and sample extracts.

3.4.1 Stock Solution Stability

Acceptance criteria: The average response for the individual analytes from the stored stock solution must be within 10% of the average response from the new stock solution.

Results: Stock standard solutions (individual analytes at 100 µg/mL in absolute ethanol) were stored at < 10 °C for 201 days. The stability determination was made by diluting each stock solution to 0.5 µg/mL and comparing analyte peak areas of the aged solution to the peak areas of the new solution (eight injections each). The percent difference was calculated as 100 - ((mean old area/mean new area)*100). There was no sample matrix present in this test. This comparison showed that the differences in analyte concentrations between the new stock solution freshly prepared from neat materials and the stored stock solution ranged from -0.6% for DCGA to 5.41% for 5-hydroxydicamba indicating acceptable stability of the stock solutions.

3.4.2 Calibration Standard Spiking Solution Stability

Acceptance criteria: SOP ES-PO-0897-01 “Quantitative Analytical Reference Standard Solution Stability”. If the percent difference in analyte response between old and new solutions is within ±10% at each standard level and there are no outliers, the expiration date can be set to reflect the time interval between preparation of the old and new solutions.

Results: Calibration standard spiking solutions (mixed analytes at 0.01 µg/mL, 0.1 µg/mL and 0.5 µg/mL in acetonitrile) were stored at < 10 °C for 201 days. The stability determination was made by comparing analyte peak areas of the aged solutions to the peak areas of the new solutions (3 injections at each level). Acceptance criteria as outlined in the SOP were met for all analytes at all concentration levels tested. The maximum percent differences for dicamba, 5-hydroxydicamba, DCSA and DCGA, respectively, were -0.39, 2.81, 3.03 and 9.18%.

3.4.3 Working Standards 72 Hour Reinjection Stability

Acceptance criteria: Separate standard curves for each analyte must each have a correlation coefficient (r) of ≥ 0.99 and yield back-calculated data points within the range of 70 – 120% for a minimum of five calibration levels

Results: Chromatographic sets were reinjected 72 hours after each initial analysis. In set 1, the largest difference in correlation coefficient occurred with the dicamba curve. The correlation coefficient for dicamba went from 0.9999 to 0.9992 after 72 hours. In set 2, the dicamba curve correlation coefficient went from 0.9994 to 0.9986 after 72 hours. In

set 3, DCSA had the most instability with the correlation coefficient decreasing from 0.9996 to 0.9977 after 72 hours (refer to Tables 1-4).

The back-calculated percent of theoretical concentrations for the reinjected calibration standards were within the range of 82.4 – 116% for all analytes in all chromatographic sets for standard levels 2-9 (1-500 µg/L). The lowest concentration working calibration standard (Level 1, 0.5 µg/L) failed for at least one analyte in every reinjected set. Working calibration standard curves for each analyte in each set had acceptable correlation coefficients of greater than 0.99 after 72 hours. The calibration standard curves met the acceptance criteria in all matrices for each analyte.

3.4.4 Sample Extracts 72 Hour Reinjection Stability

The final analyte solutions were reanalyzed after storage for approximately 72 hours. Refer to Tables 5-7 for the results of the reinjection analyses.

Acceptance criteria: Average recovery must be between 70 and 120% with RSD of ≤ 20% at each fortification level (0.01 µg/g to 0.100 µg/g). The accuracy for fortifications at concentrations above the calibration standard range of the method (2.00 µg/g) must be between 70 and 120%.

Results (0.010, 0.020 and 0.100 µg/g fortifications)

Acceptance criteria were achieved for all analytes at the 0.010 µg/g level and above in soybean forage. The average reinjection recoveries ranged from 92.2% to 112% with RSD of 3.17- 15.7%.

In soybean hay, the precision and accuracy acceptance criteria were achieved at the 0.010 µg/g level and above for all analytes. For all analytes, the average reinjection recoveries were 91.0% to 110% with RSD of 3.84-11.9%.

In soybean seed, the precision and accuracy acceptance criteria were achieved at the 0.010 µg/g level and above for dicamba, DCSA and DCGA. The maximum %RSD for these analytes at any fortification level was 7.47%. The spiked matrix recoveries for these analytes ranged from 82.9 to 106%. The acceptance criterion for accuracy was not achieved at the 0.010 and 0.020 µg/g fortification levels for 5-hydroxydicamba in soybean seed. The acceptance criterion for precision was achieved at the 0.020 µg/g fortification level, but not at the 0.010 µg/g level. At 0.020 µg/g, the percent accuracy was 63.2% with a percent RSD of 4.16%. Compared to an original percent accuracy of 71.3% for 5-hydroxydicamba at this level, this represents a small reduction in accuracy. It should be noted that the 0.010 and 0.020 µg/g fortifications are below or at the LOQ (Table 8) of 0.021 µg/g for 5-hydroxydicamba in seed.

Results (2.00 µg/g fortification)

The accuracy criterion was met for all analytes in forage, hay and seed at the 2.00 µg/g fortification level. Average reinjection recoveries were 78.0-98.9%.

In summary, good 72-hour reinjection stability was observed for all analytes in the samples at fortification levels at or above the LOQ.

3.5 Radiovalidation

A radiovalidation, which demonstrated the extraction efficiency and recovery of the method, was conducted using soybean hay and seed samples from study 06-98-M-1, "Metabolism of Dicamba in Dicamba-Tolerant Soybeans", MSL0022659, in which [^{14}C]-dicamba was utilized as test substance. Specifically, endogenous radioactive residues were extracted, hydrolyzed and quantitated from soybean hay collected from a preemergence (PRE-T) treatment, and soybean hay and seed collected from a postemergence (POE-T) treatment of [^{14}C]-dicamba to soybeans. Duplicate samples of each matrix were carried through the analytical method. Aliquots were removed at each major step of the method and analyzed by liquid scintillation counting (LSC) to determine radioactivity recoveries. The final analyte solutions were analyzed by HPLC with radioactivity detection and by LC/MS/MS to quantitate the residues. For details of the radiovalidation analysis, see Appendix II.

3.5.1 Extraction Efficiency

The analytical method incorporates a single extraction of soybean matrices with 40:60 acetonitrile:water [10:1 (volume:weight) solvent to sample ratio] followed by centrifugation. For the PRE-T hay, POE-T hay and POE-T seed samples, the amount of radioactive residues extracted averaged 94.7%, 94.2% and 48.1%, respectively. These extractabilities compare well to the corresponding extractabilities obtained in the metabolism study using multiple acetonitrile:water extractions (90.6%, 95.7% and 55.5%, for preemergence hay, postemergence hay and postemergence seed, respectively).

3.5.2 Method Recovery

Average overall recoveries of radioactivity through the method, including the extraction, hydrolysis, partitioning, evaporation and final filtration steps, were 62.9%, 64.3% and 25.5% for PRE-T hay, POE-T hay and POE-T seed, respectively. The low overall radioactivity recovery for seed was caused by the low extractability, due to significant unextracted (bound) residues, and larger losses in the partitioning step likely due to the presence of a significant amount of water-soluble residues (e.g, sugars) in the seed extract. Recoveries for the hydrolysis, evaporation and final filtration steps were near quantitative (>90% each) for all matrices. Average recoveries for the partitioning step were 74.3% and 74.5% for the hay samples, and 58.7% for the seed sample. Losses in the partitioning step may be due to 1) water solubility of a percentage of the residues (e.g., polar residues formed by degradation of dicamba to small molecules and incorporation into natural products such as sugars), especially for seed, 2) incomplete partitioning of the analytes into the organic phase, and 3) procedural losses incurred in use of the phase separation filter paper. **Note:** use of internal standards in this method compensates for any procedural losses incurred in the hydrolysis, partitioning, evaporation and filtration steps.

3.5.3 Quantitation of Residues

The analytical method converts DCSA and DCGA conjugates present in dicamba-treated soybean commodities by acid hydrolysis to DCSA or DCGA, respectively. These analytes along with dicamba and 5-hydroxydicamba are quantitated by LC/MS/MS in the method (data for 5-hydroxydicamba are not included in this radiovalidation because it was not observed as a metabolite in the metabolism study).

The final analyte solutions obtained by conducting the method on the hay and seed samples containing endogenous ^{14}C-labeled residues were analyzed by HPLC with fraction collection and liquid scintillation counting of the fractions (HPLC/LSC). Refer to the ^{14}C-histograms in Figures 4-6 of Appendix II. These HPLC analyses demonstrated that DCSA, DCGA and dicamba were the only significant radioactive components of the final analyte solutions. DCSA was the major component in all three samples, constituting 89.71%, 78.50% and 69.59% of the radioactivity in the final analyte solutions for PRE-T hay, POE-T hay and POE-T seed, respectively. Together, DCSA, DCGA and dicamba constituted 96.18%, 96.54% and 89.62% of the PRE-T hay, POE-T hay and POE-T seed HPLC profiles, respectively.

Residues in the final analyte solutions from the method were quantitated by LC/MS/MS, the normal determinative step of the method. For comparison to the LC/MS/MS quantitation results, the storage stability HPLC profiles of the PRE-T hay, POE-T hay and POE-T seed obtained at the end of the analysis phase of the metabolism study were quantitated to determine the amounts (in ppm) of DCSA-forming metabolites, DCGA-forming metabolites and dicamba present in the samples. These storage stability analyses were conducted prior to the initiation of the radiovalidation within 40 days of the initiation of the radiovalidation analyses.

A comparison of the LC/MS/MS quantitation results with the HPLC metabolite quantitation results is presented in Table 9. There is generally very good agreement, especially for DCSA and dicamba, between the mass spectral quantitation results and the actual residue concentrations present in the hay and seed samples as determined by HPLC profiling of the storage stability extracts. Only the quantitation results for DCGA at very low levels of DCGA (0.025 ppm in POE-T seed and 0.032 ppm in PRE-T hay) were substantially different than the actual values (quantitation of DCGA in the POE-T hay sample, at a level of 2.28 ppm, was very good). DCGA residues in the PRE-T hay (0.032 ppm) were overestimated by approximately a factor of 2, while the DCGA residues in the POE-T seed (0.025 ppm) were underestimated by a factor of 5. It should be noted that the DCGA residue levels for these two samples were near the limit of quantitation for DCGA. The reason for the discrepancy in the seed DCGA quantitation is not known. The HPLC profile of the final analyte solution shows a substantial DCGA peak which is 17.36% of the profiled radioactivity (about $1/4^{th}$ the level of DCSA in the sample in agreement with what is expected based on the quantitation of the HPLC stability profile). Thus, the DCGA analyte was present in the final analyte solution at the expected level, but it was not accurately quantitated in the LC/MS/MS analysis. This appears to be an aberration, and not a true indication of the capability of the method to

quantitate DCGA in soybean seed at levels of approximately 0.02 ppm, because very good recoveries of DCGA spiked onto soybean seed at 0.02 ppm were obtained in the method validation (see Table 5).

4 CONCLUSIONS

The LC/MS/MS method for dicamba and its major metabolites, 5-hydroxydicamba, 3,6-dichlorosalicylic acid (DCSA) and 3,6-dichlorogentisic acid (DCGA) in soy matrices has been validated to meet acceptance criteria described in the validation plan (see Appendix III).

The calibration standard curves met the criteria [correlation coefficient (r) of ≥ 0.99 and back-calculated data points within the range of 70 – 120% for a minimum of five calibration levels] at all levels in all chromatographic sets for each analyte.

The analytical method met acceptance criteria (average recovery must be between 70 and 120% with RSD of ≤ 20% at each fortification level) at the 0.010 µg/g fortification level and above for all analytes in soybean forage and hay. In soybean seed, the precision and accuracy for dicamba, DCSA and DCGA at the 0.010 µg/g fortification level and above met acceptance criteria. The acceptance criterion for accuracy was not met for 5-hydroxydicamba at the 0.010 µg/g level, which is below the statistically-determined LOQ, in soybean seed (66.9% recovery). In addition, it was noted in the soybean residue study REG-08-096 ("Magnitude of Residues of Dicamba in Soybean Raw Agricultural and Processed Commodities after Application to MON 87708", MSL0022660) that recoveries of 70-120% were difficult to achieve consistently for dicamba and 5-hydroxydicamba at the 0.010 µg/g fortification level. The limit of quantitation (LOQ) of the method was determined in study REG-08-096 by statistical analysis of recovery results from low-level fortifications. The LOQs for the four analytes in soybean forage, hay and seed ranged from 0.005 to 0.021 µg/g.

The precision and accuracy of the spiked matrices at concentrations outside of the calibration standard limit of the method (2.00 µg/g) demonstrate that the dilution scheme developed for this method is acceptable.

The method was evaluated in samples of soybean hay from the pre- and post-emergence treatments and soybean seed from the post-emergence treatment of study 06-98-M-1, "Metabolism of Dicamba in Dicamba-Tolerant Soybeans", MSL0022659, in which [^{14}C]-dicamba was utilized as test substance. Average extractabilities for pre- and postemergence hay and postemergence seed samples were 94.7%, 94.2% and 48.1%, respectively, and were quite comparable to the extractabilities obtained in the metabolism study. The average total recoveries of radioactivity through the method for pre- and postemergence ^{14}C-hay samples and postemergence ^{14}C-seed samples were 62.9%, 64.3% and 25.5%, respectively. There was generally good agreement between the LC/MS/MS quantitation results and actual residue levels except for the case of low-level DCGA residues in seed.

The analytical method described in AG-ME-1321-01 is suitable for use in Monsanto Regulatory studies as an accurate means of quantifying dicamba and its endogenous metabolites, analyzed as chemophores 5-hydroxydicamba, 3,6-dichlorosalicylic acid (DCSA) and 3,6-dichlorogentisic acid (DCGA) in soybean raw agricultural commodities.

5 TABLES

Table 1: Dicamba Calibration Standard Back-Calculated Percent of Theoretical

Analyte: Dicamba							
		Set 1		Set 2		Set 3	
Level	Concentration	Initial	72 hr	Initial	72 hr	Initial	72 hr
Level 1	0.5 µg/L	101	97.2	119	131	91.0	105
Level 2	1 µg/L	105	106	96.3	113	103	101
Level 3	5 µg/L	94.2	96.3	93.0	84.6	98.0	92.5
Level 4	10 µg/L	103	92.3	103	82.4	99.9	101
Level 5	25 µg/L	100	114	94.0	94.2	101	108
Level 6	50 µg/L	94.4	90.3	89.0	86.7	105	103
Level 7	100 µg/L	101	107	105	105	106	102
Level 8	250 µg/L	101	97.4	102	105	94.1	87.0
Level 9	500 µg/L	99.7	100	99.5	98.5	101	99.2
Correlation coefficient:		**0.9999**	**0.9992**	**0.9994**	**0.9986**	**0.9992**	**0.9992**

Table 2: 5-Hydroxydicamba Calibration Standard Back-Calculated Percent of Theoretical

Analyte: 5-Hydroxydicamba							
		Set 1		Set 2		Set 3	
Level	Concentration	Initial	72 hr	Initial	72 hr	Initial	72 hr
Level 1	0.5 µg/L	109	116	114	91.6	102	93.4
Level 2	1 µg/L	98.1	92.9	97.3	90	99.9	103
Level 3	5 µg/L	95.5	93.9	95.5	104	97.6	104
Level 4	10 µg/L	101	94.4	94.6	109	101	96.8
Level 5	25 µg/L	96.0	98.2	102	107	103	92.3
Level 6	50 µg/L	98.2	100	96.6	98.5	96.4	102
Level 7	100 µg/L	102	108	97.8	101	99.8	107
Level 8	250 µg/L	100	95.4	103	98.3	101	102
Level 9	500 µg/L	99.9	101	99.4	100	99.8	99.6
Correlation coefficient:		**0.9999**	**0.9993**	**0.9998**	**0.9998**	**0.9999**	**0.9998**

Table 3: DCSA Calibration Standard Back-Calculated Percent of Theoretical

Analyte: DCSA							
		Set 1		Set 2		Set 3	
Level	Concentration	Initial	72 hr	Initial	72 hr	Initial	72 hr
Level 1	0.5 µg/L	98.7	103	103	107	74.0	67.5
Level 2	1 µg/L	99.0	98.2	94.7	101	96.8	89.9
Level 3	5 µg/L	99.3	97.7	100	96.9	111	106
Level 4	10 µg/L	100	99.6	99.5	99.1	111	106
Level 5	25 µg/L	104	101	101	98.7	108	112
Level 6	50 µg/L	98.9	102	101	96.9	102	90.2
Level 7	100 µg/L	101	98.3	102	97.9	101	116
Level 8	250 µg/L	98.8	100	98.2	103	96.2	112
Level 9	500 µg/L	100	100	100	99.2	101	102
Correlation coefficient:		**0.9999**	**1.0000**	**0.9999**	**0.9998**	**0.9996**	**0.9977**

Table 4: DCGA Calibration Standard Back-Calculated Percent of Theoretical

Analyte: DCGA							
		Set 1		Set 2		Set 3	
Level	Concentration	Initial	72 hr	Initial	72 hr	Initial	72 hr
Level 1	0.5 µg/L	100	69.7	115	139	86.5	90.6
Level 2	1 µg/L	95.8	86.4	98.7	115	97.4	90.6
Level 3	5 µg/L	103	106	94.3	106	106	105
Level 4	10 µg/L	99.1	94.6	96.1	102	107	100
Level 5	25 µg/L	100	99.9	98.1	101	101	102
Level 6	50 µg/L	102	98.2	95.1	92.9	103	100
Level 7	100 µg/L	102	102	100	103	103	101
Level 8	250 µg/L	98.2	99.5	103	99.7	96.5	97.2
Level 9	500 µg/L	100	100	99.2	100	101	100
Correlation coefficient:		**0.9999**	**1.0000**	**0.9999**	**1.0000**	**1.0000**	**1.0000**

Table 5: LC/MS/MS Analysis of Dicamba and Its Major Metabolites in Soybean Seed

	5-OH Dicamba		DCGA		DCSA		Dicamba	
Level Spiked	**Initial**	**72 hr reinjection**	**Initial**	**72 hr reinjection**	**Initial**	**72 hr reinjection**	**Initial**	**72 hr reinjection**
µg/g	**µg/g**	**µg/g**	**µg/g**	**µg/g**	**µg/g**	**µg/g**	**µg/g**	**µg/g**
0	ND	ND	0.00136	0.000175	ND	0.00226	ND	ND
0	ND	ND	0.000772	0.00	ND	0.00189	ND	ND
0	ND	ND	0.000604	0.00	ND	0.00173	ND	ND
0	ND	ND	0.000752	0.0000146	ND	0.0016	ND	ND
0	ND	ND	0.000969	0.00	ND	0.00154	ND	ND
0	ND	ND	0.000479	0.00	ND	0.00123	ND	ND
0	ND	ND	0.00105	0.00	ND	0.00157	ND	ND
Mean =	**NA**	**N/A**	**0.000855**	**0.0000271**	**NA**	**0.00169**	**NA**	**N/A**
0.005	0.00353	ND	0.00434	0.00334	0.00525	0.00458	0.00531	0.00381
0.005	0.00317	ND	0.00466	0.00336	0.00555	0.00468	0.00489	0.00378
0.005	0.00333	ND	0.00419	0.00335	0.00544	0.00443	0.005	0.00421
0.005	0.00277	ND	0.00465	0.00357	0.00477	0.00449	0.00533	0.00463
0.005	0.00371	ND	0.00410	0.00335	0.00537	0.00409	0.00547	0.00310
0.005	0.00294	ND	0.00463	0.00344	0.00560	0.00506	0.00566	0.00394
0.005	0.00286	ND	0.00421	0.00351	0.00530	0.00432	0.00455	0.00523
Mean =	**0.00319**	**N/A**	**0.00440**	**0.00342**	**0.00533**	**0.00452**	**0.00517**	**0.00410**
%RSD =	**11.1**	**N/A**	**5.54**	**2.69**	**5.18**	**6.72**	**7.35**	**16.6**
Accuracy =	**63.7**	**N/A**	**88.0**	**68.4**	**107**	**90.5**	**103**	**82.0**
0.01	0.00656	0.00425	0.00954	0.00808	0.0113	0.0108	0.00958	0.00850
0.01	0.00609	0.00294	0.0100	0.00860	0.0102	0.00971	0.0105	0.00903
0.01	0.00777	0.0045	0.00974	0.00856	0.0102	0.00951	0.00997	0.00790
0.01	0.00663	0.00611	0.00914	0.00847	0.0105	0.00931	0.00949	0.00859
0.01	0.00655	0.00545	0.00944	0.00798	0.0107	0.00991	0.00990	0.00895
0.01	0.00604	0.00386	0.00954	0.00817	0.0107	0.00951	0.00941	0.00964
0.01	0.00722	0.00368	0.00964	0.00817	0.0105	0.00981	0.0110	0.00827
Mean =	**0.00669**	**0.00440**	**0.00959**	**0.00829**	**0.0106**	**0.00980**	**0.00998**	**0.00870**
%RSD =	**9.19**	**24.6**	**2.88**	**3.00**	**3.55**	**5.02**	**5.85**	**6.52**
Accuracy =	**66.9**	**44.0**	**95.9**	**82.9**	**106**	**98.0**	**99.8**	**87.0**

Values reported as actual analyte concentrations.

Table 5: LC/MS/MS Analysis of Dicamba and Its Major Metabolites in Soybean Seed (Continued)

	5-OH Dicamba		DCGA		DCSA		Dicamba	
Level Spiked	**Initial**	**72 hr reinjection**	**Initial**	**72 hr reinjection**	**Initial**	**72 hr reinjection**	**Initial**	**72 hr reinjection**
µg/g	**µg/g**	**µg/g**	**µg/g**	**µg/g**	**µg/g**	**µg/g**	**µg/g**	**µg/g**
0.02	0.0143	0.0130	0.0190	0.0197	0.0204	0.0184	0.0192	0.0192
0.02	0.0135	0.0124	0.0200	0.0212	0.0218	0.0182	0.0205	0.0197
0.02	0.0141	0.0117	0.0195	0.0200	0.0206	0.0204	0.0201	0.0178
0.02	0.0141	0.0124	0.0206	0.0210	0.0213	0.0194	0.0196	0.0161
0.02	0.0161	0.0131	0.0187	0.0212	0.0209	0.0200	0.0188	0.0167
0.02	0.0132	0.0132	0.0188	0.0198	0.0211	0.0206	0.0181	0.0170
0.02	0.0145	0.0127	0.0195	0.0201	0.0205	0.0192	0.0207	0.0173
Mean =	**0.0143**	**0.0126**	**0.0195**	**0.0204**	**0.0209**	**0.0195**	**0.0196**	**0.0177**
%RSD =	**6.53**	**4.16**	**3.52**	**3.31**	**2.39**	**4.81**	**4.81**	**7.47**
Accuracy =	**71.3**	**63.2**	**97.4**	**102**	**105**	**97.3**	**97.9**	**88.4**
0.1	0.101	0.100	0.0965	0.103	0.104	0.106	0.0943	0.0983
0.1	0.103	0.102	0.103	0.105	0.103	0.103	0.0968	0.0877
0.1	0.103	0.0983	0.101	0.105	0.104	0.111	0.101	0.0912
0.1	0.100	0.0968	0.0990	0.105	0.103	0.098	0.0995	0.0961
0.1	0.105	0.105	0.102	0.107	0.107	0.109	0.0926	0.0989
0.1	0.102	0.103	0.105	0.107	0.105	0.106	0.0973	0.0884
0.1	0.098	0.106	0.102	0.105	0.0998	0.106	0.0980	0.0817
Mean =	**0.102**	**0.102**	**0.101**	**0.105**	**0.104**	**0.106**	**0.0971**	**0.0918**
%RSD =	**2.25**	**3.35**	**2.76**	**1.31**	**2.12**	**4.05**	**2.97**	**6.92**
Accuracy =	**102**	**102**	**101**	**105**	**104**	**106**	**97.1**	**91.8**
2	2.09	1.69	1.76	1.79	2.01	1.85	1.72	1.91
2	2.07	1.73	1.75	1.66	1.88	1.74	1.79	1.59
Mean =	**2.08**	**1.71**	**1.75**	**1.72**	**1.95**	**1.79**	**1.76**	**1.75**
Accuracy =	**104**	**86.5**	**87.7**	**83.0**	**97.3**	**89.7**	**87.8**	**87.5**

Values reported as actual analyte concentrations.

Table 6: LC/MS/MS Analysis of Dicamba and Its Major Metabolites in Soybean Hay

	5-OH Dicamba		DCGA		DCSA		Dicamba	
Level Spiked	**Initial**	**72 hr reinjection**	**Initial**	**72 hr reinjection**	**Initial**	**72 hr reinjection**	**Initial**	**72 hr reinjection**
µg/g	**µg/g**	**µg/g**	**µg/g**	**µg/g**	**µg/g**	**µg/g**	**µg/g**	**µg/g**
0	ND	ND	0.000175	0.00217	ND	0.00113	ND	ND
0	ND	ND	0	0.00192	ND	0.000926	ND	ND
0	ND	ND	0	0.00187	ND	0.00106	ND	ND
0	ND	ND	0.0000146	0.00197	ND	0.000904	ND	ND
0	ND	ND	0	0.00200	ND	0.000981	ND	ND
0	ND	ND	0	0.00205	ND	0.00105	ND	ND
0	ND	ND	0	0.00207	ND	0.000803	ND	ND
Mean =	**NA**	**N/A**	**0.0000271**	**0.00201**	**NA**	**0.000979**	**NA**	**N/A**
0.005	0.00370	0.00297	0.00334	0.00387	0.00473	0.00445	ND	0.00520
0.005	0.00357	0.00364	0.00336	0.00372	0.00502	0.00421	ND	0.00633
0.005	0.00458	0.00421	0.00335	0.00407	0.00482	0.00462	ND	0.00500
0.005	0.00365	0.00343	0.00357	0.00416	0.00466	0.00423	ND	0.00538
0.005	0.00390	0.00228	0.00335	0.00377	0.00494	0.00374	ND	0.00556
0.005	0.00392	0.00335	0.00344	0.00361	0.00465	0.00439	ND	0.00623
0.005	0.00377	0.00290	0.00351	0.00424	0.00526	0.00439	ND	0.00500
Mean =	**0.00387**	**0.00325**	**0.00342**	**0.00392**	**0.00487**	**0.00429**	**NA**	**0.00553**
%RSD =	**8.73**	**18.8**	**2.69**	**6.10**	**4.55**	**6.50**	**NA**	**10.0**
Accuracy =	**77.4**	**65.1**	**68.4**	**78.5**	**97.4**	**85.8**	**NA**	**111**
0.01	0.00711	0.00875	0.00808	0.00969	0.0110	0.00972	0.00801	0.00967
0.01	0.00881	0.00976	0.00860	0.00969	0.0106	0.00962	0.00967	0.0106
0.01	0.00823	0.00811	0.00856	0.00859	0.0104	0.00861	0.00812	0.00991
0.01	0.00769	0.00902	0.00847	0.00919	0.0102	0.0101	0.00872	0.0115
0.01	0.00830	0.0116	0.00798	0.00949	0.0101	0.00942	0.00876	0.00961
0.01	0.00800	0.00930	0.00817	0.00859	0.0104	0.00912	0.00827	0.0106
0.01	0.00786	0.00891	0.00817	0.00939	0.0107	0.00922	0.00883	0.00974
Mean =	**0.00800**	**0.00935**	**0.00829**	**0.00924**	**0.0105**	**0.00941**	**0.00863**	**0.0102**
%RSD =	**6.67**	**11.9**	**3.00**	**5.11**	**2.94**	**5.14**	**6.56**	**6.83**
Accuracy =	**80.0**	**93.5**	**82.9**	**92.4**	**105**	**94.1**	**86.3**	**102**

Values reported as actual analyte concentrations.

Table 6: LC/MS/MS Analysis of Dicamba and Its Major Metabolites in Soybean Hay (Continued)

	5-OH Dicamba		DCGA		DCSA		Dicamba	
Level Spiked	**Initial**	**72 hr reinjection**	**Initial**	**72 hr reinjection**	**Initial**	**72 hr reinjection**	**Initial**	**72 hr reinjection**
µg/g	**µg/g**	**µg/g**	**µg/g**	**µg/g**	**µg/g**	**µg/g**	**µg/g**	**µg/g**
0.02	0.0145	0.0178	0.0177	0.0184	0.0217	0.0191	0.0176	0.0195
0.02	0.0153	0.0158	0.0185	0.0185	0.0213	0.0187	0.0187	0.0176
0.02	0.0167	0.0201	0.0186	0.0174	0.0208	0.0174	0.0211	0.0171
0.02	0.0168	0.0205	0.0179	0.0180	0.0215	0.0187	0.0223	0.0199
0.02	0.0176	0.0224	0.0181	0.0185	0.0217	0.0188	0.0171	0.0173
0.02	0.0176	0.0216	0.0182	0.0184	0.0209	0.0179	0.0201	0.0169
0.02	0.0191	0.0193	0.0179	0.0199	0.0207	0.0207	0.0174	0.0191
Mean =	**0.0168**	**0.0196**	**0.0181**	**0.0184**	**0.0212**	**0.0188**	**0.0192**	**0.0182**
%RSD =	**9.13**	**11.5**	**1.82**	**4.09**	**2.01**	**5.53**	**10.5**	**6.90**
Accuracy =	**84.0**	**98.2**	**90.5**	**92.2**	**106**	**93.9**	**95.9**	**91.0**
0.1	0.107	0.115	0.0975	0.0968	0.106	0.0928	0.108	0.0979
0.1	0.101	0.104	0.104	0.0955	0.107	0.102	0.0984	0.0963
0.1	0.106	0.102	0.103	0.0884	0.111	0.101	0.103	0.0938
0.1	0.0947	0.114	0.0964	0.0972	0.108	0.105	0.106	0.0790
0.1	0.103	0.115	0.0957	0.0898	0.105	0.101	0.104	0.0933
0.1	0.101	0.108	0.0984	0.101	0.111	0.098	0.112	0.0857
0.1	0.102	0.110	0.105	0.0917	0.103	0.101	0.104	0.0943
Mean =	**0.102**	**0.110**	**0.100**	**0.0943**	**0.107**	**0.100**	**0.105**	**0.0915**
%RSD =	**3.94**	**4.84**	**3.88**	**4.80**	**2.78**	**3.84**	**4.05**	**7.34**
Accuracy =	**102**	**110**	**100**	**94.3**	**107**	**100**	**105**	**91.5**
2	1.81	1.72	1.86	2.06	1.77	1.71	1.43	1.60
2	1.78	2.07	1.74	1.90	1.75	1.75	1.47	1.64
Mean =	**1.80**	**1.90**	**1.80**	**1.98**	**1.76**	**1.73**	**1.45**	**1.62**
Accuracy =	**89.8**	**94.8**	**90.0**	**98.9**	**88.0**	**86.5**	**72.5**	**81.0**

Values reported as actual analyte concentrations.

Table 7: LC/MS/MS Analysis of Dicamba and Its Major Metabolites in Soybean Forage

	5-OH Dicamba		DCGA		DCSA		Dicamba	
Level Spiked	**Initial**	**72 hr reinjection**	**Initial**	**72 hr reinjection**	**Initial**	**72 hr reinjection**	**Initial**	**72 hr reinjection**
µg/g	**µg/g**	**µg/g**	**µg/g**	**µg/g**	**µg/g**	**µg/g**	**µg/g**	**µg/g**
0	ND	ND	0.00133	0.000432	ND	ND	ND	ND
0	ND	ND	0.00141	0.000353	ND	ND	ND	ND
0	ND	ND	0.00149	0.000577	ND	ND	ND	ND
0	ND	ND	0.00134	0.000464	ND	ND	ND	ND
0	ND	ND	0.00135	0.000312	ND	ND	ND	ND
0	ND	ND	0.00151	0.000390	ND	ND	ND	ND
0	ND	ND	0.00148	0.000371	ND	ND	ND	ND
Mean =	**NA**	**N/A**	**0.00142**	**0.000414**	**NA**	**NA**	**NA**	**N/A**
0.005	0.00477	0.00566	0.00328	0.00387	0.00551	0.00475	0.00529	0.00407
0.005	0.00461	0.00578	0.00374	0.00448	0.00510	0.00438	0.00518	0.00540
0.005	0.00554	0.00594	0.00349	0.00409	0.00535	0.00558	0.00442	0.00555
0.005	0.00482	0.00554	0.00355	0.00435	0.00472	0.00554	0.00596	0.00445
0.005	0.00447	0.00529	0.00368	0.00445	0.00511	0.00457	0.00570	0.00515
0.005	0.00485	0.00520	0.00377	0.00430	0.00487	0.00491	0.00522	0.00474
0.005	0.00522	0.00539	0.00388	0.00466	0.00514	0.00500	0.00506	0.00570
Mean =	**0.00490**	**0.00554**	**0.00363**	**0.00431**	**0.00511**	**0.00496**	**0.00526**	**0.00501**
%RSD =	**7.49**	**4.84**	**5.57**	**6.10**	**5.22**	**9.23**	**9.30**	**12.1**
Accuracy =	**97.9**	**111**	**72.6**	**86.2**	**102**	**99.2**	**105**	**100**
0.01	0.00989	0.00916	0.00788	0.00928	0.0127	0.0128	0.00985	0.00984
0.01	0.00976	0.00968	0.00802	0.00812	0.0114	0.0121	0.0103	0.00867
0.01	0.0104	0.00943	0.00820	0.0101	0.0108	0.0111	0.00969	0.0102
0.01	0.00927	0.0115	0.00816	0.00913	0.0107	0.0109	0.00957	0.0109
0.01	0.0105	0.00882	0.00817	0.00989	0.0102	0.0102	0.00955	0.00938
0.01	0.00952	0.0112	0.00848	0.00950	0.0106	0.0105	0.00963	0.0118
0.01	0.0102	0.00993	0.00770	0.00858	0.0104	0.0111	0.0122	0.0109
Mean =	**0.00993**	**0.00996**	**0.00809**	**0.00922**	**0.0110**	**0.0112**	**0.0101**	**0.0102**
%RSD =	**4.60**	**10.2**	**3.10**	**7.56**	**7.75**	**8.08**	**9.45**	**10.3**
Accuracy =	**99.3**	**99.6**	**80.9**	**92.2**	**110**	**112**	**101**	**102**

Values reported as actual analyte concentrations.

Table 7: LC/MS/MS Analysis of Dicamba and Its Major Metabolites in Soybean Forage (Continued)

	5-OH Dicamba		DCGA		DCSA		Dicamba	
Level Spiked	**Initial**	**72 hr reinjection**	**Initial**	**72 hr reinjection**	**Initial**	**72 hr reinjection**	**Initial**	**72 hr reinjection**
µg/g	**µg/g**	**µg/g**	**µg/g**	**µg/g**	**µg/g**	**µg/g**	**µg/g**	**µg/g**
0.02	0.0194	0.0199	0.0176	0.0212	0.0205	0.0223	0.0196	0.0197
0.02	0.0209	0.0199	0.0183	0.0193	0.0214	0.0203	0.0193	0.0194
0.02	0.0200	0.0207	0.0174	0.0219	0.0221	0.0224	0.0211	0.0209
0.02	0.0206	0.0233	0.0171	0.0191	0.0219	0.0215	0.0219	0.0211
0.02	0.0204	0.0217	0.0181	0.0202	0.0213	0.0230	0.0186	0.0201
0.02	0.0192	0.0223	0.0170	0.0214	0.0217	0.0225	0.0192	0.0187
0.02	0.0192	0.0211	0.0178	0.0206	0.0216	0.0213	0.0203	0.0199
Mean =	**0.0200**	**0.0213**	**0.0176**	**0.0205**	**0.0215**	**0.0219**	**0.0200**	**0.0200**
%RSD =	**3.52**	**5.91**	**2.77**	**5.17**	**2.42**	**4.19**	**5.83**	**4.18**
Accuracy =	**99.8**	**106**	**88.0**	**103**	**108**	**110**	**100**	**99.9**
0.1	0.104	0.106	0.0882	0.0945	0.102	0.102	0.113	0.0976
0.1	0.107	0.104	0.0863	0.114	0.109	0.115	0.105	0.0872
0.1	0.103	0.103	0.0900	0.0990	0.106	0.114	0.0967	0.0883
0.1	0.104	0.112	0.0929	0.102	0.105	0.107	0.109	0.0752
0.1	0.101	0.106	0.0894	0.101	0.101	0.125	0.107	0.106
0.1	0.106	0.111	0.0911	0.100	0.110	0.110	0.103	0.110
0.1	0.107	0.106	0.0919	0.0977	0.109	0.111	0.101	0.120
Mean =	**0.105**	**0.107**	**0.0900**	**0.101**	**0.106**	**0.112**	**0.105**	**0.0978**
%RSD =	**2.13**	**3.17**	**2.51**	**5.98**	**3.36**	**6.44**	**5.11**	**15.7**
Accuracy =	**105**	**107**	**90.0**	**101**	**106**	**112**	**105**	**97.8**
2	1.90	1.50	1.97	1.78	1.73	1.71	1.80	1.87
2	1.93	1.62	1.93	1.82	1.81	1.83	1.91	1.98
Mean =	**1.92**	**1.56**	**1.95**	**1.80**	**1.77**	**1.77**	**1.86**	**1.93**
Accuracy =	**95.8**	**78.0**	**97.4**	**90.0**	**88.5**	**88.5**	**92.8**	**96.3**

Values reported as actual analyte concentrations.

Table 8: LOD and LOQ of the Analyses of Forage, Hay and Seed for Dicamba Residues

		LOD and LOQ[a] (ppm[b])			
Matrix	**Parameter**	**DCGA**	**DCSA**	**Dicamba**	**5-Hydroxy-dicamba**
Forage	**LOD**	0.004	0.012	0.015	0.004
	LOQ	0.006	0.013	0.021	0.005
Hay	**LOD**	0.006	0.006	0.011	0.010
	LOQ	0.013	0.007	0.014	0.014
Seed	**LOD**	0.006	0.004	0.010	0.014
	LOQ	0.011	0.005	0.013	0.021

[a] The LOQ is the $LOQ_{20\%}$, corresponding to a coefficient of variation of 20%.

[b] The LOD and LOQ are expressed as the concentration of the individual analyte.

Table 9: Comparison of LC/MS/MS and Metabolite HPLC Quantitation Results from the Radiovalidation

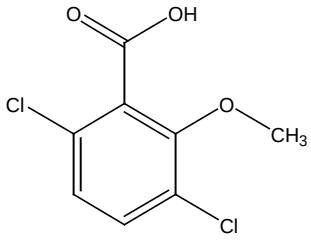
Sample	Metabolite/Analyte	HPLC Metabolite Quantitation (µg/g)*	Radiovalidation LC/MS/MS Analysis (µg/g)**
PRE-T Hay	DCGA	0.032	0.068 (0.068, 0.067)
	DCSA	0.862	0.863 (0.878, 0.849)
	Dicamba	0.012	0.016 (0.015, 0.016)
POE-T Hay	DCGA	2.28	2.44 (2.59, 2.29)
	DCSA	28.0	27.7 (25.1, 30.3)
	Dicamba	4.88	3.74 (4.30, 3.18)
POE-T Seed	DCGA	0.025	0.005 (0.007, 0.004)
	DCSA	0.099	0.088 (0.090, 0.085)
	Dicamba	0.003	Not Detected

* DCGA values are the sum of DCGA glucoside and DCGA malonylglucoside; DCSA values are the sum of DCSA glucoside, DCSA HMGglucoside and free DCSA as determined in the metabolism study final storage stability profiles.

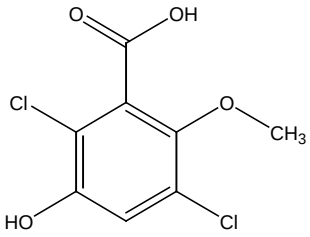
** Duplicate samples were carried through the method and analyzed by LC/MS/MS. Results are the average of the duplicate analyses (values in parentheses are the results for the individual replicates). All values are dicamba equivalents.

6 FIGURES

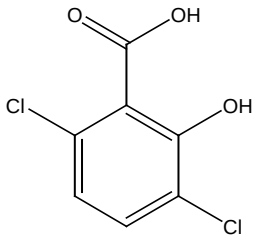
Figure 1: Analyte Structures



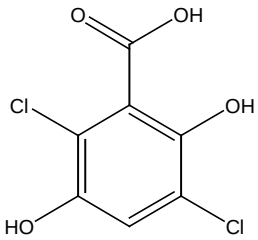
dicamba



5-hydroxydicamba

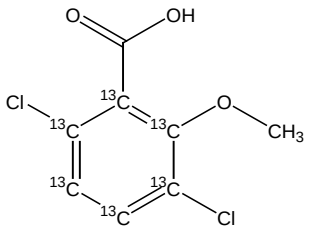


3,6-dichlorosalicylic acid

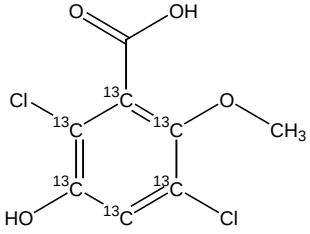


3,6-dichlorogentisic acid

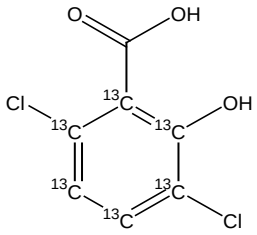
Figure 2: Internal Standard Structures



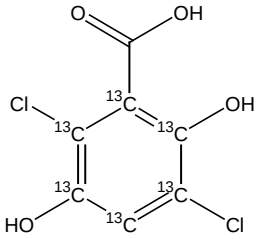
$^{13}C_6$-dicamba



$^{13}C_6$-5-hydroxydicamba



$^{13}C_6$-3,6-dichlorosalicylic acid



$^{13}C_6$-3,6-dichlorogentisic acid

Figure 3: Representative Dicamba Calibration Standard Curve

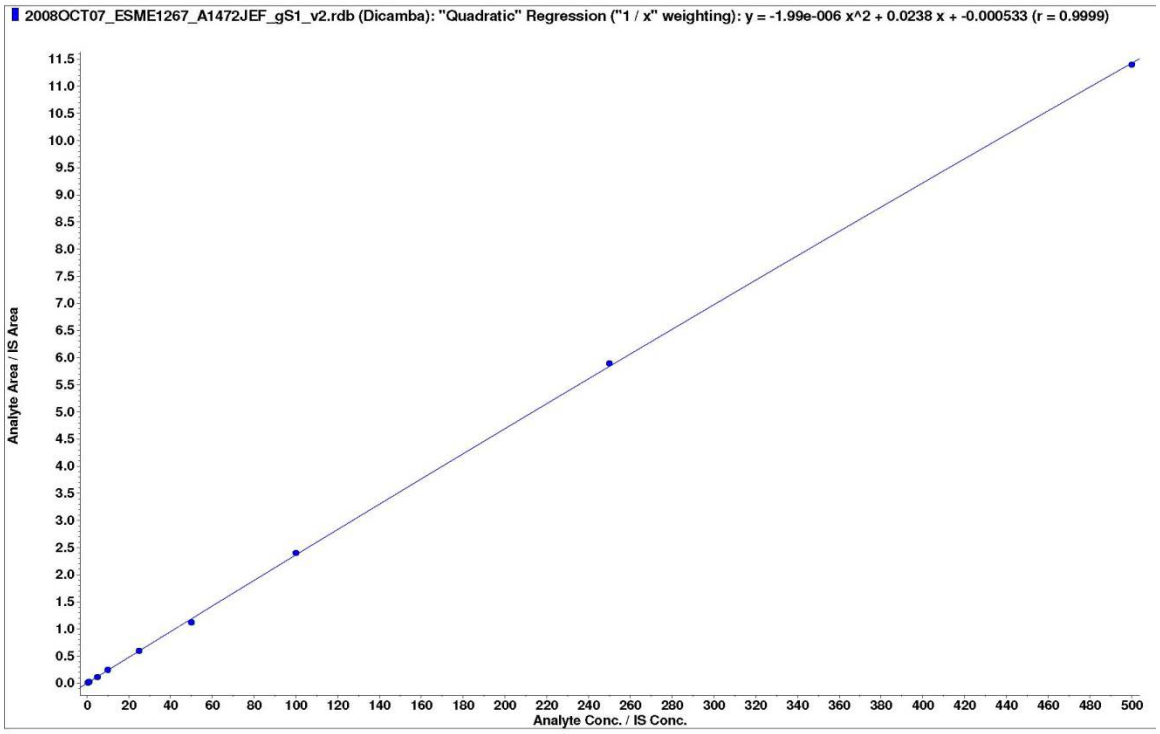


Figure 4: Representative 5-Hydroxydicamba Calibration Standard Curve

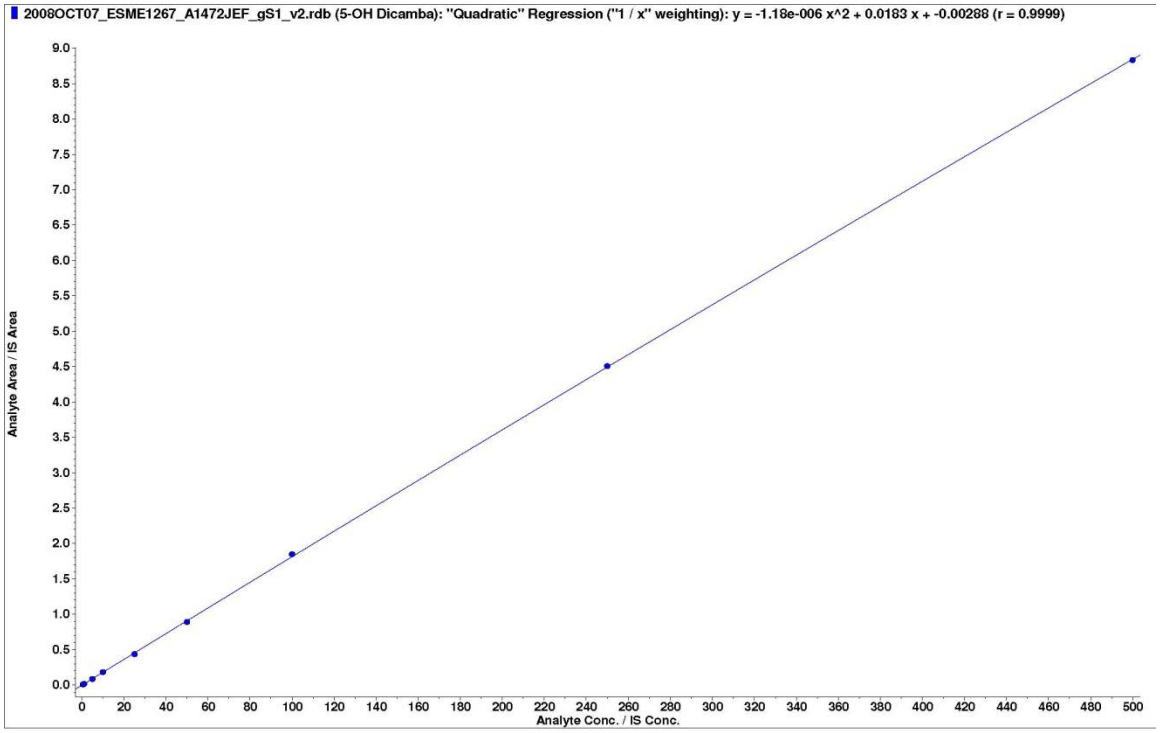


Figure 5: Representative DCSA Calibration Standard Curve

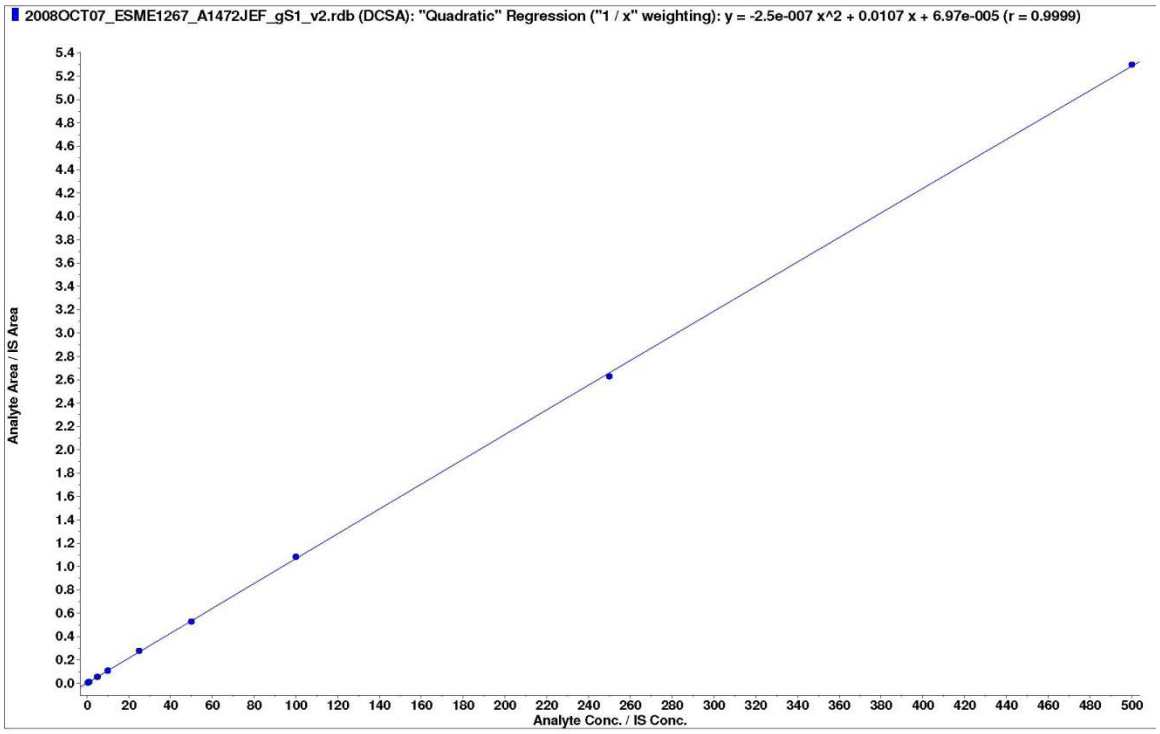
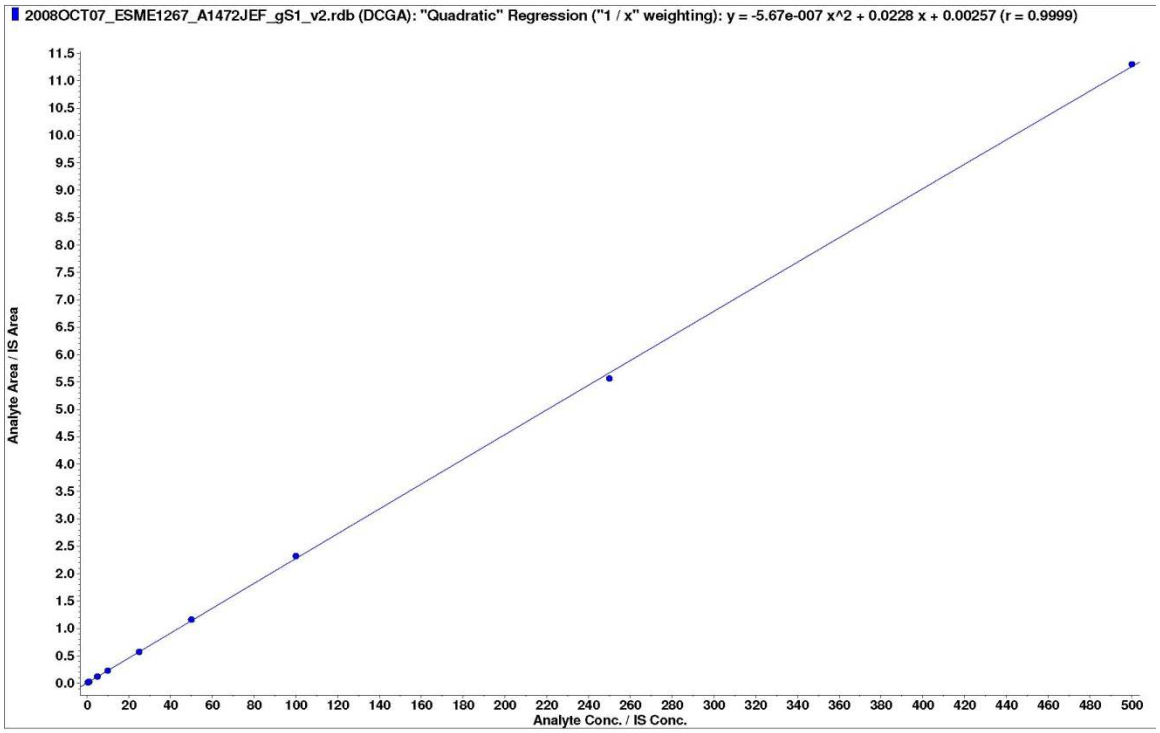


Figure 6: Representative DCGA Calibration Standard Curve



7 APPENDIX I. ANALYTICAL METHOD (WITH AMENDMENTS)

Monsanto Company Standard Operating Procedure

Effective Date: July 22, 2009 **AG-ME-1321-01** Page 1 of 42

Determination of Dicamba and Its Major Metabolites in Soybean Matrices by LC/MS/MS

AMENDMENTS: ☐ ☐ ☐ ☐ ☐

Overview

Purpose & scope

This SOP describes the method used by ESTC personnel to determine the residues of dicamba and its endogenous metabolites, analyzed as chemophores 5-hydroxydicamba, DCSA and DCGA, in soybean matrices. Analyte-specific stable labeled ISs are used to compensate for matrix effects and procedural recovery. (Refer to Appendix A for analyte and standard compound structures.) The radiovalidation, which demonstrates the extraction efficiency and recovery of the method, is conducted using soybean hay and seed samples from study 06-98-M-1, "Metabolism of Dicamba in Dicamba-Tolerant Soybeans", in which [^{14}C]-dicamba was used as the test substance.

Principles/ summary

Soybean matrices are extracted using 40:60 ACN:water. An aliquot of the extract is hydrolyzed in 1 N HCl at 95 °C in a water bath. The hydrolysate is partitioned with 40:60 ethyl acetate:isooctane and the organic phase is partially concentrated. Water is added to the organic phase and the sample is concentrated until only the aqueous solution remains. Following evaporation of the organic layer, the samples are filtered, acidified, and quantitated by LC/MS/MS with turbo ion spray ionization in negative ion mode. The lower LLMV for DCSA and DCGA is 0.010 µg/g. The LLMV for dicamba and 5-hydroxydicamba is 0.020 µg/g. In defatted flour, the LLMV for 5-hydroxydicamba is 0.050 µg/g.

Safety precautions

Follow current Monsanto safety policies. Important precautions include:

- Some solvents **are volatile and/or flammable.** Care must be taken to keep them away from **any source of ignition.**
- Ensure proper ventilation **to avoid excessive exposure to toxic solvent vapors.**
- Read and follow all safety warnings on reagent containers.
- HCl **is very corrosive.** If any chemical gets on the skin, wash affected area with soap and water. If any splashes in the eye, rinse immediately with water and seek medical attention.

Abbreviations

The following abbreviations are used in this SOP:

Abbreviation	Definition
ACN	acetonitrile
ACS	American Chemical Society
amu	atomic mass unit
API	atmospheric pressure ionization
DCGA	3,6-dichlorogentisic acid
DCSA	3,6-dichlorosalicylic acid
DI	deionized
g	gram
HPLC	high-performance liquid chromatography

IS	internal standard
L	liter
LC/MS/MS	liquid chromatography tandem mass spectrometry
LLMV	lower limit of method validation
LOQ	limit of quantitation
M	molar
mL	milliliter
mm	millimeter
mM	millimolar
MRM	multiple reaction monitoring
N/A	not applicable
ND	not detected
Q	quadrupole
QC	quality control
RSD	relative standard deviation
RT	room temperature
µg	microgram
µm	micrometer
UPLC	ultra-performance liquid chromatography
V	volt

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Materials

Equipment & apparatus

Specific brands are listed to aid the analyst in finding items. Generally, equivalent equipment obtained from other vendors may be substituted for the specified product.

Equipment/apparatus	Number/specification
UPLC system	Waters ACQUITY™
Mass spectrometer	Applied Biosystems API 5000 MS/MS with Windows based workstation using Analyst™ software

Balance: • Analytical • Top-loading	Mettler: • AE 163 • PM 4800
Centrifuge	Sorvall RC-5B, RC-5C & RC-6
HPLC column	• Analytical: Thermo Scientific Betasil Phenyl (100 mm *x* 4.6 mm *x* 5 µm), Xpertek cat. no. 948402 • In-line filter: ACQUITY™ (2.1 mm diameter frit, 0.2 µm porosity), Waters cat. no. 700002775
Pipettes	*Fixed and variable*
RapidVap™ evaporator	Labconco cat. no. 7900002
Sample shaker	Eberbach Corporation, VWR cat. no. 57007-101
Vacuum pump	Welch Duo Seal cat. no. 188471
Phase separator filter paper	Whatman™ cat. no. 2200 150
75 mm glass funnel	Fisher cat. no. 10-346B
Vial with Teflon-lined septum: • 2 mL glass screw-cap • 2 mL amber glass screw-cap • 12 mL amber glass screw-cap • 60 mL glass screw-cap	 • National Scientific cat. no. C4000-1W • National Scientific cat. no. C4000-2W • National Scientific cat. no. B7999-12A • Qorpak cat. no. GLC-07876
4 oz. amber glass bottle	VWR cat. no. 16153-102
Teflon lined caps	VWR cat. no. 16161-188
250 mL polypropylene wide-mouth bottle	VWR cat. no. 414004-125
15 mL polypropylene disposable centrifuge tube	Corning cat. no. 430052

Reagents & standards

Specific brands are listed to aid the analyst in finding items. Generally, equivalent reagents and standards obtained from other vendors may be substituted for the specified product.

Reagent/standard	Number/specification
REAGENTS	
ACN (HPLC grade)	Burdick & Jackson cat. no. 015-4
Ammonium acetate (ACS grade)	Fisher cat. no. A639-500
DI water	Milli-Q
Ethanol (ACS grade, 200 proof)	Aaper cat. no. E200
Ethyl acetate (ACS grade)	EMD cat. no. EX0240P-4
Formic acid (ACS grade)	EMD cat. no. FX0440-7
Hydrochloric acid (ACS grade)	JT Baker cat. no. 9535-00
Isooctane (2,2,4-trimethylpentane, HPLC grade)	Sigma-Aldrich cat. no. 6504-39-4L
Isopropanol (ACS grade)	EMD cat. no. PX1835-9
Methanol (HPLC grade)	EMD cat. no. MX0488-1

ANALYTICAL STANDARDS	
Dicamba	CAS # 1918-00-9, purity, >95%
5-hydroxydicamba	CAS # 7600-50-2, purity >95%
DCSA	CAS # 3401-80-7, purity >95%
DCGA	CAS # 18688-01-2, purity >95%
INTERNAL STANDARDS	
Dicamba, 3,6-dichloro-2-methoxybenzoic-1,2,3,4,5,6-$^{13}C_6$ acid	
5-hydroxydicamba, 2,5-dichloro-3-hydroxy-6-methoxybenzoic-1,2,3,4,5,6-$^{13}C_6$ acid	
DCSA, 3,6-dichloro-2-hydroxybenzoic-1,2,3,4,5,6-13C_6 acid	
DCGA, 2,5-dichloro-3,6-dihydroxybenzoic-1,2,3,4,5,6-$^{13}C_6$ acid	

Spiked recovery experiments conducted during method development revealed an issue for DCGA and $^{13}C_6$-DCGA. The recoveries were poor when the analytes were spiked directly onto hay and forage as well as when the analytes were spiked into a container containing matrix and extraction solution. The issue was circumvented by spiking dicamba, 5-hydroxydicamba, DCSA, and the corresponding $^{13}C_6$ analogs directly onto the matrix and extracting. DCGA and $^{13}C_6$-DCGA were spiked into the 10 mL extraction aliquot (See "**Sample Analysis**").

Solution Preparation

Mobile phases

Mobile Phase A - 5 mM aqueous ammonium acetate:

- Combine 0.385 g of ammonium acetate and 1000 mL of DI water.
- Adjust to pH 5.0 using glacial acetic acid and ammonium hydroxide.
- Mix well and store at RT.

Note: The pH may be adjusted to optimize the chromatography.

Mobile Phase B - 100% ACN: Store at RT.

The absolute volume of the mobile phases may be varied at the discretion of the analyst as long as the correct proportions of the components are maintained.

Needle washes

Strong needle wash - 1/1/1 (v/v/v) methanol/ACN/isopropyl alcohol:

- Using a graduated cylinder(s), add 300 mL of methanol to 300 mL of ACN to 300 mL of isopropyl alcohol in a suitable container.
- Mix well and store at RT.

Weak needle wash - 5% ACN (aqueous):

- Using a graduated cylinder(s), add 50 mL of ACN to 950 mL of DI water in a suitable container.
- Mix well and store at RT.

Reagent solutions

40:60 ACN:water:

- Combine 1600 mL of ACN and 2400 mL of DI water.
- Mix well and store at RT.

40:60 ethyl acetate:isooctane:

- Combine 1600 mL of ethyl acetate and 2400 mL of isooctane.
- Mix well and store at RT.

9% formic acid (aqueous):

- Add 9 mL of concentrated formic acid to 91 mL of DI water.
- Mix well and store at RT.

The absolute volume of the reagent solutions may be varied at the discretion of the analyst provided that the correct proportions of the components are maintained.

Stock solutions

100 µg/mL Individual Analyte:

- Weigh 0.01000 g ± 0.0001 g of each analytical grade analyte.
- Transfer into individual amber glass bottles. **Note:** The weighing container may be rinsed with a known volume of absolute ethanol to ensure complete transfer of the analyte from the container to the bottle.
- Dilute with absolute ethanol (volume adjusted for purity) to create a 100 µg/mL solution.
- Mix thoroughly.
- Solutions must be stored at ≤ 10 °C.

100 µg/mL Individual $^{13}C_6$ IS:

- Weigh 0.0100 g ± 0.001 g of each analytical grade labeled IS.
  Transfer into individual amber glass bottles. **Note:** The weighing container may be rinsed with a known volume of absolute ethanol to ensure complete transfer ofthe IS from the container to the bottle.
- Dilute with absolute ethanol to create a 100 µg/mL solution.
- Mix thoroughly.
- Solutions must be stored at ≤ 10 °C.

The absolute weight of the analyte(s), including the $^{13}C_6$ analog(s), may be varied at the discretion of the analyst provided that the correct proportions of the components are maintained.

At times, it may become necessary to create high concentration fortification spiking solutions that cannot be achieved with a 100 µg/mL stock solution. Higher concentration stock solutions may be prepared as long as the preparation is documented.

Fortification solutions

In order to estimate the analytical accuracy of the method within a given set of soybean matrix samples, it is necessary to fortify a certain number of control soybean matrix samples with a known amount of each analyte. Control samples are fortified at different analyte levels across the range of anticipated concentrations in the samples. There are two fortification solutions needed to perform analyses. One solution is a three-analyte solution containing dicamba, 5-hydroxydicamba, and DCSA. The other solution contains only DCGA. The solutions may be prepared in the following manner. Other concentrations may be used provided that the preparation is documented.

Individual stock concentration (µg/mL)	Volume of stock used (mL)	Fortification spiking solution total volume (mL)	Fortification spiking solution concentration (µg/mL)	Matrix fortification (µg/g)
100	2.0	50	4.00*	0.400
100	1.0	50	2.00*	0.200
100	0.5	50	1.00*	0.100
Fortification spiking solution concentration (µg/mL)	**Volume of spiking solution used (mL)**	**Fortification spiking solution total volume (mL)**	**Fortification spiking solution concentration (µg/mL)**	**Matrix fortification (µg/g)**
4.00	5.0	50	0.400	0.040
2.00	5.0	50	0.200	0.020
1.00	5.0	50	0.100	0.010
0	50	50	0.00	0.00

** Aliquots of these fortification solutions are diluted to create lower concentration levels.*

Example preparation of high concentration fortification spiking solution prepared from stock (4.00 µg/mL):

- Into a 50 mL volumetric flask, pipet 2.0 mL of each 100 µg/mL stock solution.
- Fill the flask to volume with ACN.
- Transfer the solution to an amber glass bottle.
- Mix well.

Example preparation of low concentration fortification spiking solution prepared by serial dilution of higher concentration fortification spiking solution (0.020 µg/mL):

- Into a 50 mL volumetric flask, pipet 5.0 mL of the 2.00 µg/mL fortification spiking solution.
- Fill the flask to volume with ACN.
- Transfer the solution to an amber glass bottle.
- Mix well.

Each example may be used to prepare two different fortification spiking solutions: 1) a three-analyte fortification spiking solution containing dicamba, 5-hydroxydicamba, and DCSA or 2) the DCGA spiking solution. Fortification solutions are prepared in ACN, and are stored in amber glass with airtight Teflon caps at ≤ 10 °C to avoid evaporation.

Calibration standards

The instrument calibration standards are made at convenient concentrations of each analyte. The solutions may be prepared in the following manner. Other concentrations may be used provided that the preparation is documented.

Individual stock concentration (µg/mL)	Volume of stock used (mL)	Calibration standard spiking solution total volume (mL)	Calibration standard spiking solution concentration (µg/mL)	Working calibration standard concentration (µg/L)
100	5.0	20	25.0*	500
100	2.5	20	12.5*	250
100	1.0	20	5.00*	100
Calibration standard spiking solution concentration (µg/mL)	**Volume of spiking solution used (mL)**	**Calibration standard spiking solution total volume (mL)**	**Calibration standard spiking solution concentration (µg/mL)**	**Injected calibration standard concentration (µg/L)**
25.0	2.0	20	2.50*	50.0
12.5	2.0	20	1.25	25.0
5.00	2.0	20	0.500*	10.0
2.50	2.0	20	0.250*	5.0
0.500	2.0	20	0.050	1.0

* *Aliquots of these calibration standard solutions are diluted to create lower concentration levels.*

The calibration standard solutions are prepared in ACN, and are stored in amber glass with airtight Teflon caps at ≤ 10 °C to avoid evaporation.

Working calibration standard:

- Pipet 0.10 mL of the appropriate concentration calibration standard spiking solution into a suitable container.
- Pipet 0.10 mL of the 2.00 µg/mL three-analyte IS solution into every container.
- Pipet 0.10 mL of the 2.00 µg/mL DCGA IS solution into every container.
- Pipet 2.20 mL of DI water into every container to achieve a total volume of 2.5 mL.
- Mix well.
- Dilute an aliquot of the 2.5 mL solution 1:1 with 9% formic acid solution in an autosampler vial. Mix well. **Note**: This is typically done by pipetting 900 µL of the calibration standard solution into an autosampler vial that already contains 900 µL of 9% formic acid solution. The absolute volume of the calibration standard solution and the 9% formic acid solution may be varied at the discretion of the analyst provided that the correct proportions of the components are maintained.

Stable labeled isotope IS solutions

There are two different working IS solutions needed to perform analyses: one solution is a multi-analyte solution containing $^{13}C_6$-dicamba, $^{13}C_6$-5-hydroxydicamba, and $^{13}C_6$-DCSA; the other solution contains only $^{13}C_6$-DCGA. The stock solution aliquots are diluted using ACN to create the working IS solutions. The absolute volume of the solutions may be varied at the discretion of the analyst provided that the correct proportions of the components are maintained. The solutions must be stored at ≤ 10 °C.

Stock solution concentration (µg/mL)	Volume of stock used (mL)	IS solution total volume (mL)	IS solution concentration (µg/mL)
100	1	50	2.00

IS diluent solution:

- Add 1 mL of the three-analyte IS solution and 1 mL of the DCGA IS solution into 98 mL of 40:60 ACN:DI water.
- Mix well.

This solution must be prepared fresh each day of use. The absolute volume of the solution may be varied at the discretion of the analyst provided that the correct proportions of the components are maintained.

Sample Analysis

Sample preparation

The following applies to soybean seed, hay, forage, and all processed fractions except tofu and lecithin. The fractions included soybean hulls, defatted flour, toasted defatted meal, protein isolate, protein concentrate, crude lecithin, degummed oil, refined bleached deodorized oil, soymilk, and tofu. See Appendix B for the modifications needed to prepare tofu and lecithin for sample analysis.

Step	Action
1	Weigh out 10 ± 0.1 g of ground dicamba-tolerant matrix into a 250 mL polypropylene bottle or other suitable container. **Note:** If samples are pre-weighed, they should be returned to frozen storage (≤ 20 °C) pending completion of the analysis.
2	For fortified samples, pipet 1.0 mL of the appropriate concentration three-analyte fortification solution into the respective container (See "**Fortification solutions**" in "**Solution Preparation**" for typical concentration levels). **Suggestion:** Turn on the water bath and allow it to begin heating prior to executing this step.
3	Pipet 1.0 mL of the 2.00 µg/mL three-analyte IS solution into every container.
4	Add approx. 98 mL of 40:60 ACN:DI water.
5	Cap the bottle tightly and place on a shaker for approx. 30 minutes.
6	Centrifuge at approx. 8000 rpm for approx. 10 minutes. **Note:** The purpose of this step is to separate the supernatant from the matrix cake. The rpm's may be varied to achieve this purpose.

7	Add approx. 17 mL of DI water and 2.7 mL of concentrated HCl to an amber glass bottle. **Note:** This step may be executed while steps 5 and 6 are being performed.
8	The steps below are followed depending on the sample type (see summary table below). Preparations must be documented. **8a) For undiluted field samples and control blanks:** A) Pipet 10 mL of the supernatant from step 6 into the amber glass bottle. B) Pipet 0.10 mL of the 2.00 µg/mL DCGA IS solution into the amber glass bottle. **8b) For undiluted fortified samples:** A) Pipet 10 mL of the supernatant from step 6 into an amber glass bottle. B) Pipet 0.10 mL of the appropriate concentration DCGA fortification solution into the respective amber glass bottle. C) Pipet 0.10 mL of the 2.00 µg/mL DCGA IS solution into the respective amber glass bottle. **8c) For diluted field samples** (**Note:** These volumes correspond to a dilution ratio of 1:10; ratio may be varied as needed (total volume = 10 mL)): A) Pipet 10 mL of the supernatant from step 6 into an intermediate container. B) Pipet 0.10 mL of the 2.00 µg/mL DCGA IS solution into the container. Mix well. C) Pipet 1.0 mL of the solution from B and 9.0 mL of the IS diluent solution into an amber glass bottle. **8d) For diluted fortified samples** (**Note:** These volumes correspond to a dilution ratio of 1:10; ratio may be varied as needed (total volume = 10 mL)): A) Pipet 10 mL of the supernatant from step 6 into a suitable container. B) Pipet 0.10 mL of the appropriate concentration DCGA fortification solution into the respective container. C) Pipet 0.10 mL of the 2.00 µg/mL DCGA IS solution into the container. Mix well. D) Pipet 1.0 mL of the fortified solution and 9.0 mL of the IS diluent solution into the amber glass bottle.

	Undiluted samples/ blanks	**Undiluted fortified samples**	**Diluted field samples**	**Diluted fortified samples**
Added directly to the amber glass bottle	Yes	Yes	No	No
Volume of supernatant	10 mL	10 mL	10 mL	10 mL
Volume of DCGA fortification solution added	N/A	0.1 mL	N/A	0.1 mL
Volume of DCGA IS solution added	0.1 mL	0.1 mL	0.1 mL	0.1 mL
Dilution aliquot taken	No	No	Yes	Yes
Dilution aliquot volume added to the amber glass bottle (as per 1:10 dilution example above)	N/A	N/A	1.0 mL	1.0 mL
IS diluent solution added	No	No	Yes	Yes
IS diluent solution volume added to the amber glass bottle (as per 1:10 dilution example above)	N/A	N/A	9.0 mL	9.0 mL

9	Loosely cap the bottle with a Teflon cap and hydrolyze in a water bath for approx. 1 hour at approx. 95 °C.
10	Cool the bottle using a container of cool water or by leaving it in a fume hood.
11	Add approx. 30 mL of 40:60 ethyl acetate:isooctane to the amber glass bottle.
12	Place the mixture on a shaker for approx. 10 minutes. Suggestion: Turn on the evaporator and allow it to preheat.
13	Collect the organic phase in a suitable evaporator vial using phase separator paper and a funnel.
14	Rinse the glass bottle with approx. 5 mL of 40:60 ethyl acetate:isooctane and add it to its respective funnel containing the phase separator paper and the aqueous layer. **Note:** At this point, the samples may be stored overnight at approx. 4 °C if necessary.
15	Evaporate the organic phase for approx. 35 minutes in an evaporator fitted with a cold trap containing dry ice. Typical RapidVap™ Evaporator settings: Temperature = 45 °C, Vacuum = 225 mBar, Speed = 35%, Time = 35 minutes.
16	Evaporate the organic layer until only the aqueous solution remains. Avoid evaporating to dryness. Typical RapidVap™ settings: Temperature = 45 °C, Vacuum = 100 mBar, Speed = 35%, Time = 35 minutes. **Suggestion:** Prepare the working calibration standards once the RapidVap™ has been activated (See "**Calibration standards**" in "**Sample Preparation**").
17	Pipet 2.5 mL of DI water into the evaporator vial.
18	Filter the solution through a 0.2 µm filter into a suitable container. **Note:** The evaporator container may be too large for a syringe to reach the sample it contains. The solution may be transferred directly from the evaporator container into a syringe, fitted with a 0.2 µm filter, that has had the plunger removed. The plunger is then replaced and the solution is filtered into a new container.
19	Pipet an equal volume of the sample and 9% aqueous formic acid into an autosampler vial. Mix well.
20	Analyze the solution by LC/MS/MS (inject 10 µL).

Mass spectrometric analysis

A sample set is a group of field (treated, unknown) samples and QC samples prepared together. QC samples are control and fortified control samples. A chromatographic set contains calibration standards, QC samples, and field samples.

Requirements for sample analyses include, but are not limited to, the following:

- **Sample/chromatographic set:**
  - At least one control and one fortified control sample will be prepared and analyzed in every sample set.
  - A chromatographic set must be arranged such that the set begins and ends with a calibration standard (i.e., QC samples, and field samples are bracketed by calibration standards).
  - No more than seven QC and/or treated samples are analyzed between calibration standards.

- **Calibration:**
  - Analyte calibration must be performed for each chromatographic set using a multi-point calibration curve.
  - Each calibration standard level will be injected one time only in a chromatographic set to provide equal weighting across the response curve.
  - The minimum correlation coefficient (r) for the calibration curve of each analyte is 0.99.
  - The lowest standard concentration level must be below the relevant LOQ or LLMV.
  - The calibration standards are arranged in a non-systematic order, relative to standard concentration, throughout the chromatographic set.
  - The analyte response of all treated and fortified controls must not exceed the upper range of the standard calibration curve. When the individual analyte response of any treated sample and fortified control is greater than this range, the sample must be diluted and reanalyzed for that analyte. The diluted sample may be analyzed in any sample set provided that the set contains a control and a fortified control sample and meets the other requirements.
- **QC samples:**
  - The average analytical recovery of fortified samples (corrected for the background found in the unfortified site-specific controls) for all analytes in each set must range between 70 and 120% of the amount fortified. Individual recovery results of fortified samples must range between 65 and 125% of the amount fortified.
  - For each matrix and analyte, the range of fortification levels over all the sets must span the range of concentrations from the LLMV to the highest concentration in the treated samples.

LC/MS/MS operating parameters

The instruments comprising the analytical system may be divided into two areas:

- UPLC pump and autosampler controlled by Analyst™ or Empower 2™ software.
- Mass spectrometer controlled by Analyst™ software.

Mass spectrometer acquisition parameters are provided for analysis using turbo ion spray ionization for four analytes and four ^{13}C IS analogs in negative ion MRM mode.

Parameters for control of the UPLC pump and autosampler are specified in methods established in the ACQUITY™ method editor. Parameters for control of the mass spectrometer are specified in the acquisition method established in the Analyst™ acquisition batch file. Data is collected and stored by the Analyst™ software. The acquisition method containing all mass spectrometer operating parameters is prepared specifically for the target analytes. The typical precursor and product ions for the analytes are shown below. Alternate ions may be used if they provide better data (sensitivity and/or specificity). It is assumed that the instrument has been properly tuned and mass calibrated prior to analysis. The following equipment and conditions are instrument dependent and may be modified to obtain optimal instrument performance and maximize sensitivity. Actual method parameters must be documented in the raw data.

LC parameters:

- HPLC column: Thermo Scientific Betasil Phenyl (100 mm *x* 4.6 mm *x* 5 µm)
- In-line filter: ACQUITY™ (2.1 mm diameter frit, 0.2 µm porosity)
- Column temperature: 60 °C
- Sample temperature: 7 °C
- Injection volume: 10 µL
- Flow rate: 600 µL/minute
- Split ratio: None
- Mobile phases: A = 5 mM ammonium acetate, pH 5.0. B = ACN
- Strong needle wash: 1/1/1 (v/v/v) methanol/ACN/isopropyl alcohol
- Weak needle wash: 5% ACN (aqueous)
- Gradient:
  - Initial: 8% A : 2% B
  - 1.0 minutes: 98% A : 2% B (hold)
  - 5.0 minutes: 95% A : 5% B (linear gradient)
  - 7.0 minutes: 80% A : 20% B(linear gradient)
  - 9.0 minutes: 80% A : 20% B (hold)
  - 10 minutes: 68% A : 32% B (linear gradient)
  - 11.5 minutes: 68% A : 32% B (linear gradient)
  - 12 minutes: 0% A : 100% B (linear gradient)
  - 15 minutes: 0% A : 100% B (hold)
  - 15.5 minutes: 98% A : 2% B (linear gradient)
  - 18 minutes: 98% A : 2% B (hold)

Unique interferences in particular specimens may require modification of the gradient. If modifications are necessary, they must be documented in the raw data.

Retention Time (Approximate minutes):

- Dicamba: 8.0
- 5-hydroxydicamba: 4.5
- DCSA: 10.1
- DCGA: 5.3

The total run time is approx. 19 minutes, injection to injection.

Mass spectrometer parameters - negative ion:

- Scan Type: Negative MRM
- Resolution Q1: Unit
- Resolution Q3: Unit
- Ion source: Turbo spray
- Collision gas (CAD): 2 (N_2)
- Curtain gas (CUR): 10 (N_2)
- Gas sources: GS1 = 30 (N_2). GS2: 35 (N_2)
- Ion spray voltage (IS): -1300 V
- Temperature (TEM): 350 °C
- Interface heater (IH): On
- Collision energy (CE)
- Declustering potential (DP)
-

- Declustering Potential (DP)
- Entrance potential (EP)
- Collision cell exit potential (CXP)

Period 1 Experiment 1

Duration of MS Acquisition: Approx. 12 minutes

Analyte	Precursor ion Q1 (amu)	Product ion Q3 (amu)	Scan time (ms)	DP (V)	EP (V)	CE (V)	CXP (V)
Dicamba	219	175	200	-135	-10	-5	-17
5-hydroxydicamba	235	191	250	-70	-10	-10	-11
DCSA	205	161	50	-25	-10	-6	-3
DCGA	221	177	200	-60	-10	-16	-29
$^{13}C_6$-dicamba	225	181	75	-135	-10	-5	-17
$^{13}C_6$-5-hydroxydicamba	241	197	75	-60	-10	-10	-9
$^{13}C_6$-DCSA	211	167	75	-45	-10	-8	-13
$^{13}C_6$-DCGA	227	183	75	-65	-10	-14	-13

Data processing

Process the data using the Analyst™ quantitation wizard. The wizard processes the data for the MRM transition pairs established in the acquisition method. The method detects and integrates the analyte peaks based on retention time and MRM transition. Chromatograms may be smoothed prior to integration as long as the smoothing is consistent throughout the entire chromatographic set. Manual peak integration must be used when the automated procedure is not effective due to baseline noise. The quantitation method uses IS calibration. For example chromatograms of each analyte in the tested matrices, see Appendix C.

Interferences

Sample matrix

Evidence of analyte response suppression or enhancement due to the sample matrix has been observed in the matrices tested. Stable labeled isotope ISs are used to correct for matrix effects.

Chromatographic interferences

Chromatographic interferences may be a problem in some matrices. Gradient conditions and the use of MS with MRM analysis greatly reduce the interferences for these samples.

Pesticide interferences

There are no interferences from other pesticides 2,4-dichlorophenoxyacetic acid (2,4-D) or 2,4-dichlorophenylacetic acid, which have the same mass transitions as dicamba and DCSA, respectively. Interferences from other pesticides are unknown. However, none are expected due to the high level of specificity of the LC/MS/MS analysis.

Analyte carryover

Analyte carryover has not been observed in this method.

Solvents A solvent blank may be included in an analytical set to confirm the suitability of a solvent used.

Labware Disposable labware should be utilized where possible.

Residue Calculations

Calibration curve The Analyst™ software automatically derives the calibration curve using the area ratio versus the concentration of the standards (µg/mL analyte) from all standards injected with the chromatographic set. A weighted quadratic curve (1/analyte concentration) is used. The resulting equation defining the standard curve is shown below:

$$AR_{analyte} = A\ (\mu g/mL\ analyte)^2 + B\ (\mu g/mL\ analyte) + C, \quad \text{where}$$

- $AR_{analyte}$ is the detector response (area ratio)
- *A*, *B*, and *C* are curve constants

The results are calculated automatically by the Analyst™ software. The calculation may be checked manually by applying the solution for quadratic equations as shown below. (**Note:** Subtract the response $AR_{analyte}$ from C first.)

$$\text{Injected concentration (ng/mL analyte)} = -B \pm \sqrt{(B^2 - 4AC)} / 2A$$

Calculations using alternative calculations (e.g., linear or exponential curve fits) are acceptable if they provide improved precision and/or accuracy.

Analyte concentrations The analytical method contains sample dilution and the resulting ppm value taken directly from the regression curve must be multiplied by a 5X dilution factor. Enter the dilution factor into the Analyst™ "dilution factor" column to automatically calculate the final concentration. The calculated value represents the concentration of the analyte in the initial sample. The sample concentration is calculated by the software as shown in the equation below:

$$\mu g/g\ (analyte) = \frac{[(\mu g/mL\ analyte\ found)(final\ volume)]}{sample\ weight\ (g)} \times \frac{extract\ volume}{extract\ aliquot\ volume}$$

Analytical recovery Successful method performance for each analytical set is assessed by the determination of percent recovery of known amounts of the analytes fortified into control samples. The percent recovery of each analyte is calculated as shown below:

$$\%\ recovery = [(100)(\mu g/g\ analyte\ found)] / \mu g/g\ analyte\ added$$

For a large study, there should be near equal numbers of fortifications at each level so the estimated analytical accuracy will not be disproportionately weighted.

Results & Discussion

Stability & matrix effects

The stability of the analytes was tested in stocks, calibration standards, and final sample extracts. Stock solutions prepared in absolute ethanol and stored at approx. 4 °C were stable for at least 201 days. Calibration standards prepared in ACN and stored at approx. 4 °C were stable for at least 201 days. The final sample extracts were stable on the autosampler for at least 72 hours.

Evidence of analyte response suppression or enhancement due to the sample matrix has been observed in the matrices tested. During the method validation, analyte-specific ^{13}C ISs were used to compensate for matrix effects.

Calibration curves

The calibration standard curves met the criteria at all levels in all matrices for each analyte.

Precision & accuracy

During validation, the analytical method provided very good precision and accuracy at the 0.010 µg/g LLMV for all analytes in soybean forage and hay. In soybean seed, the precision and accuracy for dicamba, DCSA, and DCGA at the 0.010 µg/g LLMV was acceptable. The acceptance criteria for accuracy was not met for 5-hydroxydicamba at the 0.010 µg/g level in soybean seed. For validation results in soybean seed, hay, and forage, see Appendix D.

The precision and accuracy of dicamba, 5-hydroxydicamba, DCSA, and DCGA determinations were verified in soybean processed fractions. The fractions included soybean hulls, defatted flour, toasted defatted meal, protein isolate, protein concentrate, crude lecithin, degummed oil, RBD oil, soymilk, and tofu. The precision and accuracy was very good for all analytes at all tested concentration levels in the processed fractions, except for defatted flour. Dicamba, DCSA, and DCGA achieved acceptance criteria in defatted flour at all concentration levels tested. Acceptance criteria for 5-hydroxydicamba was achieved in defatted flour at 0.050 µg/g fortification and above. For verification results in soybean processed fractions, see Appendix E.

Superseded SOP(s): None
Author(s) / prepared by: James E. Foster

Management:

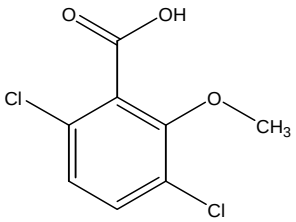
Mary P. Gasper
(TFM, Monsanto Company)

Date: 7 / 22 / 09

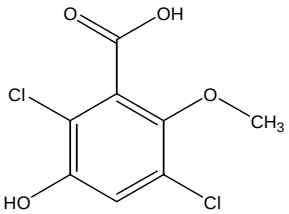
Appendices

Appendix A: Compound Structures

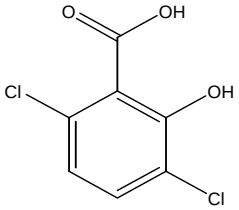
Figure 1: analyte structures



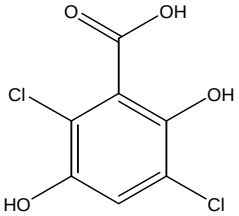
dicamba



5-hydroxydicamba

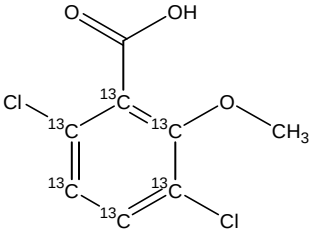


3, 6-dichlorosalicylic acid

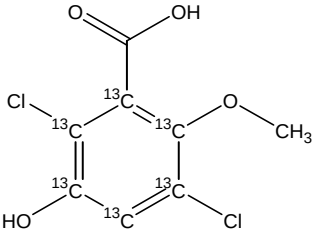


3, 6-dichlorogentisic acid

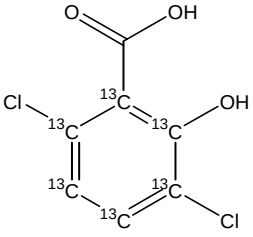
Figure 2: IS structures



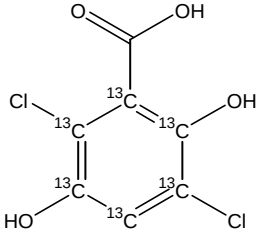
$^{13}C_6$-dicamba



$^{3}C_6$-5-hydroxydicamba



$^{13}C_6$-3, 6-dichlorosalicylic acid



$^{13}C_6$-3, 6-dichlorogentisic acid

Appendix B: Preparation Modifications for Processed Fractions

Overview

Steps indicated below refer to the "**Sample preparation**" procedural steps (in "**Sample Analysis**").

Tofu

The tofu sample must be well dispersed in the extraction solvent before shaking. While weighing, the tofu sample is chopped in small pieces in the polypropylene extraction bottle. Following spiking and addition of the extraction solvent (step 4) the matrix sample and extraction solvent are thoroughly mixed through the use of a commercial tissue homogenizer. Processing of the samples then proceeds to step 5 and follows the method as for other matrices.

Lecithin

Lecithin samples are processed (dispersed, homogenized) in similar manner to the procedure used for tofu, except that 27 mL of concentrated HCl are added to each sample before the extraction shaking. After shaking (step 5) and centrifugation (step 6), the addition of 2.7 mL of concentrated HCl in step 7 is omitted. In step 8a) (undiluted samples), a 12.7 mL aliquot is used in place of the 10 mL aliquot specified for other matrices. For the dilutions (steps 8b)), a 12.7 mL aliquot is also used, with the volumes of DCGA fortification solutions and IS solution unchanged. The diluted lecithin samples are then treated the same as other matrices until step 18. To dilute lecithin samples, extra control sample may be needed. Dilute samples as in the examples below:

- **1:5 dilution:** Take 0.18 mL of the filtered sample + 0.72 mL of filtered control sample, and mix with 0.9 mL of 9% formic acid solution. Mix well and proceed to step 20.
- **1:45 dilution:** Take 0.02 mL of the filtered sample + 0.88 mL of filtered control sample, and mix with 0.9 mL of 9% formic acid solution. Mix well and proceed to step 20.

Note: Good recoveries for fortified samples were obtained by not diluting these levels until the final sample volume is obtained (step 18). Diluting the sample at this point with excess composite control sample was found to give excellent recoveries.

Partitioning

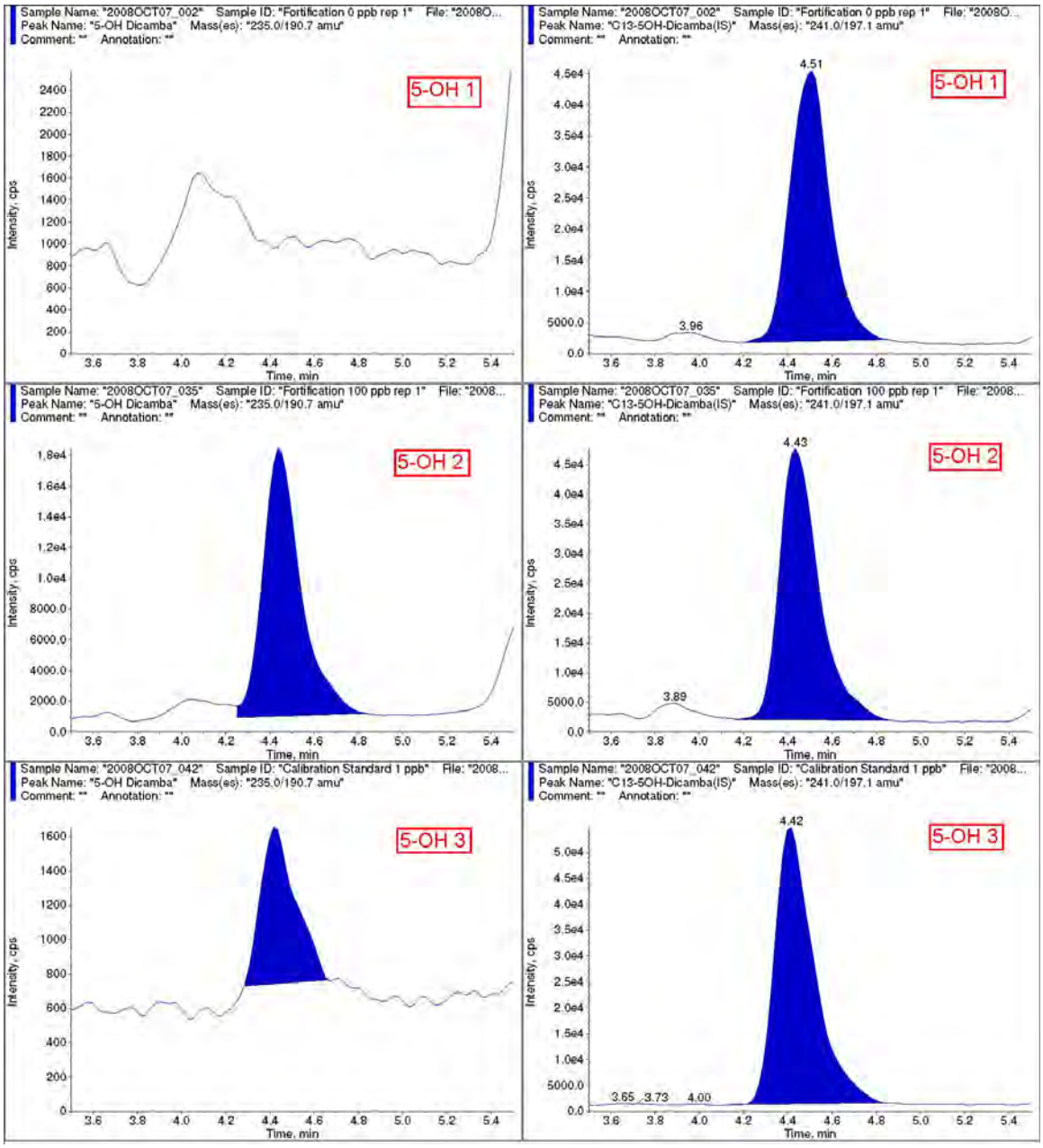
After partitioning with 40:60 ethyl acetate/isooctane (steps 11 and 12) some process fraction samples (e.g., protein isolates) may form an emulsion and not separate. When this occurs, the entire sample is poured into a polypropylene centrifuge bottle and centrifuged at 11,000 rpm for 10 minutes. The samples separate well when centrifuged. Processing of the samples then proceeds to step 13 and continues as for other matrices.

Additional equipment

- IKA T-25 High-Speed Homogenizer, IKA cat. no. EW-04739-01
- IKA T-25 High-Speed Homogenizer Rotor-Stator Generator, IKA cat. no. EW-04720-12

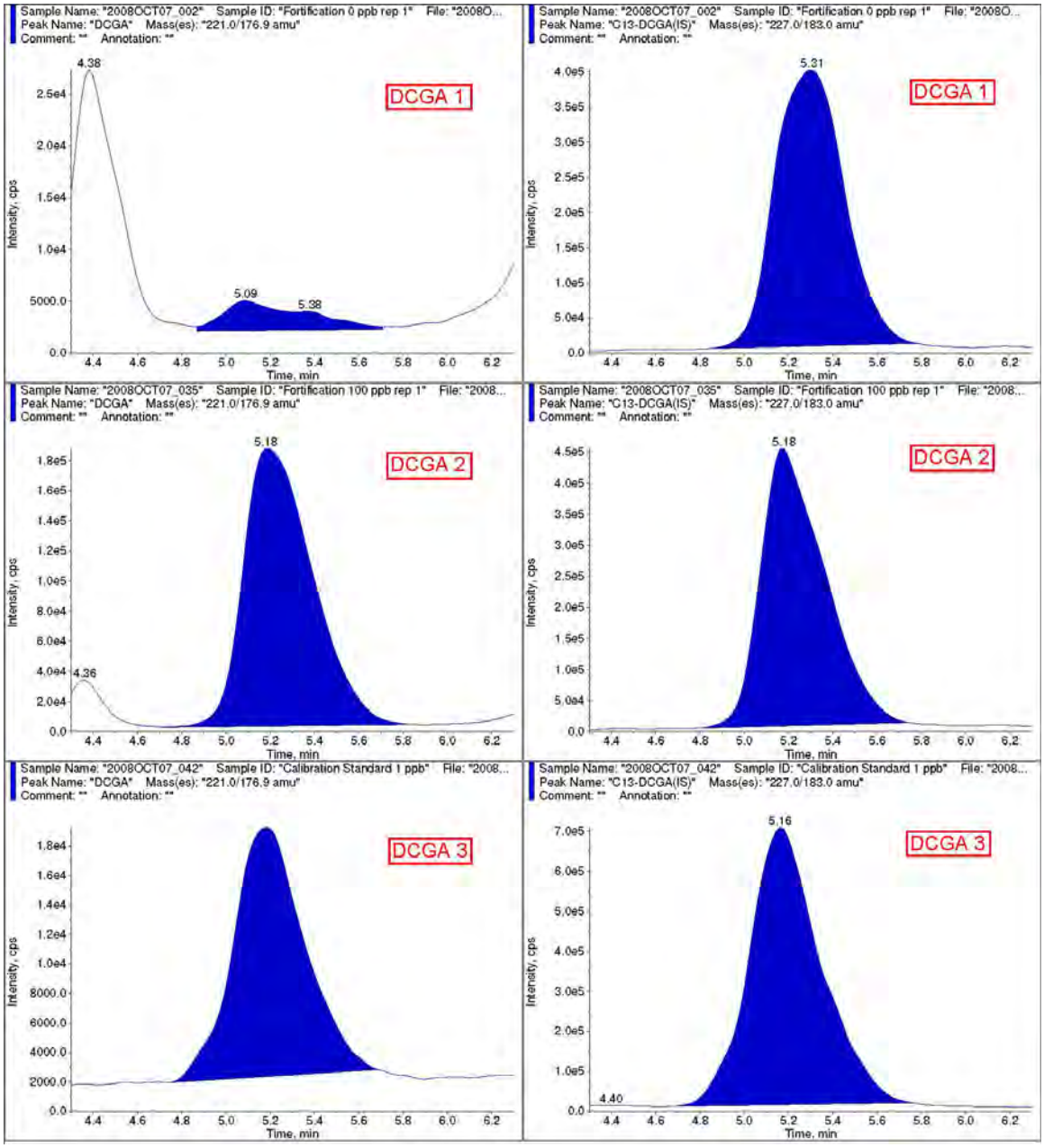
Appendix C: Example Chromatograms (Mobile Phase A, pH = 5.0)

5-hydroxydicamba in soybean seed



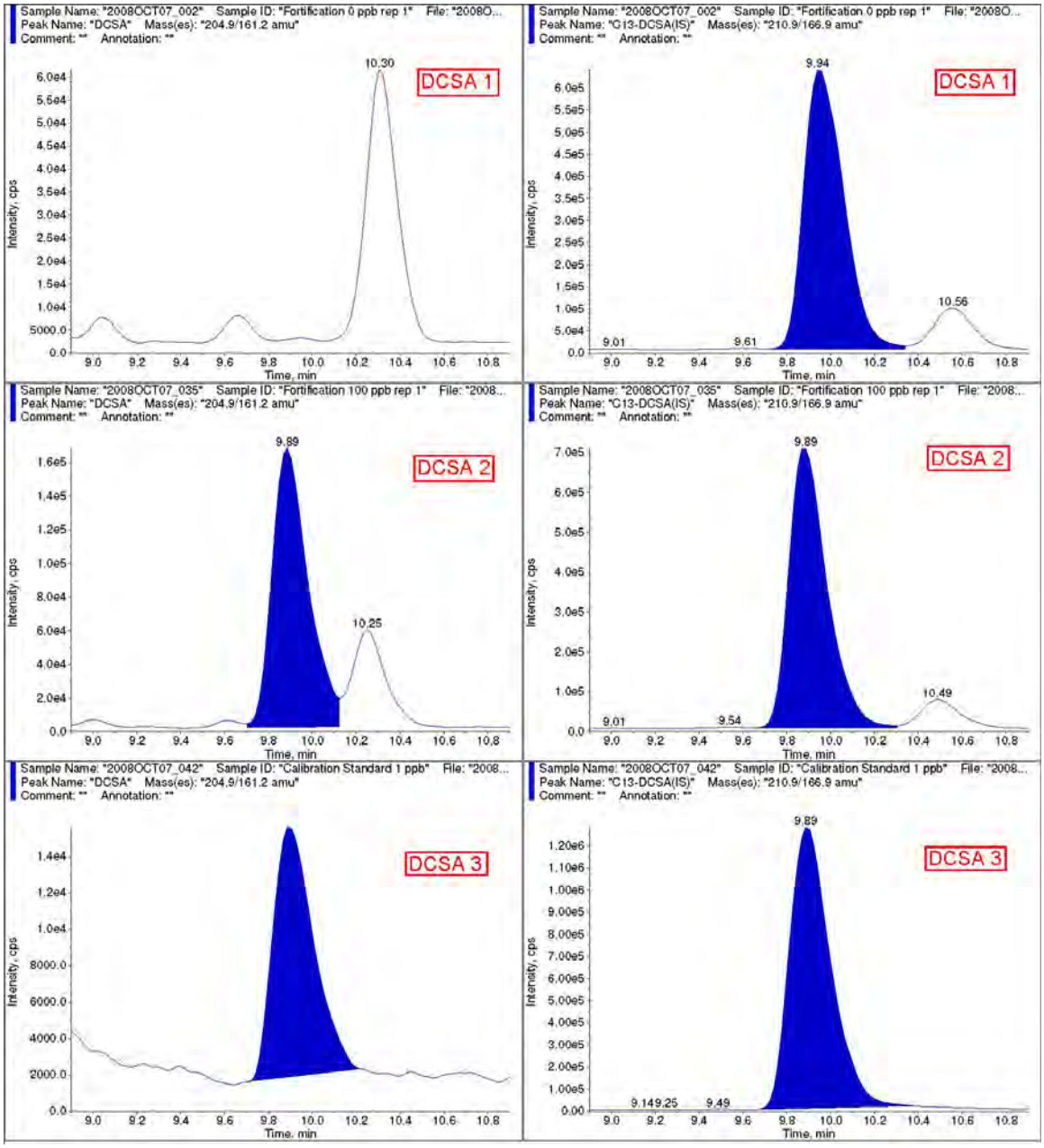
5-OH 1: untreated control (left): IS 0.040 µg/g (right)
5-OH 2: fortified control 0.10 µg/g (left): IS 0.040 µg/g (right)
5-OH 3: calibration standard 0.001 µg/mL (left): IS 0.040 µg/g (right)

DCGA in soybean seed



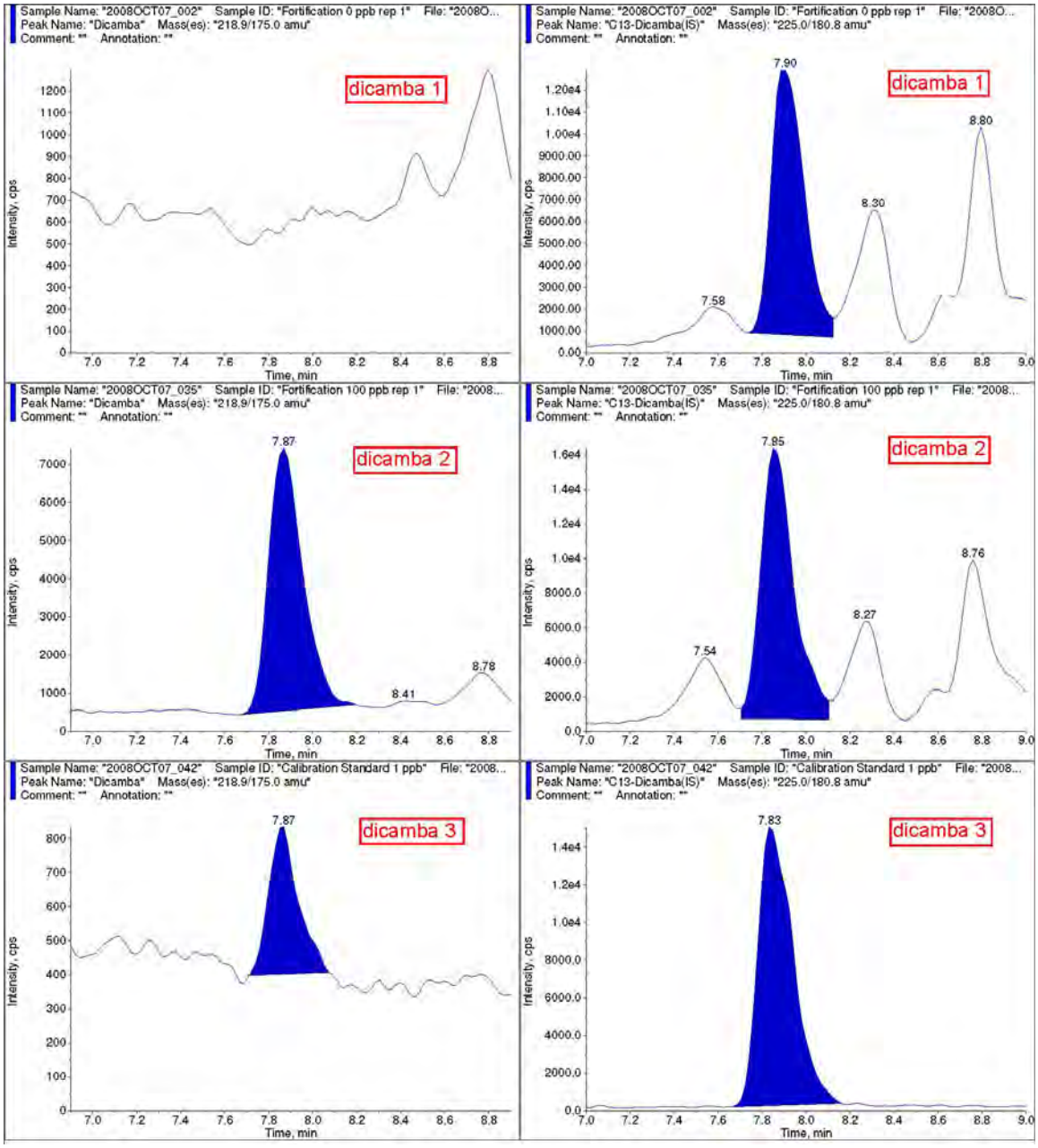
DCGA 1: untreated control (left): IS 0.040 µg/g (right)
DCGA 2: fortified control 0.10 µg/g (left): IS 0.040 µg/g (right)
DCGA 3: calibration standard 0.001 µg/mL (left): IS 0.040 µg/g (right)

DCSA in soybean seed



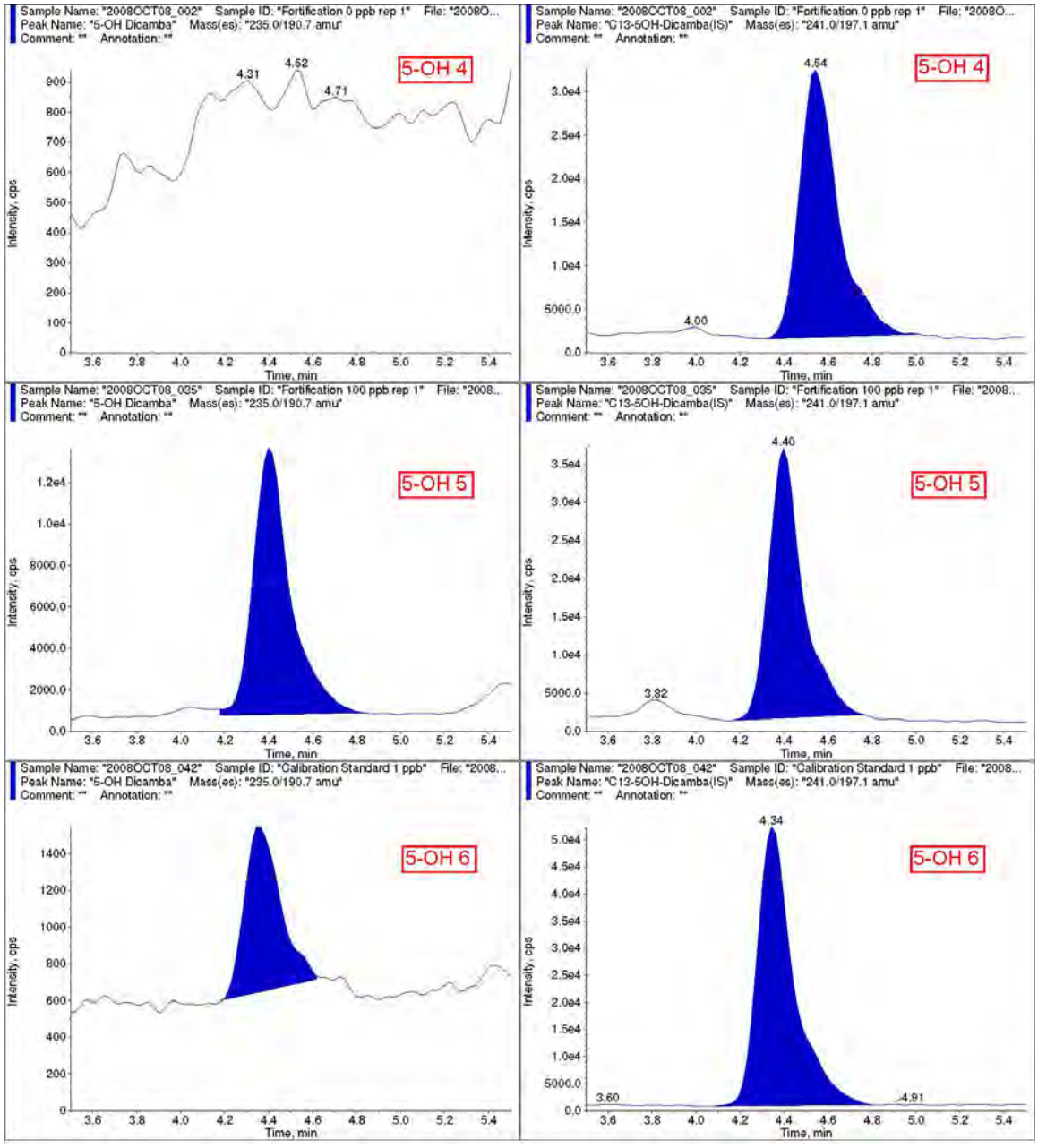
DCSA 1: untreated control (left): IS 0.040 µg/g (right)
DCSA 2: fortified control 0.10 µg/g (left): IS 0.040 µg/g (right)
DCSA 3: calibration standard 0.001 µg/mL (left): IS 0.040 µg/g (right)

Dicamba in soybean seed



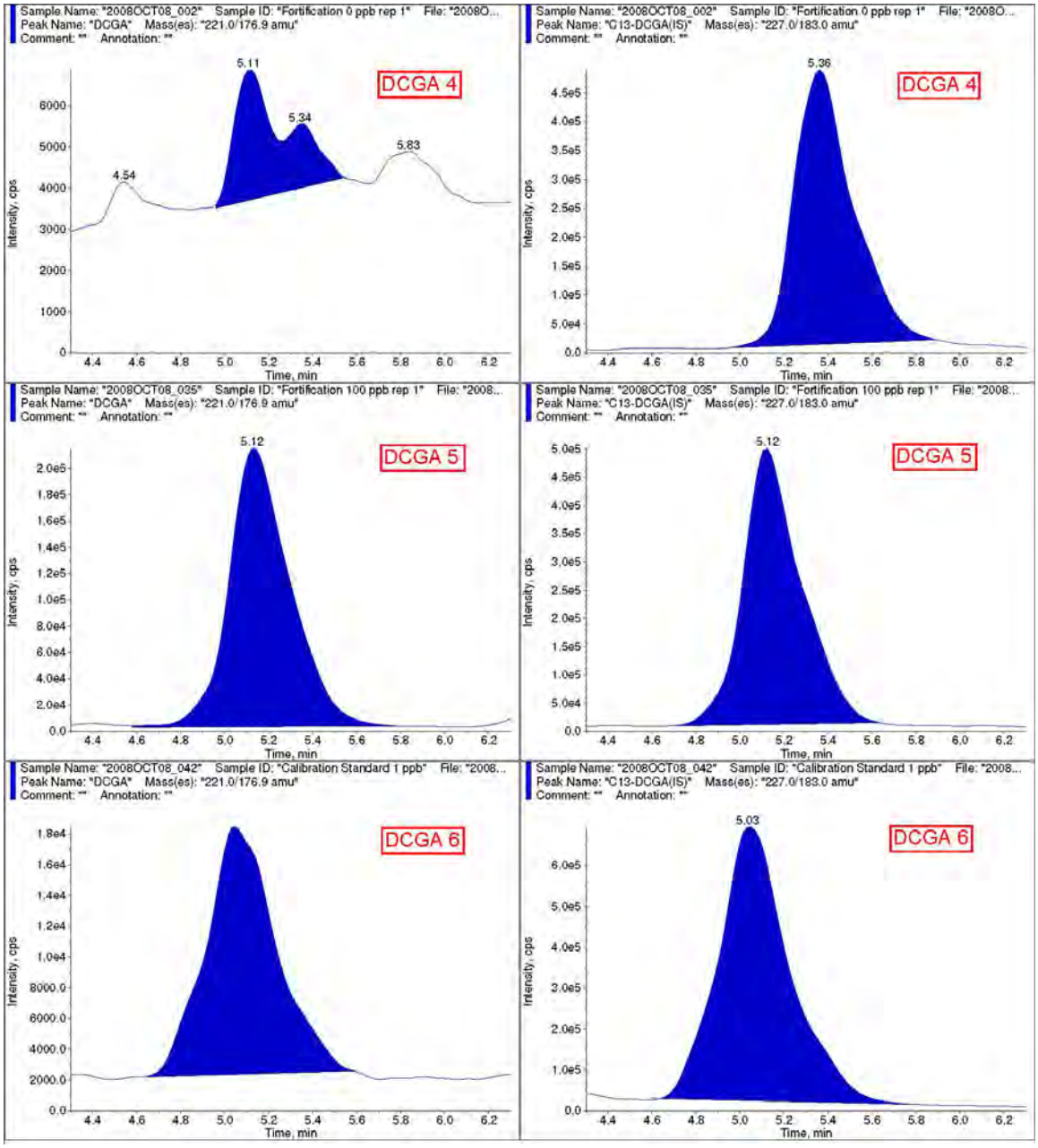
Dicamba 1: untreated control (left): IS 0.040 µg/g (right)
Dicamba 2: fortified control 0.10 µg/g (left): IS 0.040 µg/g (right)
Dicamba 3: calibration standard 0.001 µg/mL (left): IS 0.040 µg/g (right)

5-hydroxydicamba in soybean hay



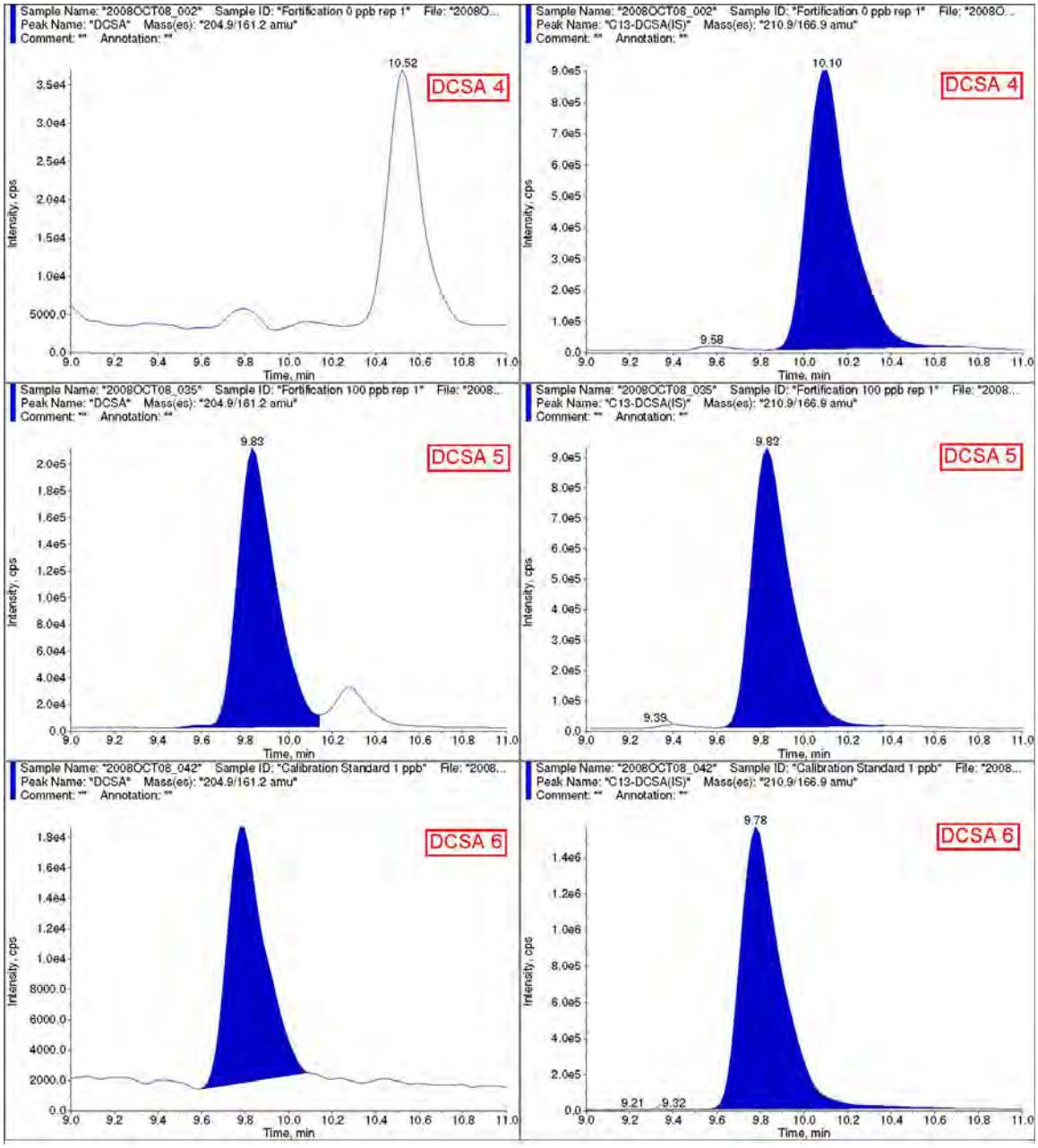
5-OH 4: untreated control (left): IS 0.040 µg/g (right)
5-OH 5: fortified control 0.10 µg/g (left): IS 0.040 µg/g (right)
5-OH 6: calibration standard 0.001 µg/mL (left): IS 0.040 µg/g (right)

DCGA in soybean hay



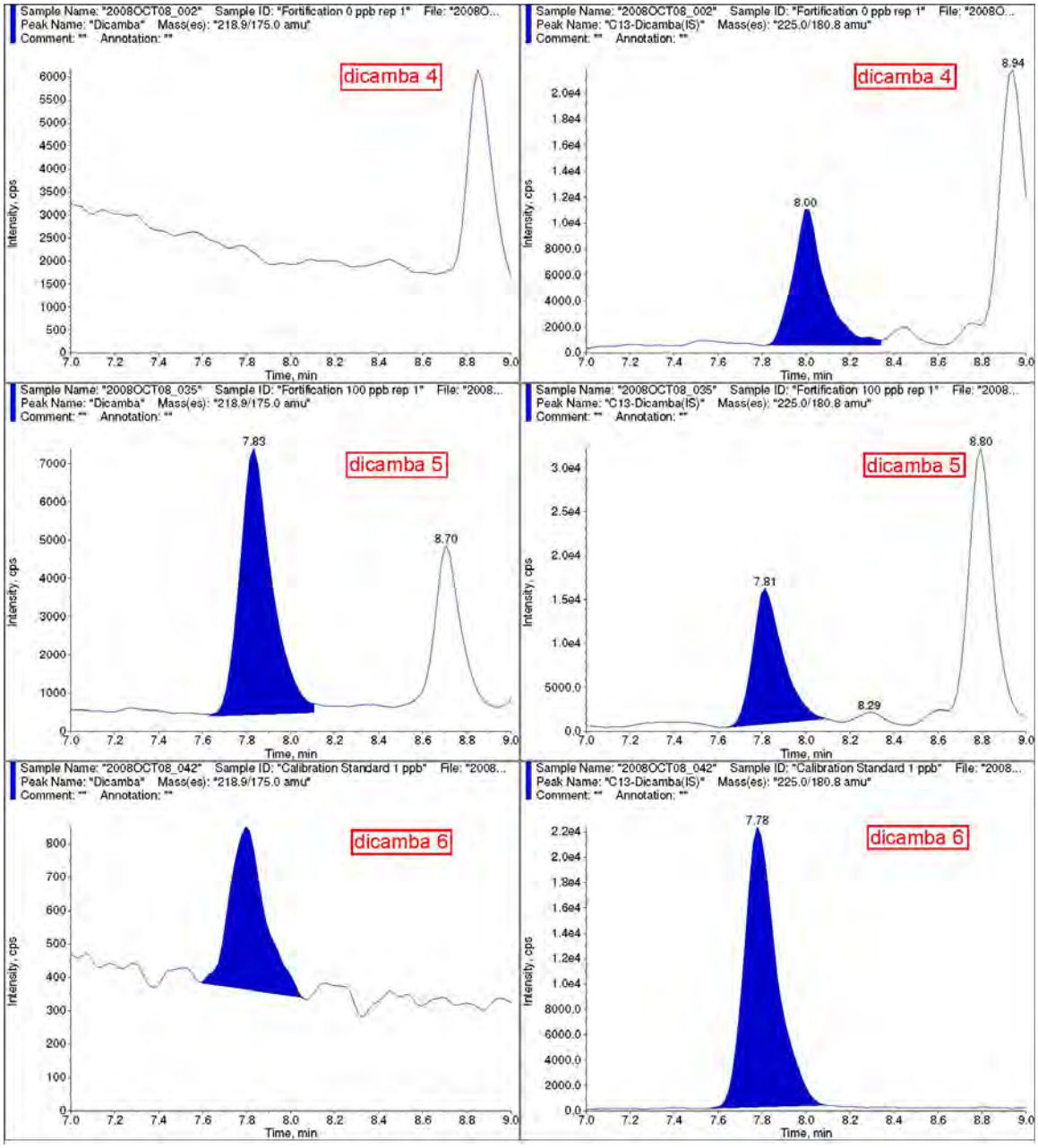
DCGA 4: untreated control (left): IS 0.040 µg/g (right)
DCGA 5: fortified control 0.10 µg/g (left): IS 0.040 µg/g (right)
DCGA 6: calibration standard 0.001 µg/mL (left): IS 0.040 µg/g (right)

DCSA in soybean hay



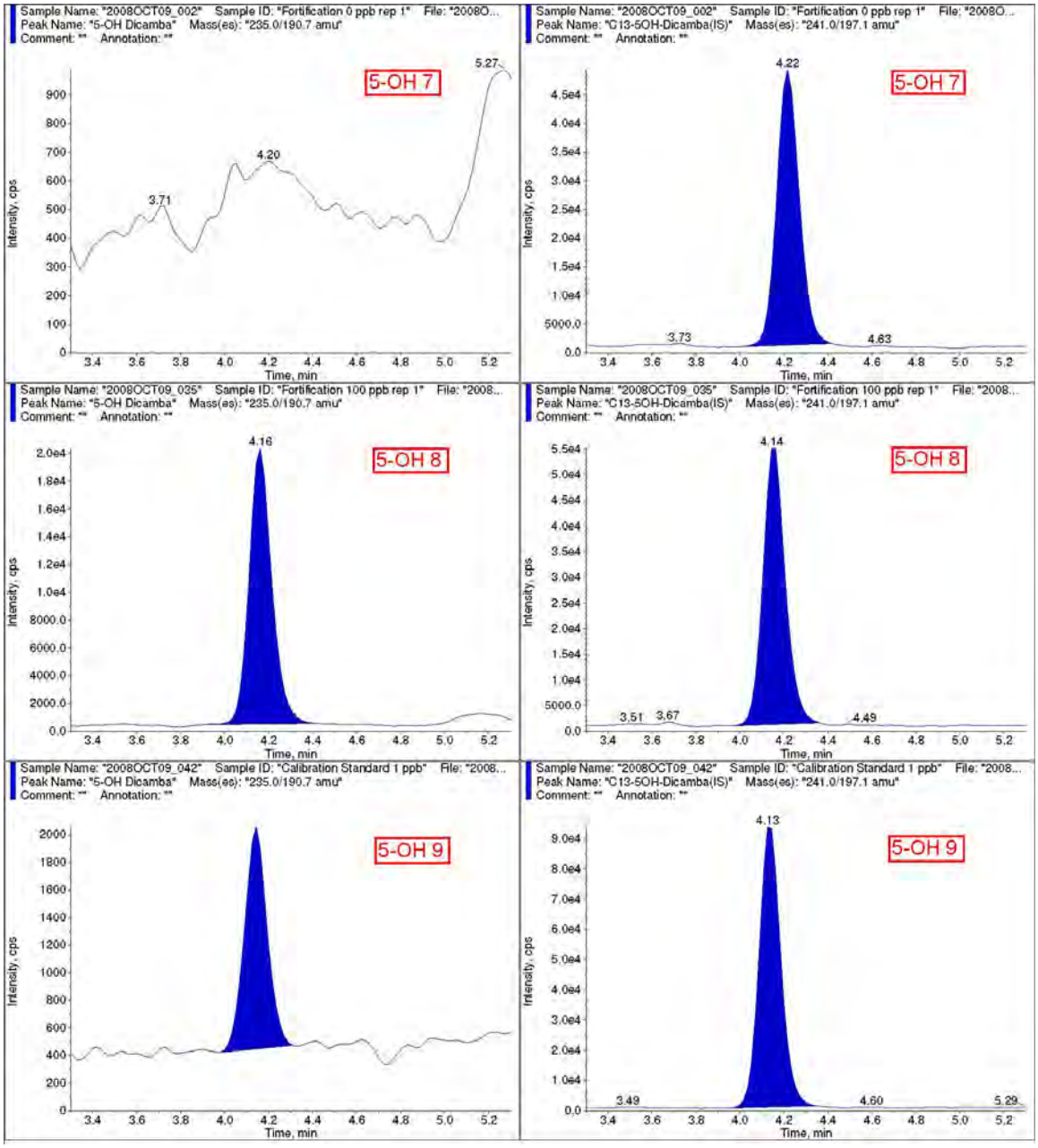
DCSA 4: untreated control (left): IS 0.040 µg/g (right)
DCSA 5: fortified control 0.10 µg/g (left): IS 0.040 µg/g (right)
DCSA 6: calibration standard 0.001 µg/mL (left): IS 0.040 µg/g (right)

Dicamba in soybean hay



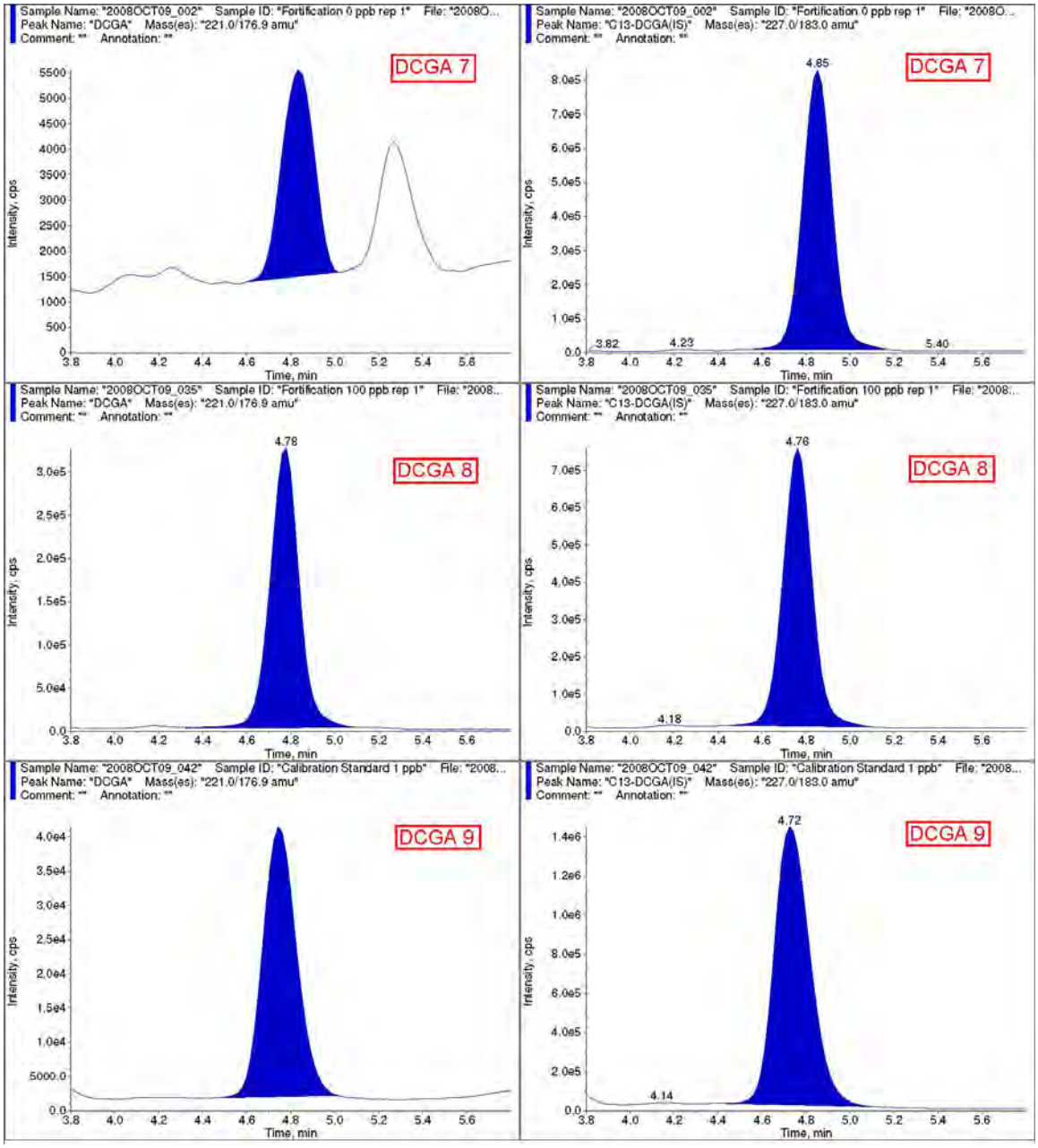
Dicamba 4: untreated control (left): IS 0.040 µg/g (right)
Dicamba 5: fortified control 0.10 µg/g (left): IS 0.040 µg/g (right)
Dicamba 6: calibration standard 0.001 µg/mL (left): IS 0.040 µg/g (right)

5-hydroxydicamba in soybean forage



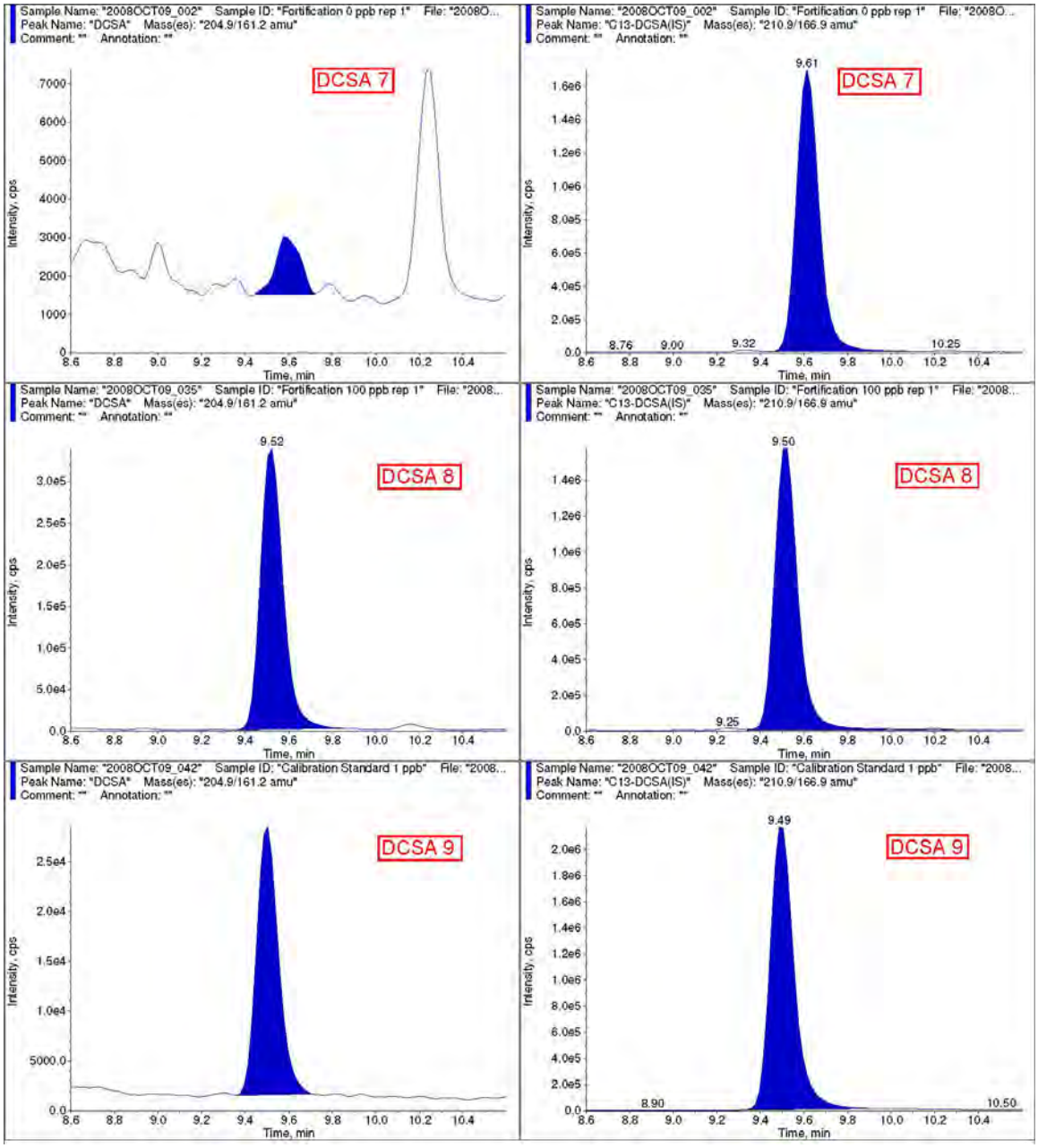
5-OH 7: untreated control (left): IS 0.040 µg/g (right)
5-OH 8: fortified control 0.10 µg/g (left): IS 0.040 µg/g (right)
5-OH 9: calibration standard 0.001 µg/mL (left): IS 0.040 µg/g (right)

DCGA in soybean forage



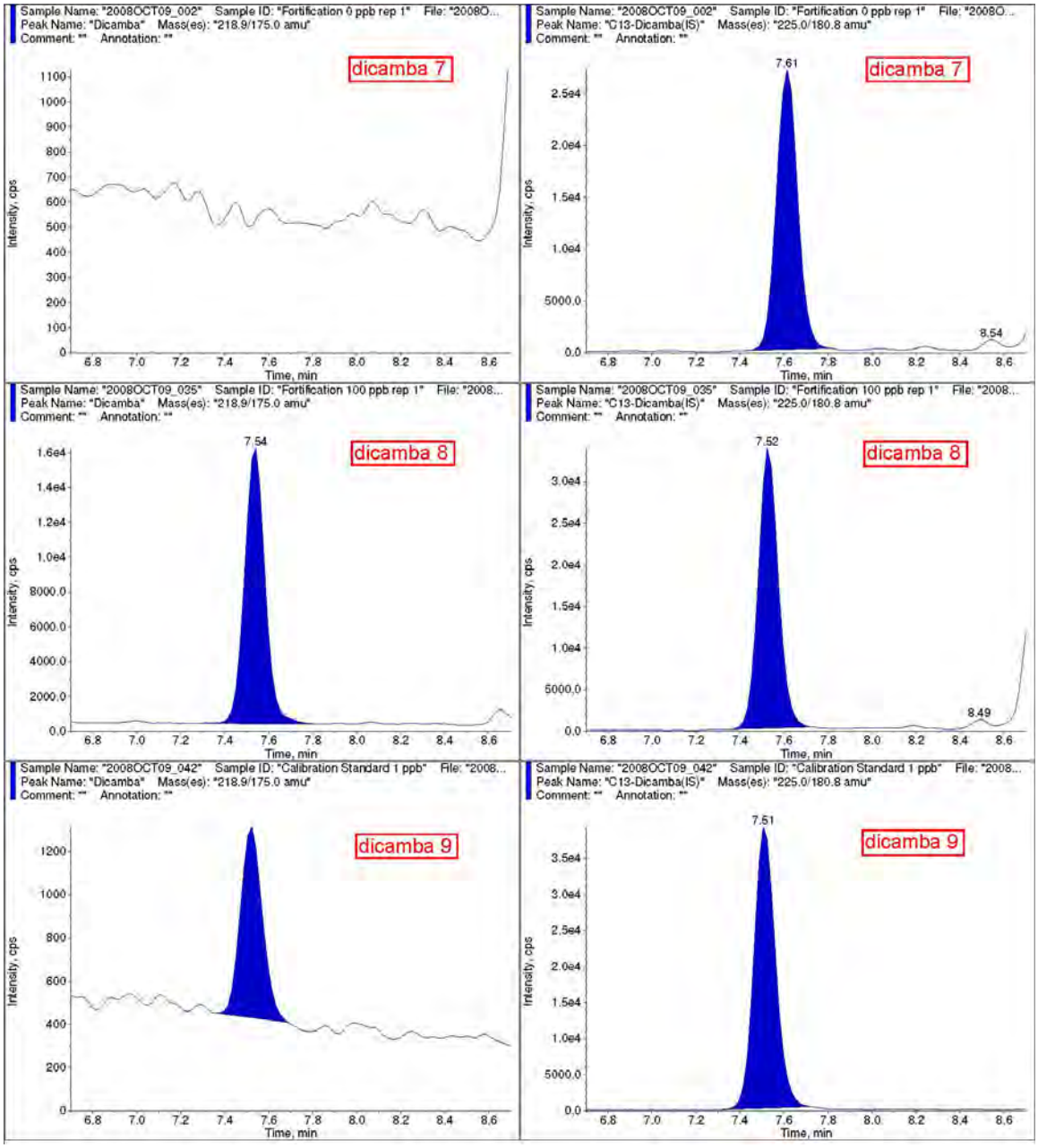
DCGA 7: untreated control (left): IS 0.040 µg/g (right)
DCGA 8: fortified control 0.10 µg/g (left): IS 0.040 µg/g (right)
DCGA 9: calibration standard 0.001 µg/mL (left): IS 0.040 µg/g (right)

DCSA in soybean forage



DCSA 7: untreated control (left): IS 0.040 µg/g (right)
DCSA 8: fortified control 0.10 µg/g (left): IS 0.040 µg/g (right)
DCSA 9: calibration standard 0.001 µg/mL (left): IS 0.040 µg/g (right)

Dicamba in soybean forage



Dicamba 7: untreated control (left): IS 0.040 µg/g (right)
Dicamba 8: fortified control 0.10 µg/g (left): IS 0.040 µg/g (right)
Dicamba 9: calibration standard 0.001 µg/mL (left): IS 0.040 µg/g (right)

Appendix D: Validation Results

Table 1: LC/MS/MS analysis of dicamba and its major metabolites in soybean seed

Level spiked (µg/g)	5-hydroxydicamba	DCGA	DCSA	Dicamba
0	ND	0.00136	ND	ND
0	ND	0.000772	ND	ND
0	ND	0.000604	ND	ND
0	ND	0.000752	ND	ND
0	ND	0.000969	ND	ND
0	ND	0.000479	ND	ND
0	ND	0.00105	ND	ND
Mean =	**N/A**	**0.000855**	**N/A**	**N/A**
0.005	0.00353	0.00434	0.00525	0.00531
0.005	0.00317	0.00466	0.00555	0.00489
0.005	0.00333	0.00419	0.00544	0.00500
0.005	0.00277	0.00465	0.00477	0.00533
0.005	0.00371	0.00410	0.00537	0.00547
0.005	0.00294	0.00463	0.00560	0.00566
0.005	0.00286	0.00421	0.00530	0.00455
Mean =	**0.00319**	**0.00440**	**0.00533**	**0.00517**
%RSD =	**11.1**	**5.54**	**5.18**	**7.35**
Accuracy =	**63.7**	**88.0**	**107**	**103**
0.010	0.00656	0.00954	0.0113	0.00958
0.010	0.00609	0.01004	0.0102	0.0105
0.010	0.00777	0.00974	0.0102	0.00997
0.010	0.00663	0.00914	0.0105	0.00949
0.010	0.00655	0.00944	0.0107	0.00990
0.010	0.00604	0.00954	0.0107	0.00941
0.010	0.00722	0.00964	0.0105	0.0110
Mean =	**0.00669**	**0.00959**	**0.0106**	**0.00998**
% RSD =	**9.19**	**2.88**	**3.55**	**5.85**
Accuracy =	**66.9**	**95.9**	**106**	**99.8**
0.020	0.0143	0.0190	0.0204	0.0192
0.020	0.0135	0.0200	0.0218	0.0205
0.020	0.0141	0.0195	0.0206	0.0201
0.020	0.0141	0.0206	0.0213	0.0196
0.020	0.0161	0.0187	0.0209	0.0188
0.020	0.0132	0.0188	0.0211	0.0181
0.020	0.0145	0.0195	0.0205	0.0207
Mean =	**0.0143**	**0.0195**	**0.0209**	**0.0196**
% RSD =	**6.53**	**3.52**	**2.39**	**4.81**
Accuracy =	**71.3**	**97.4**	**105**	**97.9**
0.100	0.101	0.0965	0.104	0.0943
0.100	0.103	0.103	0.103	0.0968
0.100	0.103	0.101	0.104	0.101
0.100	0.100	0.099	0.103	0.0995
0.100	0.105	0.102	0.107	0.0926
0.100	0.102	0.105	0.105	0.0973
0.100	0.0980	0.102	0.0998	0.0980
Mean =	**0.102**	**0.101**	**0.104**	**0.0971**
% RSD =	**2.25**	**2.76**	**2.12**	**2.97**
Accuracy =	**102**	**101**	**104**	**97.1**
2.00	2.09	1.76	2.01	1.72
2.00	2.07	1.75	1.88	1.79
Mean =	**2.08**	**1.75**	**1.95**	**1.76**
Accuracy =	**104**	**87.7**	**97.3**	**87.8**

Table 2: LC/MS/MS analysis of dicamba and its major metabolites in soybean hay

Level spiked (µg/g)	5-hydroxydicamba	DCGA	DCSA	Dicamba
0	ND	0.000175	ND	ND
0	ND	0	ND	ND
0	ND	0	ND	ND
0	ND	0.0000146	ND	ND
0	ND	0	ND	ND
0	ND	0	ND	ND
0	ND	0	ND	ND
Mean =	**N/A**	**0.0000271**	**N/A**	**N/A**
0.005	0.00370	0.00334	0.00473	ND
0.005	0.00357	0.00336	0.00502	ND
0.005	0.00458	0.00335	0.00482	ND
0.005	0.00365	0.00357	0.00466	ND
0.005	0.00390	0.00335	0.00494	ND
0.005	0.00392	0.00344	0.00465	ND
0.005	0.00377	0.00351	0.00526	ND
Mean =	**0.00387**	**0.00342**	**0.004869**	**N/A**
% RSD =	**8.73**	**2.69**	**4.55**	**N/A**
Accuracy =	**77.4**	**68.4**	**97.4**	**N/A**
0.010	0.00711	0.00808	0.0110	0.00801
0.010	0.00881	0.00860	0.0106	0.00967
0.010	0.00823	0.00856	0.0104	0.00812
0.010	0.00769	0.00847	0.0102	0.00872
0.010	0.00830	0.00798	0.0101	0.00876
0.010	0.00800	0.00817	0.0104	0.00827
0.010	0.00786	0.00817	0.0107	0.00883
Mean =	**0.00800**	**0.00829**	**0.0105**	**0.00863**
% RSD =	**6.67**	**3.00**	**2.94**	**6.56**
Accuracy =	**80.0**	**82.9**	**105**	**86.3**
0.020	0.0145	0.0177	0.0217	0.0176
0.020	0.0153	0.0185	0.0213	0.0187
0.020	0.0167	0.0186	0.0208	0.0211
0.020	0.0168	0.0179	0.0215	0.0223
0.020	0.0176	0.0181	0.0217	0.0171
0.020	0.0176	0.0182	0.0209	0.0201
0.020	0.0191	0.0179	0.0207	0.0174
Mean =	**0.0168**	**0.0181**	**0.0212**	**0.0192**
% RSD =	**9.13**	**1.82**	**2.01**	**10.5**
Accuracy =	**84.0**	**90.5**	**106**	**95.9**
0.100	0.107	0.0975	0.106	0.108
0.100	0.101	0.104	0.107	0.0984
0.100	0.106	0.103	0.111	0.103
0.100	0.0947	0.0964	0.108	0.106
0.100	0.103	0.0957	0.105	0.104
0.100	0.101	0.0984	0.111	0.112
0.100	0.102	0.105	0.103	0.104
Mean =	**0.102**	**0.100**	**0.107**	**0.105**
% RSD =	**3.94**	**3.88**	**2.78**	**4.05**
Accuracy =	**102**	**100**	**107**	**105**
2.00	1.81	1.86	1.77	1.43
2.00	1.78	1.74	1.75	1.47
Mean =	**1.80**	**1.80**	**1.76**	**1.45**
Accuracy =	**89.8**	**90.0**	**88.0**	**72.5**

Table 3: LC/MS/MS analysis of dicamba and its major metabolites in soybean forage

Level spiked (µg/g)	5-hydroxydicamba	DCGA	DCSA	Dicamba
0	ND	0.00133	ND	ND
0	ND	0.00141	ND	ND
0	ND	0.00149	ND	ND
0	ND	0.00134	ND	ND
0	ND	0.00135	ND	ND
0	ND	0.00151	ND	ND
0	ND	0.00148	ND	ND
Mean =	**N/A**	**0.00142**	**N/A**	**N/A**
0.005	0.00477	0.00328	0.00551	0.00529
0.005	0.00461	0.00374	0.00510	0.00518
0.005	0.00554	0.00349	0.00535	0.00442
0.005	0.00482	0.00355	0.00472	0.00596
0.005	0.00447	0.00368	0.00511	0.00570
0.005	0.00485	0.00377	0.00487	0.00522
0.005	0.00522	0.00388	0.00514	0.00506
Mean =	**0.00490**	**0.00363**	**0.00511**	**0.00526**
% RSD =	**7.49**	**5.57**	**5.22**	**9.30**
Accuracy =	**97.9**	**72.6**	**102**	**105**
0.010	0.00989	0.00788	0.0127	0.00985
0.010	0.00976	0.00802	0.0114	0.0103
0.010	0.0104	0.00820	0.0108	0.00969
0.010	0.00927	0.00816	0.0107	0.00957
0.010	0.0105	0.00817	0.0102	0.00955
0.010	0.00952	0.00848	0.0106	0.00963
0.010	0.0102	0.00770	0.0104	0.0122
Mean =	**0.00993**	**0.00809**	**0.0110**	**0.0101**
% RSD =	**4.60**	**3.10**	**7.75**	**9.45**
Accuracy =	**99.3**	**80.9**	**110**	**101**
0.020	0.0194	0.0176	0.0205	0.0196
0.020	0.0209	0.0183	0.0214	0.0193
0.020	0.0200	0.0174	0.0221	0.0211
0.020	0.0206	0.0171	0.0219	0.0219
0.020	0.0204	0.0181	0.0213	0.0186
0.020	0.0192	0.0170	0.0217	0.0192
0.020	0.0192	0.0178	0.0216	0.0203
Mean =	**0.0200**	**0.0176**	**0.0215**	**0.0200**
% RSD =	**3.52**	**2.77**	**2.42**	**5.83**
Accuracy =	**99.8**	**88.0**	**108**	**100**
0.100	0.104	0.0882	0.102	0.113
0.100	0.107	0.0863	0.109	0.105
0.100	0.103	0.0900	0.106	0.0967
0.100	0.104	0.0929	0.105	0.109
0.100	0.101	0.0894	0.101	0.107
0.100	0.106	0.0911	0.11	0.103
0.100	0.107	0.0919	0.109	0.101
Mean =	**0.105**	**0.0900**	**0.106**	**0.105**
% RSD =	**2.13**	**2.51**	**3.36**	**5.11**
Accuracy =	**105**	**90.0**	**106**	**105**
2.00	1.90	1.97	1.73	1.80
2.00	1.93	1.93	1.81	1.91
Mean =	**1.92**	**1.95**	**1.77**	**1.86**
Accuracy =	**95.8**	**97.4**	**88.5**	**92.8**

Appendix E: Verification Results of Processed Fractions

Table 4: LC/MS/MS analysis of dicamba and its major metabolites in soybean hulls

Level spiked (µg/g)	Dicamba	DCSA	5-hydroxydicamba	DCGA
Control	ND	ND	ND	ND
Control	ND	ND	ND	ND
Control	0.00	0.000910	0.000333	0.00132
0.010	95.3	117	62.4	74.3
0.010	89.7	114	86.4	82.4
Mean =	**92.5**	**115**	**74.4**	**78.4**
0.020	99.8	110	63.4	91.9
0.020	92.7	111	66.9	92.9
0.020	84.5	109	86.8	79.9
0.020	94.0	108	82.3	79.9
Mean =	**92.8**	**109**	**74.9**	**86.2**
% RSD =	**6.80**	**1.42**	**15.3**	**8.39**
0.050	95.1	107	62.1	92.4
0.050	93.1	110	62.2	97.2
0.050	100	94.2	100	75.4
Mean =	**96.1**	**104**	**74.8**	**88.3**
% RSD =	**3.81**	**8.09**	**29.3**	**13.0**
0.200	96.5	99.5	100	86.8
0.400	99.3	101	92.2	91.6
0.400	103	95.6	91.0	90.1
Mean =	**101**	**98.3**	**91.6**	**90.9**
1.500	106	97.9	101	97.9

Table 5: LC/MS/MS analysis of dicamba and its major metabolites in soybean defatted flour

Level spiked (µg/g)	Dicamba	DCSA	5-hydroxydicamba	DCGA
Control	ND	ND	0.0101	ND
Control	ND	ND	0.0112	ND
Control	0.00145	0.00197	0.00345	0.000181
Control	0.00	0.00187	0.00464	0.00
0.010	86.4	105.8	31.9	78.4
0.010	72.1	98.8	30.0	87.6
Mean =	**79.3**	**102**	**31.0**	**83.0**
0.020	79.4	94.9	70.0	101
0.020	76.9	101	58.8	99.1
Mean =	**78.2**	**98.2**	**64.4**	**99.9**
0.050	96.3	103	84.3	84.8
0.200	94.1	94.0	91.5	91.0
0.400	103	98.0	96.7	102
0.400	109	101	102	90.0
0.400	97.0	112	99.6	96.4
Mean =	**103**	**104**	**99.4**	**96.0**
% RSD =	**5.82**	**7.11**	**2.57**	**6.10**
2.000	89.7	91.0	98.5	100
2.000	74.1	87.3	88.5	95.4
Mean =	**81.9**	**89.2**	**93.5**	**97.7**
3.000	97.6	92.3	98.5	96.3

Table 6: LC/MS/MS analysis of dicamba and its major metabolites in soybean toasted defatted meal

Level spiked (µg/g)	Dicamba	DCSA	5-hydroxydicamba	DCGA
Control	ND	ND	ND	ND
Control	ND	ND	ND	ND
Control	0.00	0.00356	0.000114	0.00154
0.010	83.4	96.4	75.9	78.0
0.010	93.9	89.4	65.5	85.5
Mean =	**88.7**	**92.9**	**70.7**	**81.8**
0.020	104	111	94.5	99.2
0.020	100	120	94.7	97.8
0.020	94.0	97.7	76.4	89.3
0.020	92.5	92.7	80.9	84.8
Mean =	**97.5**	**105**	**86.6**	**92.8**
% RSD =	**5.42**	**11.8**	**10.8**	**7.42**
0.050	99.0	110	77.0	97.7
0.050	81.3	114	89.6	94.5
0.050	98.2	98.1	93.0	83.9
Mean =	**92.8**	**107**	**86.5**	**92.0**
% RSD =	**10.8**	**7.70**	**9.74**	**7.85**
0.200	94.0	101	101	83.2
0.400	96.7	104	85.6	90.4
0.400	96.7	99.8	85.7	95.4
Mean =	**96.7**	**102**	**85.7**	**92.9**
1.500	88.0	93.8	95.3	104

Table 7: LC/MS/MS analysis of dicamba and its major metabolites in soybean protein isolate

Level spiked (µg/g)	Dicamba	DCSA	5-hydroxydicamba	DCGA
Control	ND	ND	ND	ND
Control	ND	0.000515	ND	ND
Control	0.00	0.00127	0.00	0.000269
Control	0.00	0.00165	0.00	0.000185
0.010	83.0	92.4	80.4	79.2
0.010	77.9	86.4	93.2	79.3
Mean =	**80.5**	**89.4**	**86.8**	**79.3**
0.020	76.5	95.2	90.3	84.3
0.020	97.7	94.2	97.9	81.4
Mean =	**87.1**	**94.7**	**94.1**	**82.9**
0.400	86.8	107	103	104
0.400	76.5	78.4	92.3	74.4
Mean =	**81.7**	**92.7**	**97.7**	**89.2**
0.800	87.8	105	99.8	104
0.800	86.4	95.8	96.1	83.2
Mean =	**87.1**	**100**	**98.0**	**93.6**
1.500	88.1	92.6	101	86.7

Table 8: LC/MS/MS analysis of dicamba and its major metabolites in soybean protein concentrate

Level spiked (µg/g)	Dicamba	DCSA	5-hydroxydicamba	DCGA
control	ND	ND	ND	ND
control	ND	0.00148	ND	0.00166
control	0.00	0.00137	0.00	0.000281
0.010	77.6	97.3	81.5	79.0
0.010	84.3	95.3	79.9	86.3
Mean =	**81.0**	**96.3**	**80.7**	**82.7**
0.020	90.1	103	84.8	89.2
0.200	90.1	105	106	97.1
0.400	110	101	104	104
0.400	118	93.6	96.7	87.8
Mean =	**114**	**97.3**	**100**	**95.9**
1.500	94.3	90.6	105	89.3

Table 9: LC/MS/MS analysis of dicamba and its major metabolites in soybean crude lecithin

Level spiked (µg/g)	Dicamba	DCSA	5-hydroxydicamba	DCGA
Control	0.00	0.000889	0.00	0.00
Control	0.00	0.000102	0.00	0.00
Control	0.000946	0.00	0.000394	0.00
Control	0.00	0.00	0.000844	0.00
Control	0.00	0.00	0.00	0.00
Control	0.00	0.00	0.00	0.00
Control	0.00167	0.00120	0.00	0.00
Control	0.00	0.00	0.000321	0.00
0.010	101	93.9	81.1	68.0
0.010	77.4	98.0	79.4	68.4
0.010	72.3	88.9	91.7	77.5
Mean =	**83.5**	**93.6**	**84.1**	**71.3**
% RSD =	**18.1**	**4.90**	**7.93**	**7.54**
0.200	95.0	106	102	79.5
0.200	76.6	91.2	100	77.0
0.200	86.6	93.2	94.4	80.0
Mean =	**86.1**	**96.7**	**98.9**	**78.8**
% RSD =	**10.7**	**8.15**	**4.05**	**2.04**
0.400	109	107	104	93.5
0.400	104	108	110	88.0
Mean =	**107**	**107**	**107**	**90.8**
2.00	107	109	111	99.5
2.00	108	107	95.0	103
Mean =	**107**	**108**	**103**	**101**

Table 10: LC/MS/MS analysis of dicamba and its major metabolites in soybean degummed oil

Level spiked (µg/g)	Dicamba	DCSA	5-hydroxydicamba	DCGA
Control	ND	ND	ND	ND
Control	ND	ND	ND	ND
Control	0.00	0.00	0.00102	0.00
Control	0.00	0.000451	0.00148	0.00142
0.010	94.9	93.8	82.3	81.7
0.010	98.5	99.5	86.2	69.3
0.010	102	94.7	86.2	61.3
Mean =	**98.5**	**96.0**	**84.9**	**70.8**
% RSD =	**3.61**	**3.19**	**2.65**	**14.5**
0.020	88.0	96.5	89.4	95.5
0.020	95.5	101	85.6	70.9
0.020	97.0	99.7	88.1	67.4
Mean =	**93.5**	**99.1**	**87.7**	**77.9**
% RSD =	**5.16**	**2.42**	**2.20**	**19.6**
0.050	78.0	103	90.8	93.6
0.050	95.6	98.7	96.6	70.8
Mean =	**86.8**	**101**	**93.7**	**82.2**
0.200	119	96.5	102	105
0.400	83.5	99.8	90.0	100
0.400	106	104	106	88.8
0.400	100	107	102	88.7
Mean =	**96.5**	**104**	**99.3**	**92.6**
% RSD =	**12.1**	**3.49**	**8.38**	**7.20**
2.000	107	95.7	102	107
2.000	99.1	103	99.9	103
Mean =	**103**	**99.4**	**101**	**105**

Table 11: LC/MS/MS analysis of dicamba and its major metabolites in soybean RBD oil

Level spiked (µg/g)	Dicamba	DCSA	5-hydroxydicamba	DCGA
Control	ND	ND	ND	0.00202
Control	ND	ND	ND	ND
Control	0.00	0.000603	0.00949	0.00109
Control	0.000887	0.000733	0.00112	0.00191
0.010	85.6	94.0	73.3	60.1
0.010	88.0	95.7	88.7	64.2
0.010	93.1	95.7	79.3	66.6
Mean =	**88.9**	**95.1**	**80.4**	**63.6**
% RSD =	**4.31**	**1.03**	**9.65**	**5.17**
0.020	69.5	97.5	79.3	77.1
0.020	93.1	104	89.4	76.0
0.020	100	93.3	91.4	66.5
Mean =	**87.6**	**98.2**	**86.7**	**73.2**
% RSD =	**18.3**	**5.38**	**7.48**	**7.96**
0.050	79.6	91.8	95.3	82.4
0.050	103	101	104	76.2
Mean =	**91.1**	**96.2**	**100**	**79.3**
0.200	105	99.7	90.0	94.5
0.400	94.8	97.1	92.0	105
0.400	112	107	107	94.0
0.400	105	104	108	99.7
Mean =	**104**	**103**	**102**	**99.4**
% RSD =	**8.32**	**4.94**	**8.76**	**5.29**
2.000	102	106	101	100
2.000	101	107	98.6	106
Mean =	**102**	**107**	**100**	**103**

Table 12: LC/MS/MS analysis of dicamba and its major metabolites in soymilk

Level spiked (µg/g)	Dicamba	DCSA	5-hydroxydicamba	DCGA
Control	ND	ND	ND	ND
Control	ND	ND	ND	ND
Control	0.00	0.00	0.00	0.00
Control	0.00	0.00	0.00108	0.000870
0.010	101	95.0	84.2	74.8
0.010	95.1	105	82.0	68.8
0.010	89.1	102	84.2	74.3
Mean =	**95.1**	**101**	**83.5**	**72.6**
% RSD =	**6.26**	**5.10**	**1.52**	**4.58**
0.020	108	92.5	81.5	92.5
0.020	94.5	108	91.6	78.7
0.020	86.0	108	93.6	78.7
Mean =	**96.2**	**103**	**88.9**	**83.3**
% RSD =	**11.5**	**8.58**	**7.30**	**9.56**
0.050	67.4	102	92.8	100
0.050	100	106	96.2	72.7
Mean =	**83.9**	**104**	**94.5**	**86.5**
0.200	64.0	105	87.5	107
0.400	79.0	89.5	90.8	95.8
0.400	97.0	92.9	95.2	82.8
0.400	96.0	99.8	92.6	85.0
Mean =	**90.7**	**94.1**	**92.9**	**87.9**
% RSD =	**11.2**	**5.58**	**2.38**	**7.92**
2.000	76.0	101	96.3	96.6
2.000	79.4	94.5	88.5	93.8
Mean =	**77.7**	**97.8**	**92.4**	**95.2**

Table 13: LC/MS/MS analysis of dicamba and its major metabolites in tofu

Level spiked (µg/g)	Dicamba	DCSA	5-hydroxydicamba	DCGA
Control	ND	ND	ND	ND
Control	0.00	0.00	0.000111	0.000557
0.010	82.3	112	97.8	74.5
0.010	73.0	106	87.5	81.1
Mean =	**77.7**	**109**	**92.7**	**77.8**
0.020	80.5	101	96.4	78.2
0.050	80.9	103	100	92.6
0.200	97.6	91.6	94.7	85.6
0.400	96.2	92.3	92.5	92.5
0.400	76.1	84.8	87.5	90.5
0.400	71.2	70.5	73.5	74.1
Mean =	**81.2**	**82.5**	**84.5**	**85.7**
% RSD =	**16.3**	**13.4**	**11.7**	**11.8**
1.500	91.1	80.5	83.3	93.3

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SOP Amendment

SOP number: **AG-ME-1321-01**

Title: **Determination of Dicamba and Its Major Metabolites in Soybean Matrices by LC/MS/MS**

Amendment number: **1** Effective date: **November 19, 2009**

SOP Originally States (Include page no. &/or section.):

Purpose & scope

This SOP describes the method used by ESTC personnel to determine the residues of dicamba and its endogenous metabolites, analyzed as chemophores 5-hydroxydicamba, DCSA and DCGA, in soybean matrices. Analyte-specific stable labeled ISs are used to compensate for matrix effects and procedural recovery. (Refer to Appendix A for analyte and standard compound structures.) The radiovalidation, which demonstrates the extraction efficiency and recovery of the method, is conducted using soybean hay and seed samples from study 06-98-M-1, "Metabolism of Dicamba in Dicamba-Tolerant Soybeans", in which [^{14}C]-dicamba was used as the test substance.

"**Sample preparation**" procedure, page 10 :

16	Evaporate the organic layer until only the aqueous solution remains. Avoid evaporating to dryness. Typical RapidVap™ settings: Temperature = 45 °C, Vacuum = 100 mBar, Speed = 35%, Time = 35 minutes. **Suggestion:** Prepare the working calibration standards once the RapidVap™ has been activated (See "**Calibration standards**" in "**Sample Preparation**").
17	Pipet 2.5 mL of DI water into the evaporator vial.

SOP Amended as Follows:

Purpose & scope

This SOP describes the method used by ESTC personnel to determine the residues of dicamba and its endogenous metabolites, analyzed as chemophores 5-hydroxydicamba, DCSA and DCGA, in soybean matrices. Analyte-specific stable labeled ISs are used to compensate for matrix effects and procedural recovery. (Refer to Appendix A for analyte and standard compound structures.)

16	Pipet 2.5 mL of DI water into the evaporator vial.
17	Evaporate the organic layer until only the aqueous solution remains. Avoid evaporating to dryness. Typical RapidVap™ settings: Temperature = 45 °C, Vacuum = 100 mBar, Speed = 35%, Time = 35 minutes. **Suggestion:** Prepare the working calibration standards once the RapidVap™ has been activated (See "**Calibration standards**" in "**Sample Preparation**").

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Reason for Amendment (This may include a description of the impact of the change.):

Removed the reference in the purpose & scope about radiovalidation as that information has been provided as part of the validation summary versus the analytical method SOP.

Steps 16 and 17 of sample preparation were iandvertently switched and are being corrected.

Author(s) / prepared by: James Foster / Tessa V. Ploesser

Management:

Mary P. Gasser
(TFM, Monsanto Company)

Date: 11 / 19 / 09

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SOP Amendment

SOP number: **AG-ME-1321-01**

Title: **Determination of Dicamba and Its Major Metabolites in Soybean Matrices by LC/MS/MS**

Amendment number: **2** Effective date: **March 19, 2010**

1) SOP Originally States (Include page no. and/or section):

- **Page 7, "Solution Preparation"** section:

Calibration standards

The instrument calibration standards are made at convenient concentrations of each analyte. The solutions may be prepared in the following manner. Other concentrations may be used provided that the preparation is documented.

Individual stock concentration (µg/mL)	Volume of stock used (mL)	Calibration standard spiking solution total volume (mL)	Calibration standard spiking solution concentration (µg/mL)	Working calibration standard concentration (µg/L)
100	5.0	20	25.0*	500
100	2.5	20	12.5*	250
100	1.0	20	5.00*	100
Calibration standard spiking solution concentration (µg/mL)	**Volume of spiking solution used (mL)**	**Calibration standard spiking solution total volume (mL)**	**Calibration standard spiking solution concentration (µg/mL)**	**Injected calibration standard concentration (µg/L)**
25.0	2.0	20	2.50*	50.0
12.5	2.0	20	1.25	25.0
5.00	2.0	20	0.500*	10.0
2.50	2.0	20	0.250*	5.0
0.500	2.0	20	0.050	1.0

* *Aliquots of these calibration standard solutions are diluted to create lower concentration levels.*

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1) SOP Amended as Follows:

Calibration standards

The instrument calibration standards are made at convenient concentrations of each analyte. The solutions may be prepared in the following manner. Other concentrations may be used provided that the preparation is documented.

Individual stock concentration (µg/mL)	Volume of stock used (mL)	Calibration standard spiking solution total volume (mL)	Calibration standard spiking solution concentration (µg/mL)	Injected calibration standard concentration (µg/mL)
100	5.0	20	25.0*	0.500
100	2.5	20	12.5*	0.250
100	1.0	20	5.00*	0.100
Calibration standard spiking solution concentration (µg/mL)	**Volume of spiking solution used (mL)**	**Calibration standard spiking solution total volume (mL)**	**Calibration standard spiking solution concentration (µg/mL)**	**Injected calibration standard concentration (µg/mL)**
25.0	2.0	20	2.50*	0.050
12.5	2.0	20	1.25	0.025
5.00	2.0	20	0.500*	0.010
2.50	2.0	20	0.250*	0.005
0.500	2.0	20	0.050	0.001

* *Aliquots of these calibration standard solutions are diluted to create lower concentration levels.*

1) Reason for Amendment:

Changed the last column heading to "Injected calibration standard concentration (µg/mL)" and converted the injected calibration standard concentration values from units of µg/L to µg/mL for consistency.

2) SOP Originally States (Include page no. and/or section):

- Page 14, "**Residue Calculations**" section:

Quadratic equation in "**Calibration curve**".

$$\textit{Injected concentration (ng/mL analyte)} = -B \pm \sqrt{(B^2 - 4AC)} / 2A$$

2) SOP Amended as Follows:

$$\textit{Injected concentration (µg/mL analyte)} = [-B \pm \sqrt{(B^2 - 4AC)}] / 2A$$

2) Reason for Amendment:

The injected concentration equation was changed to µg/mL for consistency in units. The numerator in this equation was clearly defined.

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3) SOP Originally States (Include page no. and/or section):

- Page 14, "**Residue Calculations**" section:

Analyte concentrations

The analytical method contains sample dilution and the resulting ppm value taken directly from the regression curve must be multiplied by a 5X dilution factor. Enter the dilution factor into the Analyst™ "dilution factor" column to automatically calculate the final concentration. The calculated value represents the concentration of the analyte in the initial sample. The sample concentration is calculated by the software as shown in the equation below:

$$\mu g/g\ (analyte) = \frac{[(\mu g/mL\ analyte\ found)(final\ volume)]}{sample\ weight\ (g)}\ X\ \frac{extract\ volume}{extract\ aliquot\ volume}$$

---

3) SOP Amended as Follows:

Analyte concentrations

The analytical method contains sample dilution and the resulting concentration value (µg/mL analyte) derived from the regression curve must be multiplied by an appropriate dilution factor. Enter the dilution factor into the Analyst™ "dilution factor" column to automatically calculate the final concentration in the raw sample. The dilution factor is calculated manually using the following equation:

$$dilution\ factor\ (mL/g) = \frac{final\ volume\ (mL)}{sample\ weight\ (g)}\ X\ \frac{extract\ volume\ (mL)}{extract\ aliquot\ volume\ (mL)}$$

where:

- final volume is the volume of the standard solutions (5 mL)
- sample weight is the raw sample weight (10 g)
- extract volume is the volume of extraction solvent (100 mL)
- extract aliquot volume is the volume of the extract aliquot carried through the method (normally 10 mL but will be lower for samples requiring additional dilution)

Using the sample weights and volumes (above) dictated by the method, the dilution factor calculated by the above equation and entered into the Analyst™ software is '5'. For samples that require additional dilution due to responses out of the standard curve range, the sample dilution is included in the dilution factor calculation by modifying the 'extract aliquot volume' in the equation. For instance, for a 10-fold sample dilution (1-mL extract aliquot carried through the method), the calculated dilution factor is '50'. The raw sample concentration is calculated by the Analyst™ software as shown in the equation below:

$$\mu g/g\ (ppm)\ analyte\ found = (\mu g/mL\ analyte\ found)\ X\ (dilution\ factor)$$

Using this method, the raw sample concentration results (ppm analyte found) generated by the Analyst™ software are individual analyte concentrations. In order to convert the concentration values to parent dicamba equivalents, it is necessary to multiply the values by appropriate conversion factors accounting for the differences in molecular

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weight between parent dicamba and the analytes. Based on molecular weights of 221.04, 207.01, 223.01 and 237.04 for dicamba, DCSA, DCGA and 5-hydroxydicamba, respectively, the conversion factors are 1.068, 0.991, and 0.933 for DCSA, DCGA and 5-hydroxydicamba, respectively.

Note: The Residue Information Management System III (RIMS III) software allows entry of parent/analyte conversion factors in the 'P/A Conversion' tab of the analytical interface. However, the values entered are not utilized by the RIMS III software for analytical data imported from the Analyst™ data system. The calculation for conversion to parent equivalents must be conducted outside of Analyst™ and RIMS III.

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3) Reason for Amendment:

The text was modified to clarify how analyte calculations are performed and how dilution factors are entered into Analyst™. Information was added regarding parent/analyte conversions as well.

4) SOP Originally States (Include page no. and/or section):

- Page 14, "**Residue Calculations**" section:

Analytical recovery

Successful method performance for each analytical set is assessed by the determination of percent recovery of known amounts of the analytes fortified into control samples. The percent recovery of each analyte is calculated as shown below:

$$\%\ recovery = [(100)(\mu g/g\ analyte\ found)] / \mu g/g\ analyte\ added$$

For a large study, there should be near equal numbers of fortifications at each level so the estimated analytical accuracy will not be disproportionately weighted.

---

4) SOP Amended as Follows:

Analytical recovery

Successful method performance for each analytical set is assessed by the determination of percent recovery of known amounts of the analytes fortified into control samples. The percent recovery of each analyte is calculated as shown below:

$$\%\ recovery = (100)\ X\ \frac{(\mu g/g\ analyte\ found) - (\mu g/g\ analyte\ in\ control)}{\mu g/g\ analyte\ added}$$

For a large study, there should be near equal numbers of fortifications at each level so the estimated analytical accuracy will not be disproportionately weighted.

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4) Reason for Amendment:

The original equation did not show that the percent recoveries were background corrected.

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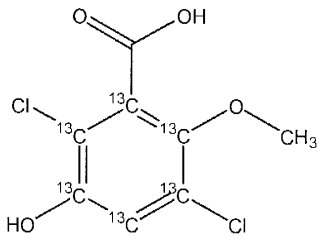
5) SOP Originally States (Include page no. and/or section):

- Page 16, "**Appendix A: Compound Structures**"

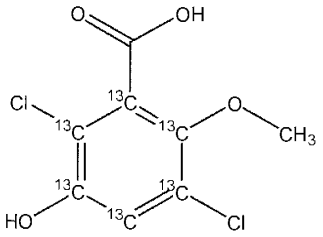
Figure 2:
IS structures



$^{3}C_6$-5-hydroxydicamba

5) SOP Amended as Follows:

Figure 2:
IS structures



$^{13}C_6$-5-hydroxydicamba

5) Reason for Amendment:

The name written for structure $^{13}C_6$-5-hydroxydicamba was incorrect.

Author(s) / prepared by: James E. Foster / Tessa V. Ploesser

Management:

Mary P. Gasper
(TFM, Monsanto Company)

Date: 3 / 19 / 10

8 APPENDIX II. RADIOVALIDATION

RADIOVALIDATION

I. Purpose and Scope of Radiovalidation

The residue analytical method AG-ME-1321 was evaluated by analyzing soybean hay and seed samples containing radioactive endogenous dicamba-derived residues. The samples were obtained from study 06-98-M-1, "Metabolism of Dicamba in Dicamba-Tolerant Soybeans", MSL0022659. In that study, dicamba-tolerant soybeans were treated either at planting (preemergence) or at the R1 growth stage (postemergence) with [phenyl-U-^{14}C]dicamba. The specific samples selected for radiovalidation analysis were soybean hay from the preemergence (PRE-T) treatment and soybean hay and seed from the postemergence (POE-T) treatment. The radioactive samples were prepared using the procedure described in residue analytical method AG-ME-1321 (draft 03DEC08), with appropriate modifications for determining recoveries of radioactivity. Radioactivity recoveries were determined for each major sample preparation step of the method. The nature and approximate quantities of the final analytes (hydrolyzed residues) generated by the method were determined by ^{14}C-HPLC profiling of the final analyte solution. Finally, quantitative analysis of the residues was performed using the liquid chromatography / tandem mass spectrometry (LC/MS/MS) procedure of the analytical method.

The suitability of the residue analytical method was assessed by 1) comparison of the amount of endogenous residues extracted using the analytical method extraction procedure to the amount extracted with the procedures utilized in the metabolism study; 2) the overall accountability (recovery) of radioactivity through the analytical procedure; and 3) comparison of the residue quantitation results obtained using the LC/MS/MS method with the actual residue levels in the samples as determined by combustion and ^{14}C-HPLC profiling.

II. Experimental Design

A draft version (draft 03DEC08) of analytical method AG-ME-1321 (see Appendix I) was used for the radiovalidation analyses. The calibration standards, fortifications, solutions and sample preparation portions of the draft version of the method were identical to the version presented in Appendix I. Other sections of the method had not been written or were edited upon subsequent review. Minor modifications were made to the procedure to accommodate the determination of radioactivity recoveries. These modifications consisted of additional weighings and aliquoting at the various steps for which recoveries of radioactivity were determined. The detailed procedure that was followed for preparation and analysis of the radioactive samples is presented in Section III (Sample Analysis). Duplicate samples of PRE-T hay, POE-T hay and POE-T seed from metabolism study 06-98-M-1 were prepared and analyzed. The samples were extracted with 40:60 (v:v) acetonitrile:water and centrifuged. An aliquot of each extract (supernatant) was submitted to acid hydrolysis and the hydrolysis solution was partitioned with ethyl acetate:isooctane. The organic partition, which contained the desired analytes, was evaporated with a water keeper and the resulting aqueous solution was filtered. For each matrix, one of the duplicate post-evaporation aqueous solutions was analyzed by HPLC with collection of fractions and liquid scintillation counting of the fractions (HPLC/LSC, Section IIIB) to determine the nature and amounts of the final analytes generated by the residue analytical method. Identities of the analytes were determined by comparison of HPLC/UV retention times of reference standards to the retention times of the analyte peaks. Quantitative analysis was performed on both replicates of the final analyte solutions generated from each of the three matrices by LC/MS/MS (Section IIIC).

The radioactive residues in each sample extract, hydrolysis solution, organic and aqueous partition fractions, post-evaporation aqueous solution, evaporation distillate and post-filtration aqueous solution were quantified by liquid scintillation counting (LSC)(Section IIID). The liquid samples were weighed before and after each analytical method step and aliquots were removed and weighed for LSC. The post-extraction solids (extraction pellets) from the extractions of the hay and seed were weighed and aliquots were analyzed by combustion and LSC (Section IIIE). LSC results and the sample/aliquot weights were used to determine the recovery of radioactivity in each step of the method.

III. Sample Analysis

A. Sample Preparation

Soybean hay and seed samples were prepared for analysis as follows:

1. Weigh 10 ± 0.1 g of ground radioactive dicamba-tolerant soybean matrix into a tared 250 mL polypropylene bottle or other suitable container.
2. Pipet 1.0 mL of the 2.00 µg/mL three-analyte internal standard solution into every container.
3. Add approximately 98 mL of 40:60 acetonitrile:water.
4. Cap the bottle tightly and place on a shaker for approximately thirty minutes.
5. Centrifuge for approximately 10 minutes at 8000-9500 rpm.
6. Obtain the total sample weight.
7. Decant the liquid into a tared plastic bottle and weigh. Count aliquots. Weigh wet pellet.
8. FOR POE-T HAY ONLY: Dilute 1/15 as follows. Remove 10 mL of the supernatant to a tared plastic bottle and weigh. Add 0.1 mL of DCGA internal standard solution and reweigh. Remove 0.67 mL into a tared amber glass bottle and weigh. Add 9.33 mL of internal standard diluents solution, 17 mL of deionized water and 2.7 mL of concentrated HCl. Proceed to step 11.
9. FOR PRE-T HAY AND POE-T SEED: Pipet 10 mL of the supernatant into a tared amber glass bottle and weigh. Add approximately 17 mL of deionized water and 2.7 mL of concentrated HCl.
10. Spike all with 0.1 mL of DCGA internal standard solution.
11. Loosely cap the amber glass bottle with a Teflon cap and heat in a water bath for approximately 1 hour at approximately 95 °C.
12. Allow the bottle to return to ambient temperature using a container of cool water or by leaving it in a fume hood for an extended time. Weigh, remove aliquots for LSC, and reweigh the remainder.
13. Add approximately 30 mL of 40:60 ethyl acetate:isooctane to the amber glass bottle.
14. Place the mixture on a shaker for at least 10 minutes.
15. Collect the organic phase in a suitable tared evaporator vial using phase separator paper and a funnel.
16. Rinse the glass bottle with approximately 5 ml of 40:60 ethyl acetate:isooctane and add it to its respective funnel containing the phase separator paper and the aqueous layer.
17. Weigh the entire organic layer, remove weighed aliquots for LSC (cap them to contain evaporation) then reweigh the remaining organic layer. Weigh the water layer into a tared plastic bottle, aliquot for LSC. Save filter paper (see step 26).

18. Evaporate the organic phase for approximately thirty-five minutes. Typical RapidVap™ Evaporator settings: Temp: 45°C, Vac: 225 mBar, Speed: 35%
19. Pipet 2.5 mL of deionized water into the evaporator vial.
20. Evaporate the organic layer until only the aqueous solution remains. Avoid evaporating to dryness. Weigh, aliquot for LSC. Typical RapidVap™ Evaporator settings: Temp: 45°C, Vac: 100 mBar, Speed: 35%, Time: 35 minutes
21. FOR ^{14}C-HPLC PROFILING – filter approximately 1.2 mL of the solution through a 0.2 μm filter into a tared vial and weigh. Remove applied aliquots to count by LSC. Weigh the vial before and after injection.
22. Inject approx. 900 uL of the filtered solution onto the HPLC using the method described in Section IIIB, below, and collect 0.3 min fractions using the Foxy fraction collector. Add 6 mL of scintillation cocktail to each vial and count by LSC for 10 minutes with the applied aliquots and three blanks (background vials).
23. FOR LC-MS ANALYSIS – filter approx. 1.2 mL of the solution through a 0.2 μm filter into a 15 mL centrifuge tube. Add equivalent volumes of the filtered sample and 9% formic acid to an autosampler vial (approx. 500 uL each). Analyze the solution by LC/MS/MS (inject 10 μL).
24. Weigh the wet pellet. Dry the pellet and reweigh it. Weigh and combust aliquots of the dried pellet.
25. Weigh and count the distillate from Steps 19-21 (combined for all samples and aliquot, if necessary). **Note:** This was not done for POE-T hay since many non-validation samples were done at the same time.
26. Extract filter papers with 15 mL acetone by shaking in a scintillation vial. Weigh the acetone and count three 1 mL aliquots.
27. FOR PRE-T HAY: Dry (in air) 1 gram of the combustion pellet for 4 days and reweigh to determine the loss on drying in order to estimate the percent moisture in hay.

Appropriate fortified and control samples were included with each sample set and were prepared as described in the analytical method.

B. ^{14}C-HPLC Analysis

^{14}C-HPLC profiles of the post-evaporative aqueous sample (final analyte solution) were obtained using the following method:

Instrument: Agilent/Hewlett Packard HP 1100 consisting of G1322A solvent degasser, G1311A quaternary pump, G1315A diode array (UV) detector, G1313A autosampler with a 900-μL loop, G1316A column heater, a Packard C150TR flow scintillation analyzer and an ISCO/Foxy fraction collector. ChemStation software (Agilent Technologies, version A.10.02) was used to control injection volumes and gradient conditions.
Column: Beckman Ultrasphere ODS, 5 um, 10 x 250 mm
Pre-column: Brownlee NewGuard RP-18
Flow Rate: 3.0 mL/min
Solvent A: 0.5% (v/v) formic acid in water
Solvent B: acetonitrile
Gradient: 10% B, hold 5 minutes then ramp to 100% B at 50 minutes

Detection:
Radioactivity: fraction collection with liquid scintillation counting (HPLC/LSC). Chromatograms (histograms) were generated from the scintillation results using the TRACE II system (Section IIID).
UV: 280 nm - data collection, analysis and report generation were performed by the Atlas 2003R1.1 chromatography data collection system (ThermoElectron).

Fraction collection: 0.3 minutes per vial

C. LC/MS/MS Analysis

LC/MS/MS analysis was performed using an Applied Biosystems/Sciex API 5000 MS/MS instrument. Samples were introduced into the mass spectrometer via a Waters ACQUITY™ UPLC. All chromatography and mass spectrometry parameters were identical to the parameters used to analyze ^{12}C-dicamba fortified soybean matrix samples as described in the analytical method (AG-ME-1321, see Appendix I).

The ^{12}C ions of the DCSA, DCGA, dicamba and 5-hydroxydicamba residues were monitored.

D. Radioactivity Analysis (Liquid Scintillation Counting)

Liquid scintillation counting was conducted using the Monsanto TRACE II (**T**he **R**adio**A**ctivity **C**ounting Syste**E**m, second generation) system, a multi-component hardware/software system for LSC data collection, storage, processing, and reporting. This system incorporates PerkinElmer Tri-Carb 2900TR liquid scintillation analyzers with QuantaSmart 2.02 software interfaced to a data collection server running Jane™ (Ver. 4.2.18.30) software, a liquid scintillation counting data acquisition, processing, and reporting application developed and modified to Monsanto Company specifications by LabLogic Systems Limited.

Samples were mixed with Ultima-Flo™ AP scintillation cocktail and were generally counted using a single five- or 10-minute pass. Samples were counted using the automatic external standard pulse method. For each set of vials counted, the background or noise value was determined by counting vials containing only scintillation cocktail (and solvent, optionally) to obtain an average cpm background level. All LSC data were corrected for background. Quench curves were established using a set of sealed vials containing a fixed quantity of ^{14}C-radioactivity ([^{14}C]toluene), increasing quantities of a quenching solvent (nitromethane), and an appropriate counting fluid. The sealed vials were purchased from PerkinElmer. The instrument performance (counting efficiency and background) was assessed each day prior to usage by counting sealed background and standard (^{14}C and ^{3}H) vials.

E. Radioactivity Analysis (Combustion)

Combustion analysis was performed using a Packard Model 307 oxidizer. Samples were weighed into combustion cones and oxidized in a continuous flow of oxygen to $^{14}CO_2$

which was trapped in a solution of CarboSorb® E and Permafluor® E^+. Performance of the oxidizer was routinely monitored by combustion assays of ^{14}C-benzoic acid. Samples prepared by the oxidizer were analyzed by LSC to determine radioactivity.

IV. Results and Discussion

Combustion data and ^{14}C-HPLC metabolite profiles from the final storage stability analyses conducted as part of metabolism study 06-98-M-1 were used as a starting reference for the radioactivity levels and amounts of metabolites present in each of the three soybean matrices (PRE-T hay, POE-T hay and POE-T seed). The ground samples had been stored at approximately –20 ºC for approximately 2-2.5 years since their collection. The final storage stability analyses showed very little change from the initial analyses indicating good stability of the samples in storage. The storage stability analyses were conducted prior to the initiation of the radiovalidation but within 40 days of the initiation of the radiovalidation analyses. The PRE-T hay, POE-T hay and POE-T seed samples were chosen for radiovalidation because they constituted a representative range of sample types and contained a representative range of dicamba-derived residues.

The metabolism final storage stability HPLC profiles (chromatograms) were used to calculate the amounts of DCGA, DCSA and dicamba analytes to be expected as a result of analysis using the residue analytical method. The storage stability chromatograms and the corresponding metabolite quantitation tables are presented in Figures 1-3. In the residue analytical method, the endogenous DCGA and DCSA conjugates are converted in the acid hydrolysis step to the analytes DCGA and DCSA. Thus, the amount of DCGA analyte expected (in ppm or mg/kg) was calculated as the sum of the amounts of DCGA glucoside and DCGA malonylglucoside metabolites present in the samples. Similarly, DCSA glucoside and DCSA 3-hydroxy-3-methylglutarylglucoside (DCSA HMGglucoside) metabolites, and free DCSA (if present), were summed to derive the amount of DCSA expected. Dicamba is unchanged on hydrolysis, so the amount expected in the residue analysis is simply the amount of dicamba present in the sample. Data for 5-hydroxydicamba are not included in this radiovalidation because it was not observed as a metabolite in the metabolism study.

The results of the radiovalidation analyses for the PRE-T hay, POE-T hay and POE-T seed samples are presented in Figures 4, 5 and 6, respectively.

A. Extraction Efficiency

The dicamba residue analytical method AG-ME-1321 incorporates a single extraction of soybean matrices with 40:60 acetonitrile:water [10:1 (volume:weight) solvent to sample ratio] followed by centrifugation. A 10-mL aliquot of the supernatant is removed directly from the centrifuge bottle and carried through the remainder of the method. Because only a single extraction was conducted with no filtration or washing of the solids, and the extract could not be quantitatively decanted from the extracted solids, the extractabilities were calculated from the radioactivity measured in aliquots of the extract supernatant and based on the total volume of extract. For seed, a very dry matrix, the volume of extract was assumed to be 100 mL (equal to the volume of solution added). For forage and hay, which contained approximately 80% moisture (the forage and hay from the metabolism study were not dried), the extract volume was estimated to be 108 mL (100 mL of extraction solution plus 8 mL of water extracted from the 10-g sample). The 80% moisture value for the forage and hay was based on a hay drying experiment of extracted hay pellets which

indicated that the hay contained 78% moisture. **Note:** in the residue analytical method the use of an aliquot of the extract, rather than the whole extract, and the use of internal standards obviates the need for careful separation of the supernatant and extracted solids or washing of the solids.

Extractabilities are summarized in part A of Figures 4-6. For the PRE-T hay, POE-T hay and POE-T seed samples, the amount of radioactive residues extracted averaged 94.7%, 94.2% and 48.1%, respectively. These extractabilities compare well to the corresponding extractabilities obtained in the metabolism study using multiple acetonitrile: water extractions (90.6%, 95.7% and 55.5%, for PRE-T hay, POE-T hay and POE-T seed, respectively, Figures 1-3).

B. Method Recovery

Average overall recoveries of radioactivity through the method, including the extraction, hydrolysis, partitioning, evaporation and final filtration steps, were 62.9%, 64.3% and 25.5% for PRE-T hay, POE-T hay and POE-T seed, respectively (part A of Figures 4-6). The low overall radioactivity recovery for seed was caused by the low extractability, due to significant unextracted (bound) residues, and larger losses in the partitioning step likely due to the presence of a significant amount of water-soluble residues (e.g, sugars) in the seed extract. Recoveries for the hydrolysis, evaporation and final filtration steps were near quantitative (>90% each) for all matrices. Very little radioactivity loss was found in the distillate of the evaporation step. Average recoveries for the partitioning step were 74.3% and 74.5% for the hay samples, and 58.7% for the seed sample. Losses in the partitioning step may be due to 1) water solubility of a percentage of the residues (e.g., polar residues formed by degradation of dicamba to small molecules and incorporation into natural products such as sugars), especially for seed, 2) incomplete partitioning of the analytes into the organic phase, and 3) procedural losses incurred in use of the phase separation filter paper. **Note:** use of internal standards in this method compensates for any procedural losses incurred in the hydrolysis, partitioning, evaporation and filtration steps.

C. Quantitation of Residues

HPLC/LSC analyses of the post-evaporation aqueous solutions, which contained the final analytes, are shown in part B of Figures 4-6. These analyses demonstrated that DCSA, DCGA and dicamba were the only significant radioactive components of the final analyte solutions. DCSA was the major component in all three samples, constituting 89.71%, 78.50% and 69.59% of the radioactivity in the final analyte solutions for PRE-T hay, POE-T hay and POE-T seed, respectively. Together, DCSA, DCGA and dicamba constituted 96.18%, 96.54% and 89.62% of the PRE-T hay, POE-T hay and POE-T seed HPLC profiles, respectively. This demonstrates the efficiency of the hydrolysis and partitioning steps of the method and indicates that DCSA, DCGA and dicamba are the only significant dicamba-related residues in the samples.

Residues in the final analyte solutions from the method were quantitated by LC/MS/MS, the normal determinative step of the method. In the metabolism study, from which the samples for this study were derived, the [^{14}C]dicamba utilized as test substance was prepared by mixing [^{14}C]dicamba of high specific activity with unlabeled dicamba to give test materials with specific activities of 5.39 and 5.43 mCi/mmol for the preemergence and postemergence treatments, respectively. As such, the test materials consisted of >90% unlabeled dicamba (the [^{14}C]dicamba was less than 10% of each of the mixtures). For this reason, only the ^{12}C ions of the analytes

were monitored in the quantitative mass spectral analyses. This resulted in an error of less than 10% in the LC/MS/MS residue analytical results for this study.

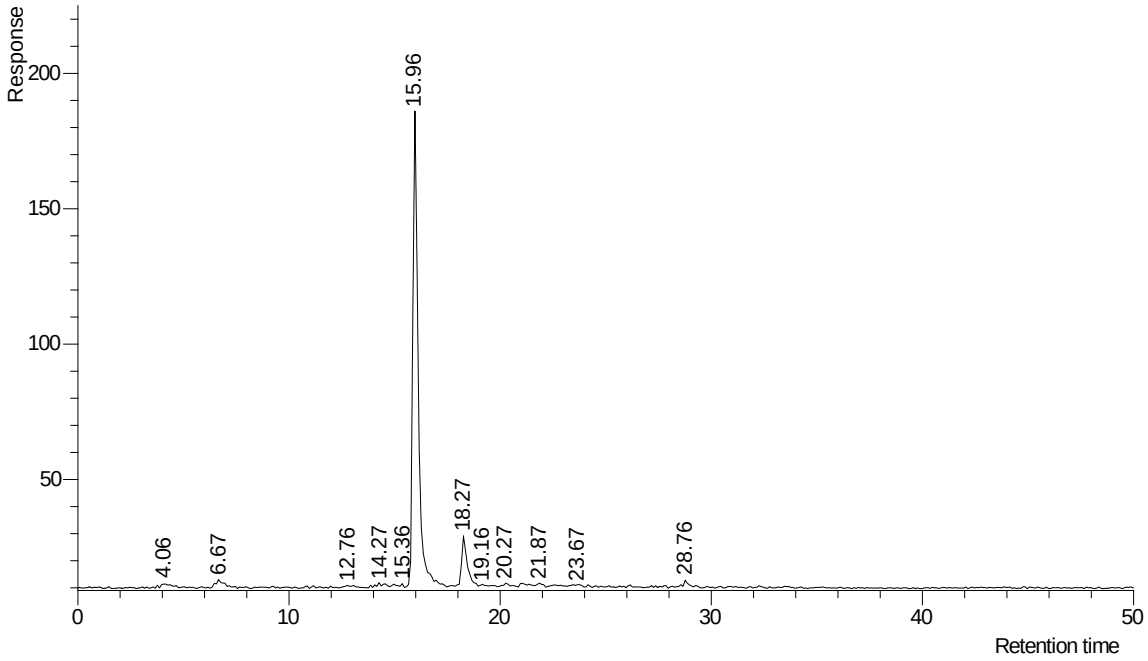
Comparisons of the LC/MS/MS quantitation results with the HPLC metabolite quantitation results are presented in part C of Figures 4-6. There is generally very good agreement, especially for DCSA and dicamba, between the mass spectral quantitation results and the actual residue concentrations present in the hay and seed samples as determined by quantitation of the storage stability HPLC profiles (Figures 1-3). Only the quantitation results for DCGA at very low levels of DCGA (0.025 ppm in POE-T seed and 0.032 ppm in PRE-T hay) were substantially different than their actual values (quantitation of DCGA in the POE-T hay sample, at a level of 2.28 ppm, was very good). DCGA residues in the PRE-T hay (0.032 ppm) were overestimated by approximately a factor of 2, while the DCGA residues in the POE-T seed (0.025 ppm) were underestimated by a factor of 5. It should be noted that the DCGA residue levels for these two samples were near the limit of quantitation for DCGA. The reason for the discrepancy in the seed DCGA quantitation is not known. The HPLC profile of the final analyte solution shows a substantial DCGA peak which is 17.36% of the profiled radioactivity (about $1/4^{th}$ the level of DCSA in the sample in agreement with what is expected based on the quantitation of the HPLC stability profile). Thus, the DCGA analyte was present in the final analyte solution at the expected level, but it was not accurately quantitated in this particular LC/MS/MS analysis set.

V. Conclusion

The analytical method for dicamba residues in soybeans, AG-ME-1321, was evaluated by analysis of soybean hay and seed samples containing radioactive dicamba-derived residues. The samples were from a study of dicamba metabolism in dicamba-tolerant soybeans.

Average extractabilities for pre- and postemergence hay and postemergence seed samples were 94.7%, 94.2% and 48.1%, respectively, and were quite comparable to the extractabilities obtained in the metabolism study. The average total recoveries of radioactivity through the method for pre- and postemergence ^{14}C-hay samples and postemergence ^{14}C-seed samples were 62.9%, 64.3% and 25.5%, respectively. The low extractability and low radioactivity recovery for the seed sample were due to polar and/or unextracted residues derived from extensive breakdown of dicamba to small molecules and incorporation into natural plant constituents. There was generally good agreement between LC/MS/MS quantitation results and actual residue levels, especially for DCSA and dicamba analytes, except for the case of low-level DCGA residues in seed.

Figure 1: Final Storage Stability HPLC/RAD Chromatogram from Metabolism Study 06-98-M-1 for Soybean Hay from the Preemergence Treatment (PRE-T Hay)



Matrix Total Radioactive Residue (TRR, ppm)	Extractability	Evaporation and Centrifugation Recovery	Total dpm Injected	Column Recovery
1.087	90.64%	101.06%	39741	99.60%

Retention Time (min)	Identification	Percent of Chromatogram	Percent of Matrix TRR*	Peak mg/kg (ppm)**
6.67	DCGA Glucoside	2.45	2.22	0.024
15.36	DCGA Malonylglucoside	0.77	0.70	0.008
15.96	DCSA Glucoside	78.22	70.90	0.771
18.27	DCSA HMGglucoside	9.25	8.38	0.091
28.76	Dicamba	1.20	1.09	0.012
	TOTALS	**91.89**	**83.29**	**0.905**

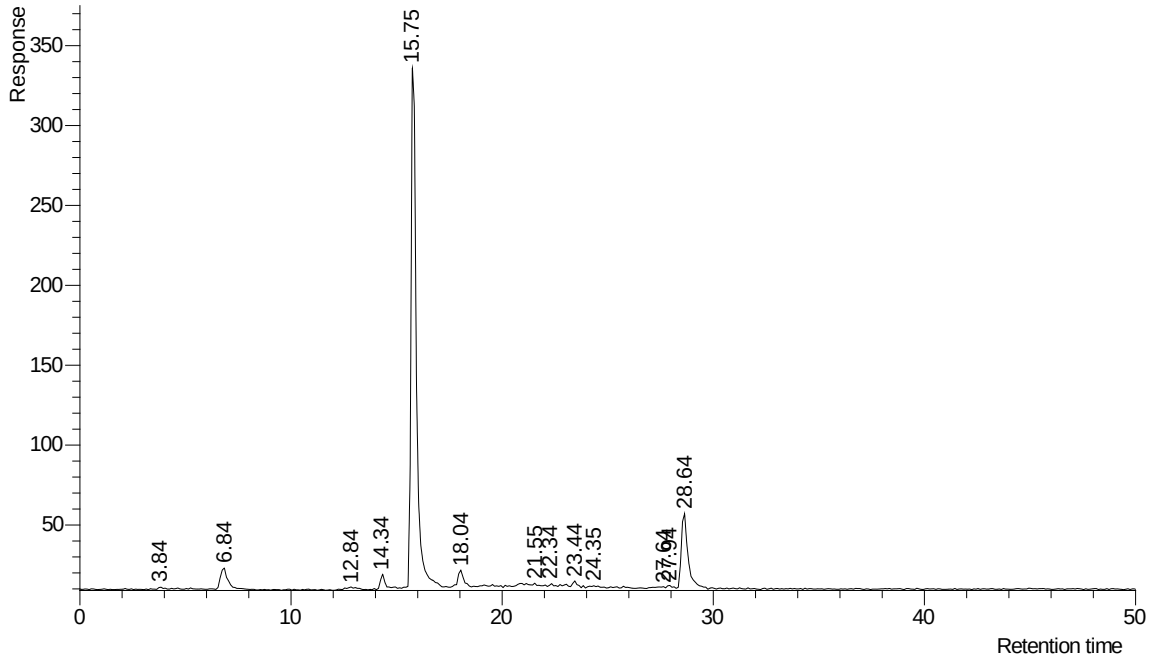
* Percent of Matrix TRR = (Percent of Chromatogram) x (Extractability)

Peak mg/kg (ppm) = (Matrix TRR) x (Percent of Matrix TRR)
**dicamba equivalents

Sum of DCGA-forming metabolites: 0.032 ppm
(DCGA Glucoside and DCGA Malonylglucoside)

Sum of DCSA-forming metabolites: 0.862 ppm
(DCSA Glucoside and DCSA HMGglucoside)

Figure 2: Final Storage Stability HPLC/RAD Chromatogram from Metabolism Study 06-98-M-1 for Soybean Hay from the Postemergence Treatment (POE-T Hay)



Matrix Total Radioactive Residue (TRR, ppm)	Extractability	Evaporation and Centrifugation Recovery	Total dpm Injected	Column Recovery
39.342	95.68%	100.62%	83410	100.37%

Retention Time (min)	Identification	Percent of Chromatogram	Percent of Matrix TRR	Peak mg/kg (ppm)*
6.84	DCGA Glucoside	4.25	4.07	1.600
14.34	DCGA Malonylglucoside	1.81	1.73	0.681
15.75	DCSA Glucoside	71.17	68.10	26.790
18.04	DCSA HMGglucoside	2.40	2.30	0.903
27.6, 27.9	DCSA	0.94	0.90	0.354
28.64	Dicamba	12.96	12.40	4.878
	TOTALS	**93.53**	**89.49**	**35.206**

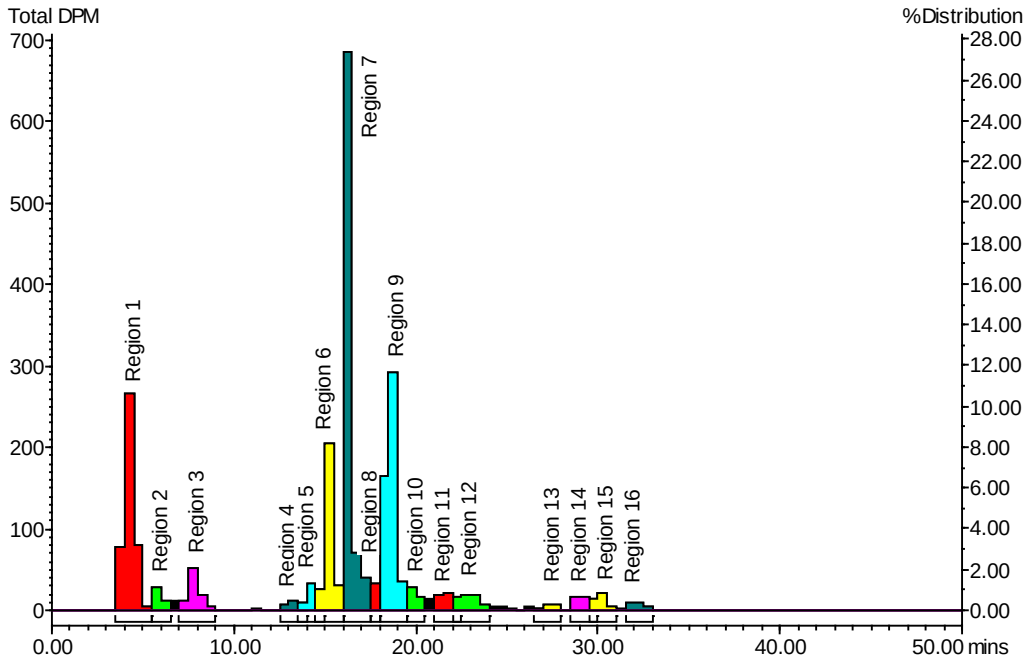
* Percent of Matrix TRR = (Percent of Chromatogram) x (Extractability)

Peak mg/kg (ppm) = (Matrix TRR) x (Percent of Matrix TRR)
**dicamba equivalents

Sum of DCGA-forming metabolites: 2.28 ppm
(DCGA Glucoside and DCGA Malonylglucoside)

Sum of DCSA-forming metabolites: 28.0 ppm
(DCSA Glucoside, DCSA HMGglucoside and DCSA)

Figure 3: Final Storage Stability HPLC/LSC Chromatogram from Metabolism Study 06-98-M-1 for Soybean Seed from the Preemergence Treatment (POE-T Seed)



Matrix Total Radioactive Residue (TRR, ppm)	Extractability	Evaporation and Centrifugation Recovery	Total dpm Injected	Column Recovery
0.383	55.52%	97.96%	2731	91.65%

Region	Retention Time (min)	Identification	Percent of Chromatogram	Percent of Matrix TRR	Peak mg/kg (ppm)*
3	7.75	DCGA Glucoside	3.43	1.71	0.007
6	15.25	DCGA Malonylglucoside	9.87	4.92	0.019
7	16.25	DCSA Glucoside	31.78	15.84	0.061
9	18.75	DCSA HMGglucoside	19.66	9.80	0.038
13	27.25	DCSA	0.63	0.31	0.001
14	29.25	Dicamba	1.60	0.80	0.003
		TOTALS	**66.97**	**33.38**	**0.128**

* Percent of Matrix TRR = (Percent of Chromatogram) x (Extractability) x (Evaporation and Centrifugation Recovery) x (Column Recovery)

Peak mg/kg (ppm) = (Matrix TRR) x (Percent of Matrix TRR)
**dicamba equivalents

Sum of DCGA-forming metabolites: 0.025 ppm**
(DCGA Glucoside and DCGA Malonylglucoside)

Sum of DCSA-forming metabolites: 0.099 ppm
(DCSA Glucoside, DCSA HMGglucoside and DCSA)
**Calculated using non-rounded values (hand-calculation using rounded values gives 0.026 ppm)

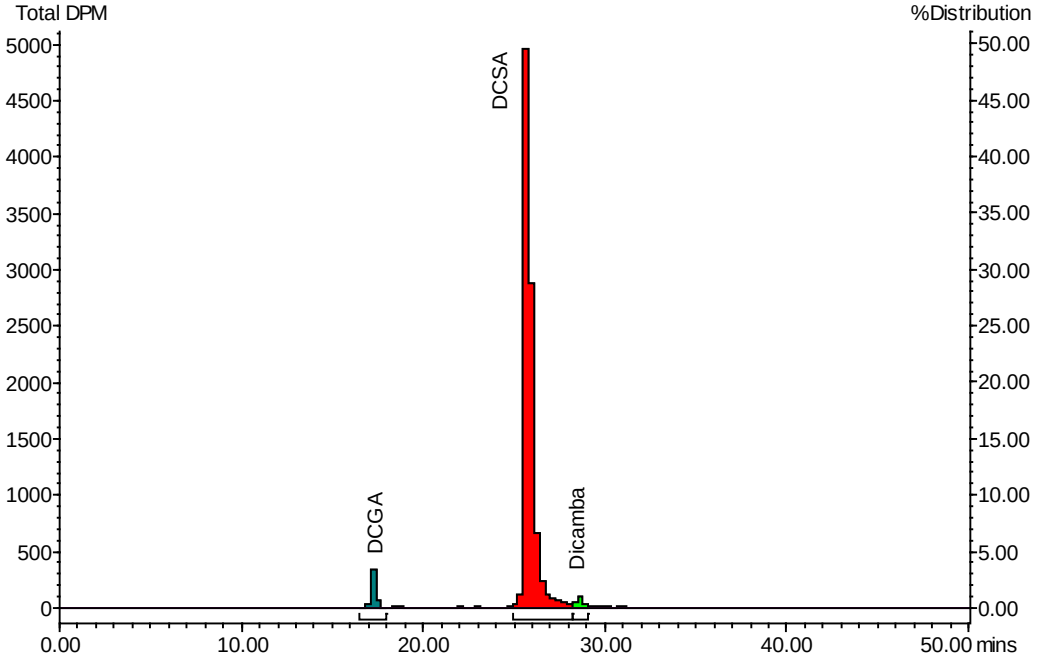
Figure 4: Summary of Radiovalidation Results for Soybean Hay from the Preemergence Treatment (PRE-T Hay)

A) Radioactivity Accountability

	Percent Recovery		
Method Step	**Replicate 1**	**Replicate 2**	**Average**
Extraction	95.3	94.1	94.7
Hydrolysis	97.7	98.9	98.3
Partition – Organic	75.1	73.4	74.3
Partition – Aqueous + filter paper*	16.1	16.0	16.1
Evaporation	95.2	95.9	95.6
Filtration	94.2	96.3	95.3
Method Recovery	**62.7**	**63.1**	**62.9**

*Not used in the calculation of the method recovery.

B) ^{14}C HPLC Profile of PRE-T Soybean Hay Analyte Solution after Final Filtration



Analyte (^{14}C)	Start (mins)	End (mins)	Retention (mins)	Height (Total DPM)	Area (Total DPM)	%Total (%)
DCGA	16.50	18.00	17.25	332	443	4.32
DCSA	24.90	27.90	25.65	4964	9198	89.71
Dicamba	27.90	29.10	28.65	107	221	2.15

C) Comparison of Analyte Concentrations from Metabolism Study Storage Stability Profiles with those Determined by LC/MS/MS

Metabolite/Analyte	Metabolism Study Storage Stability Profile (mg/kg, ppm)**	Radiovalidation LC/MS Analysis (mg/kg, ppm)
DCGA	0.032	0.068, 0.067 (avg. 0.068)
DCSA	0.862	0.878, 0.849 (avg. 0.863)
Dicamba	0.012	0.015, 0.016 (avg. 0.016)

** For the metabolism profile, DCGA and DCSA values are the sum of the respective glucoside metabolites and free DCSA.
All values in this table are dicamba equivalents.

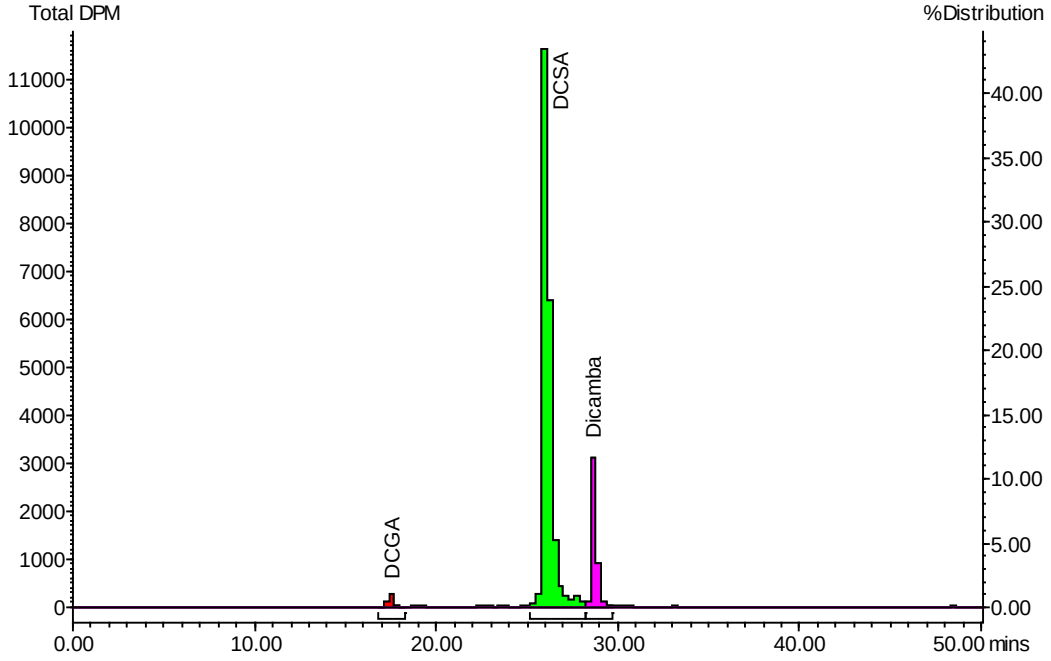
Figure 5: Summary of Radiovalidation Results for Soybean Hay from the Postemergence Treatment (POE-T Hay)

A) Radioactivity Accountability

	Percent Recovery		
Method Step	**Replicate 1**	**Replicate 2**	**Average**
Extraction	94.7	93.6	94.2
Hydrolysis	101.5	100.2	100.9
Partition – Organic	76.3	72.6	74.5
Partition – Aqueous + filter paper*	12.4	17.1	14.8
Evaporation	96.2	101.1	98.7
Filtration	91.4	93.2	92.3
Method Recovery	**64.5**	**64.1**	**64.3**

*Not used in the calculation of the method recovery

B) ^{14}C HPLC Profile of POE-T Soybean Hay Analyte Solution after Final Filtration



Analyte (^{14}C)	Start (mins)	End (mins)	Retention (mins)	Height (Total DPM)	Area (Total DPM)	%Total (%)
DCGA	16.80	18.30	17.55	294	486	1.82
DCSA	25.20	28.20	25.95	11633	21000	78.50
Dicamba	28.20	29.70	28.65	3131	4338	16.22

C) Comparison of Analyte Concentrations from Metabolism Study Storage Stability Profiles with those Determined by LC/MS/MS

Metabolite/Analyte	Metabolism Study Storage Stability Profile (mg/kg, ppm)**	Radiovalidation LC/MS Analysis (mg/kg, ppm)
DCGA	2.28	2.59, 2.29 (avg. 2.44)
DCSA	28.0	25.1, 30.3 (avg. 27.7)
Dicamba	4.88	4.30, 3.18 (avg. 3.74)

** For the metabolism profile, DCGA and DCSA values are the sum of the respective glucoside metabolites and free DCSA.
All values in this table are dicamba equivalents.

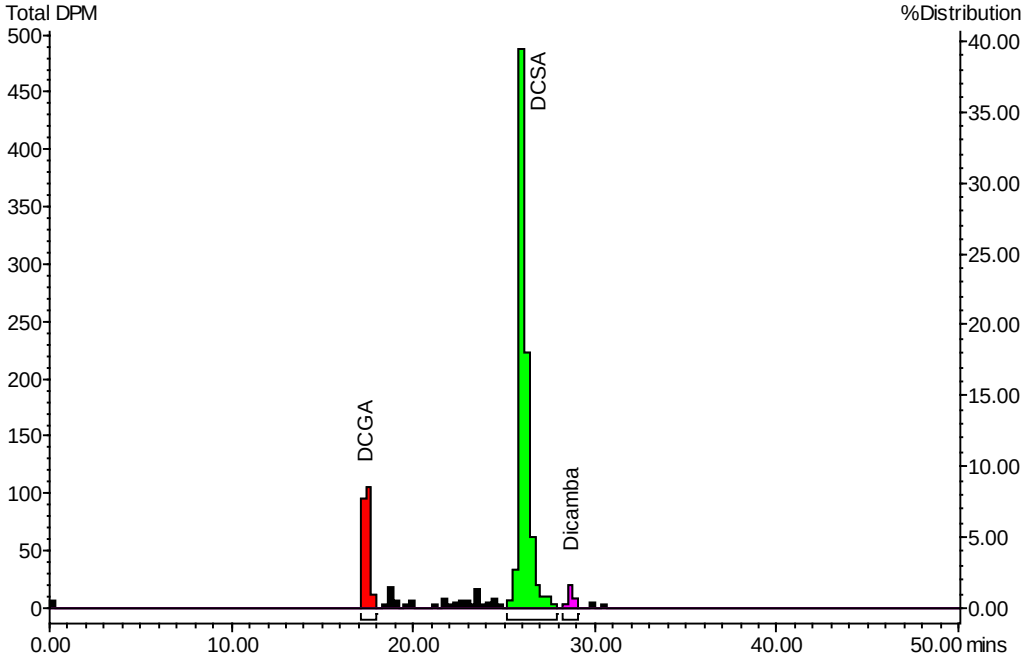
Figure 6: Summary of Radiovalidation Results for Soybean Seed from the Postemergence Treatment (POE-T Seed)

A) Radioactivity Accountability

	Percent Recovery		
Method Step	**Sample R1**	**Sample MS1**	**Average**
Extraction	47.9	48.3	48.1
Hydrolysis	98.9	98.5	98.7
Partition – Organic	55.7	61.6	58.7
Partition – Aqueous + filter paper*	31.5	30.5	31.0
Evaporation	99.6	99.3	99.5
Filtration	94.7	89.7	92.2
Method Recovery	**24.9%**	**26.0%**	**25.5%**

*Not used in the calculation of the method recovery

B) ^{14}C HPLC Profile of POE-T Soybean Seed Analyte Solution after Final Filtration



Analyte (^{14}C)	Start (mins)	End (mins)	Retention (mins)	Height (Total DPM)	Area (Total DPM)	%Total (%)
DCGA	17.10	18.00	17.55	106	215	17.36
DCSA	25.20	27.90	25.95	488	860	69.59
Dicamba	28.20	29.10	28.65	21	33	2.67

Comparison of Analyte Concentrations from Metabolism Study Storage Stability Profiles with those Determined by LC/MS/MS

Metabolite/Analyte	Metabolism Study Storage Stability Profile (mg/kg, ppm)**	Radiovalidation LC/MS Analysis (mg/kg, ppm)
DCGA	0.025	0.007, 0.004 (avg. 0.005)
DCSA	0.099	0.090, 0.085 (avg. 0.088)
Dicamba	0.003	No peaks

** For the metabolism profile, DCGA and DCSA values are the sum of the respective
glucoside metabolites and free DCSA.
All values in this table are dicamba equivalents.

9 APPENDIX III. VALIDATION PLAN (WITH AMENDMENTS)

Note: A validation plan was originally written for an earlier draft version of the analytical method SOP. An amendment (Amendment 1) was subsequently written for that version of the validation plan. At a later date, the validation plan was re-written (the later plan is included in this appendix) and the method was validated according to that plan. Amendments to the later validation plan began with Amendment 2 (there is no Amendment 1 to the later plan). The original (obsolete) validation plan and its amendment (Amendment 1) are not included in this report to avoid potential confusion.

Method Validation Plan for ES-ME-1267 (draft 29SEP08)

Method: ES-ME-1267 (draft 29SEP08)

Title: Determination of Dicamba and Its Major Metabolites in Soy Forage, Hay and Grain by LC/MS/MS

Purpose and Scope of Validation

This validation plan determines the performance of a method for quantitative analysis of dicamba and its major metabolites, 5-OH dicamba, 3,6-dichlorosalicylic acid (DCSA) and 3,6-dichlorogentisic acid (DCGA) in soy using liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS). The method includes the use of stable labeled isotope internal standards ($^{13}C_6$- dicamba, $^{13}C_6$-5-OH dicamba, $^{13}C_6$- DCSA and $^{13}C_6$- DCGA) for quantitation.

Recovery tests to demonstrate precision and accuracy across the dynamic range will be performed. The validation will show performance at levels expected during routine analysis through spike recoveries at the expected method limit of quantitation (LOQ), 2X the LOQ, and 10X the LOQ.

The validation will confirm the range, precision and accuracy of standards.
The validation will also demonstrate the stability of the analytes in sample extract and standard solutions and confirm the dilution method for out-of-range samples.

An additional spike recovery will be evaluated at 0.5X the LOQ to better define the LOD and the LOQ.

Experimental Design:

The experimental design reflects proposed ESTC standard requirements for parameters of method validation. The method will be validated at Monsanto by an analyst from the ESTC using an ABS/Sciex API 5000 LC/MS/MS instrument. The test matrices will be ground soybean hay, grain and forage from a suitable source(s). The matrix source(s) will be documented in the raw data. The proposed LOQ value for all test matrices is 10 ng/g for dicamba, 5-OH dicamba, DCSA and DCGA. The selected soybean hay, grain and forage will serve as the analytical control for the tests.

1) Standards

Establish the dynamic range, accuracy and precision of the nine calibration standards based upon single injections of each calibration standard level. Evaluate dicamba, 5-OH dicamba, DCSA and DCGA over a range of 0.5 to 500 µg/L.

Acceptance criteria:

- Separate standard curves for each analyte must each have a coefficient of determination of ≥ 0.99 and yield back-calculated data points within the range of 70 – 120% for a minimum of seven calibration levels.

2) Samples

2a) Precision and Accuracy

Using ES-ME-1267 (draft 29SEP08), analyze 7 samples per matrix of soybean hay, grain or forage as control samples. Also, analyze 7 samples per matrix of soybean hay, grain and forage fortified with analytes at 5 ng/g, 10 ng/g, 20 ng/g and 100 ng/g. Determine the background corrected percent recovery for each analyte in each sample based on the amount of analyte in the controls.

2b) Dilution of High Level Samples

Demonstrate out of range analyses and dilution mechanism as described in the analytical SOP by dilution of high concentration samples. Two replicates of each matrix type will be analyzed.

Acceptance criteria:

- For fortified samples, average background corrected recovery must be between 70-120% with RSD of ≤ 20% at each fortification level at or above the LOQ.

2c) LOD and LOQ Determination

A statistician will analyze the recovery data to estimate the LOD and the LOQ.

3) Analyte Stability

The stability of the analytes will be evaluated in standard solutions and the final analyte solutions.

- Determine the stability of the injected analyte solution when stored at ≤ 10 °C for 3 days.
  Acceptance Criteria:
  The average recovery must be between 70-120% with RSD of ≤ 20%.

- Analyze the native stock standard solutions according to ES-PO-0897-01 to determine stability for at least 7 days.

- Analyze the working calibration solutions according to ES-PO-0897-01 to determine stability for at least 7 days.

The internal standards will not be tested due to limited supply. It is assumed and expected that the stable labeled internal standards will have nearly identical chemical properties as the native analytes.

Review:

James E. Foster Date: 30 / SEP / 08
(Lead Scientist, Environmental Sciences Technology Center)

Carrie Logan Date: 9 / 30 / 08
(QAU, Biotechnology Regulatory Sciences)

Monte A. Marshall Date: 10 / 1 / 08
(TFM, Environmental Sciences Technology Center)

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Method Validation Plan Amendment

SOP number: **ES-ME-1267 (draft 17NOV08)**

Title: Determination of Dicamba and Its Major Metabolites in Dicamba Tolerant Soy by LC/MS/MS

Amendment number: **02** Effective date: **December 01, 2008**

Change 1:
Method Validation Plan Originally States:
There are no changes to what was stated in the original method plan. Additional experiments were added.

Method Validation Plan Amended as Follows:

Purpose and Scope of Validation

The method will be evaluated in samples of soybean hay from the pre- and post-emergence treatments and soybean seed from the postemergence treatment of study 06-98-M-1, "Metabolism of Dicamba in Dicamba-Tolerant Soybeans", in which [^{14}C]-dicamba was utilized as test substance. Combustion data and ^{14}C-HPLC metabolite profiles from the final storage stability analyses of the metabolism study will be used as a starting reference for the radioactivity levels and amounts of metabolites present in each matrix. The radioactive samples will be prepared using ES-ME-1267 and quantitative radioactive recoveries will be determined for each major sample preparation step of the residue method. Also, the nature and approximate quantities of the final residues will be determined by ^{14}C HPLC profiles of the post-evaporation filtered aqueous sample (prior to the addition of 9% formic acid). Finally, quantitative analysis will be performed by monitoring for the ^{12}C isotope in the treated samples using liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS).

Treatment	Matrices	
preemergence application	Hay	——
postemergence application	Hay	Seed

Experimental Design:

Analytical method ES-ME-1267 (draft 17NOV08) will be used to generate the samples that will be analyzed by HPLC, combustion and/or liquid scintillation counting as well as the quantitative analysis performed using an ABS/Sciex API 5000 LC/MS/MS instrument. The samples for quantitative analysis will be prepared and analyzed in duplicate. The sample sources will be documented in the raw data.

At a minimum, the radioactive residues in each sample extract, extraction centrifugation pellet, hydrolysis solution, ethyl acetate and aqueous partition fractions, post-evaporation aqueous solution and post-filtration aqueous solution will be quantified by combustion and/or liquid scintillation counting. The liquid samples will be weighed before and after each analytical method step and aliquots will be removed and weighed for liquid scintillation counting. The solid samples (extraction pellet) will be weighed and aliquots will be weighed and combusted using a Packard 307 oxidizer (SOP ES-EQ-0546). The combusted samples generated by the oxidizer will then be counted by liquid scintillation counting. The liquid scintillation counting will be conducted using a PerkinElmer Tri-Carb 2900TR (SOP AG-EQ-

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1199) liquid scintillation counter. The scintillation counting results and the sample/aliquot weights will be used to determine the recovery of radioactive residues in each step.

The post-evaporation aqueous solution will be analyzed by ^{14}C-HPLC using a Hewlett Packard/Agilent 1100 HPLC with fraction collection and liquid scintillation counting of the fractions. For ^{14}C-HPLC with fraction collection, chromatograms (histograms) will be generated from the scintillation results and processed with Trace II software (SOP AG-EQ-1200) to determine the nature and amounts of the final analytes generated by the residue analytical method. For HPLC with UV detection (for analysis of reference standards for retention time comparison to the final analytes), chromatograms will be generated using the ATLAS chromatography system (SOP AG-EQ-1051).

^{14}C- HPLC metabolite profiles of the post-evaporative aqueous sample will be obtained using the following method:

Chromatography method for Hewlett Packard HPLC (SOP AG-EQ-0785):

Column: Beckman Ultrasphere ODS, 5 um, 10 x 250 mm
Pre-column: Brownlee NewGuard RP-18
Flow Rate: 3.0 mL/min
Solvent A: 0.5% (v/v) formic acid in water
Solvent B: acetonitrile
Gradient: 10% B, hold 5 minutes then ramp to 100% B at 50 minutes
Detection:
radioactivity: fraction collection with scintillation counting
UV: 280 nm

Foxy/ISCO Fraction Collector parameters (SOP ES-EQ-0284):
0.3 minutes per vial

Reason for Amendment:
The validation plan is being updated to include a radiovalidation of the analytical method.

James Foster Date: 01 / DEC / 2008
James E. Foster
(Lead Scientist, Environmental Sciences Technology Center)

Carrie Logan Date: 12 / 1 / 08
Carrie Logan
(QAU, Biotechnology Regulatory Sciences)

Monte G. Marshall Date: 12 / 1 / 08
Monte A. Marshall
(TFM, Environmental Sciences Technology Center)

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Method Validation Plan Amendment

SOP number: **AG-ME-1321-01**

Title: Determination of Dicamba and Its Major Metabolites in Dicamba Tolerant Soy by LC/MS/MS

Amendment number: **03** Effective date: **October 29, 2009**

Change 1:
Method Validation Plan Originally States:
The SOP number was ES-ME-1267

Method Validation Plan Amended as Follows:
The SOP number is now AG-ME-1321-01

Reason for Amendment:
The SOP number was inadvertently issued twice by the testing facility management support group.

James Foster PhD Date: 29 / OCT / 09
James E. Foster
(Lead Scientist, Environmental Sciences Technology Center)

Date: 10 / 29 / 09
Steve Reale
(QAU, Monsanto ~~company~~)

Mary P. Gasper Date: 10 / 29 / 09
Mary P. Gasper
(TFM, Monsanto Company)

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Method Validation Plan Amendment

SOP number: **AG-ME-1321-01**

Title: Determination of Dicamba and Its Major Metabolites in Dicamba Tolerant Soy by LC/MS/MS

Amendment number: **04** Effective date: **November 17, 2009**

Change 1:
Method Validation Plan Amendment 2 Originally States:

Experimental Design:

Analytical method ES-ME-1267 (draft 17NOV08) will be used to generate the samples that will be analyzed by HPLC, combustion and/or liquid scintillation counting as well as the quantitative analysis performed using an ABS/Sciex API 5000 LC/MS/MS instrument.

Method Validation Plan Amended as Follows:
Analytical method ES-ME-1267 (draft 03DEC08) will be used to generate the samples that will be analyzed by HPLC, combustion and/or liquid scintillation counting as well as the quantitative analysis performed using an ABS/Sciex API 5000 LC/MS/MS instrument.

Reason for Amendment:
The current draft of the analytical method was ES-ME-1267 (draft 03DEC08) when the radiovalidation conducted.

ES-ME-1267 (draft 17NOV08) states:

VII. SAMPLE ANALYSIS

A. Sample Preparation

6. Centrifuge for approximately 10 minutes at 9500 rpm.

ES-ME-1267 (draft 03DEC08) states:

VII. SAMPLE ANALYSIS

A. Sample Preparation

6. Centrifuge for approximately 10 minutes at 8000 rpm.

This change was made to keep centrifuge vials from collapsing in the centrifuge. There is no impact to the validation data.

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James E Foster PhD Date: 18 / NOV / 09
James E. Foster
(Lead Scientist, Environmental Sciences Technology Center)

Date: 11 / 18 / 09
Steve Reale
(QAU, Monsanto/company)

Mary P Gasper Date: 11 / 19 / 09
Mary P. Gasper
(TFM, Monsanto Company)